Plant Cell, Tissue and Organ Culture **34**: 183–190, 1993.

Influence of the carbon source on growth and rosmarinic acid production in suspension cultures of *Coleus blumei*

Claudia Gertlowski & Maike Petersen*
*Institut für Entwicklungs- und Molekularbiologie der Pflanzen, Heinrich-Heine-Universität Düsseldorf, Universitätsstr. 1, D-4000 Düsseldorf 1, Germany (*requests for offprints)*

Received 14 September 1992; accepted in revised form 17 February 1993

Key words: carbon source, cell cultures, *Coleus blumei* (Lamiaceae), culture medium, rosmarinic acid, secondary product formation

Abstract

Suspension cultures of *Coleus blumei* were characterized with respect to growth and rosmarinic acid formation in media with different sugars and various sugar concentrations. Sucrose is the sugar with the highest stimulating effect on growth and rosmarinic acid accumulation, followed by glucose and fructose. The sugar alcohol mannitol cannot be metabolized by the plant cells. Sucrose is cleaved into glucose and fructose by the *Coleus* cells. Sucrose concentrations from 1 to 5% have an increasing positive effect on growth and rosmarinic acid synthesis in the cell cultures with a maximum rosmarinic acid content of 12% of the dry weight in medium with 5% sucrose; in medium with 6% sucrose rosmarinic acid accumulation obviously did not reach its highest level in the culture period of 14 days. A very high yield of rosmarinic acid (2 mg ml^{-1} suspension) could also be achieved by maintaining a sucrose concentration of 2% during the whole culture period. The start of rosmarinic acid synthesis by the cell cultures seems to be regulated by the growth limitation when a nutrient, e.g. phosphate is depleted from the medium. The rate of rosmarinic acid accumulation is related to the amount of carbon left in the medium when growth ceases.

Abbreviations: RA – rosmarinic acid

Introduction

The first reports on the production of rosmarinic acid (RA), an ester of caffeic acid and 3.4-dihydroxyphenyllactic acid, in suspension cultures of *Coleus blumei* were published by Zenk et al. (1977) and Razzaque & Ellis (1977). Zenk and co-workers reported that the sucrose concentration of the culture medium influenced the amount of RA synthesized by the cell cultures. Highest RA concentrations were found in medium with 7% sucrose. This positive effect of high sucrose concentrations on the accumulation of RA was exploited by Ulbrich et al. (1985) who established a two-phase culture system for the production of RA by cell cultures of *Coleus blumei* in bioreactors. In the growth phase, the suspension cultures produced biomass but nearly no RA in a modified B5-medium with 2% sucrose. The cell mass was then diluted into a production medium consisting of a 5% sucrose solution where the cells started to accumulate RA but grew only slowly. With this system, about 100 g of RA (purity 97%) could be isolated from 32 l of production medium after 8 days in the growth medium followed by a production period of 11 days. The RA content of the *Coleus blumei* cells reached 21% of the dry

weight. In our laboratory a similar system is used routinely for the maintenance of *Coleus blumei* suspension cultures and the stimulation of RA synthesis for investigations of the biosynthetic pathway of RA. The medium for routine subculturing is a modified B5-medium with 2% sucrose. In this medium only about 2–3% of the cell dry weight are accumulated as RA. In the production medium with 4% sucrose, however, up to 19% of the cell dry weight can be accumulated as RA during the final 5 days of the growth phase (Petersen & Alfermann 1988). This system has enabled us to investigate and clarify the pathway of RA biosynthesis in our *Coleus* cells and to characterize the enzymes involved (Petersen & Alfermann 1988; Häusler et al. 1991; Petersen 1991; Petersen et al. 1993). Recently, we have started to study the influence of different medium parameters on the accumulation of RA in suspension cultures of *Coleus blumei*. Here we report the influence of the quantity and quality of the carbon source in the culture medium on RA accumulation.

Materials and methods

Cell cultures and culture conditions

The original callus cultures of *Coleus blumei* were a gift of Dr. B. Ulbrich (Nattermann/Rhône-Poulenc-Rorer, Cologne, Germany). Suspension cultures initiated from this callus culture were maintained in a modified B5-medium (Gamborg et al. 1968) with 2% sucrose (CB2) as described previously (Petersen & Alfermann 1988).

Variation of the culture media

Quality and quantity of the sugar in the culture medium were modified as follows: 1 to 6% sucrose (CB1, CB2, CB3, CB4, CB5, CB6); 2% or 4% fructose instead of sucrose (CB2Fr, CB4Fr); 2% or 4% glucose instead of sucrose (CB2Gl, CB4Gl); 1% fructose plus 1% glucose instead of 2% sucrose (CB1Fr1Gl); 2% fructose plus 2% glucose instead of 4% sucrose (CB2Fr2Gl); 2% sucrose plus 2% mannitol (CB2S2M). In one experiment, the sucrose concentration of the medium was maintained at 2% by daily supplementation with a concentrated sterile (50%) sucrose solution ($CB2S_N$).

Characterization of the cell cultures

Fifteen 100 ml-Erlenmeyer flasks with 25 ml of the appropriate culture medium were inoculated with 10 ml each of a 7 day old suspension culture of *Coleus blumei*. The cultures were incubated in the dark at 25°C with continuous shaking (120 rpm). Each day of the culture period one flask was harvested for characterization.

For the determination of the cell fresh weight a defined volume of the suspension was harvested by vacuum filtration and weighed. Dry weight was measured in these samples after lyophilization. Cell-free media were used for the determination of pH and specific conductivity by the appropriate electrodes, osmolality by an osmometer (Gonotec, Berlin, Germany) and refractive index by an Abbe refractometer (Krüss, Hamburg, Germany). Phosphate concentration in the medium was determined colorimetrically according to Gomorri (1942). Nitrate was measured by HPLC over anion exchanger columns (BT I AN, BT S AG, 120 × 12 mm, Biotronik, Maintal, Germany) with elution by 3 mM Na_2CO_3, 3.2 mM $NaHCO_3$ at a flow rate of 1.8 ml min^{-1} and detected with a conductivity detector.

The concentrations of sucrose, glucose and fructose in the cell-free media were determined enzymatically with a test combination (Boehringer, Mannheim, Germany) according to the manufacturers protocol.

Rosmarinic acid was extracted from 20 mg aliquots of the freeze-dried cells, using 5 ml hot 70% ethanol and sonication for 20 min at 70°C. After vigorous mixing and centrifugation (10 min; 3,000 *g*), the supernatants were diluted 1:10 with 50% methanol acidified with 0.01% H_3PO_4 (85%) and analyzed by HPLC as described previously (Petersen & Alfermann 1988).

Extraction and determination of starch

Frozen *Coleus blumei* cells (1 g) were homogenized in 4 ml 0.5 M NaOH for 3 min with an Ultra-Turrax. After addition of a further 6 ml

0.5 M NaOH, the cells were incubated for 3 h at room temperature with occasional shaking. The extract was then heated at 100°C for 6 min to destroy monosaccharides, and acidified to pH 4.6 by the addition of 17 ml 0.5 M acetic acid. After centrifugation (10,000 *g*; 10 min) the starch concentration in the supernatant was determined by a test kit (Boehringer, Mannheim, Germany) according to the manufacturers protocol.

Results

Influence of the sucrose concentration on growth and RA production in cell cultures of Coleus blumei

Cell cultures of *Coleus blumei* inoculated into media with 1, 2, 3, 4, 5, or 6% sucrose were monitored over a culture period of 14 days. The growth of the cultures measured as fresh weight accumulation is slightly retarded in media with high sucrose concentrations, but this effect is not visible in the dry weight curves (Fig. 1A). This might indicate a negative osmotic effect of the high sugar concentrations on the cells. The maximum dry weight accumulated by the cell cultures is strongly and positively influenced by the sucrose concentration in the medium. This effect is not so prominent regarding fresh weight accumulation. We could not show any accumulation of starch in the cells which could have been the reason for the high dry weight accumulation in media with high sucrose concentrations. Dry weight accumulation starts from day zero on and runs linearly with about the same slope in all media. Dependent on the amount of sucrose added to the medium the cultures enter the stationary phase and decline phase.

The RA in the cells inoculated into the fresh medium is diluted by cell division and growth and the initial RA content therefore declines. Net RA accumulation then starts at day 4 to 5 of the culture period, independent of the initial sucrose concentration in the medium (Fig. 1B). The final amounts of RA synthesized, and the rate of synthesis, are related to the amount of sucrose added to the medium. No RA accumulation is seen in medium with 1% sucrose, while in CB6-medium, RA accumulation obviously has

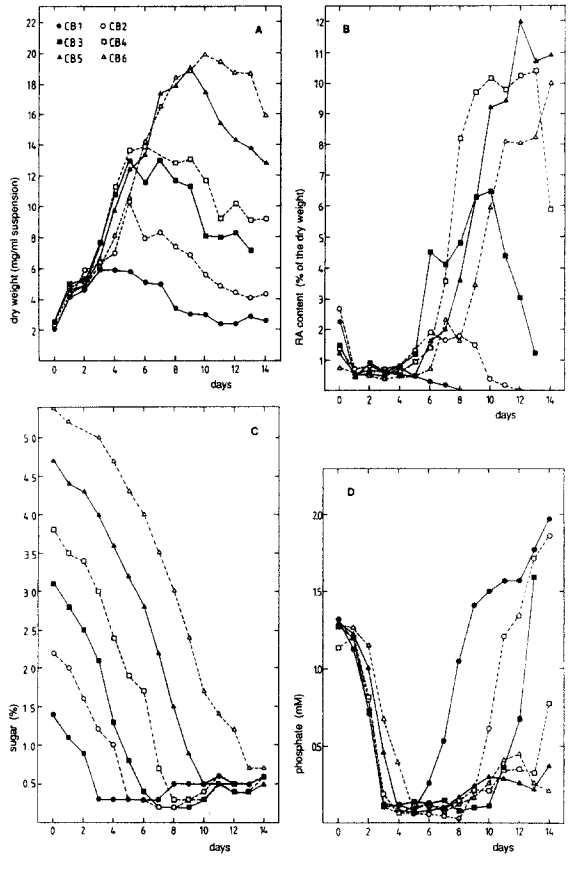

Fig. 1. Characterization of a cell culture of *Coleus blumei* in CB-media with 1% (●, CB1), 2% (○, CB2), 3% (■, CB3), 4% (□, CB4), 5% (▲, CB5) and 6% (△, CB6) sucrose. (*A*) dry weight (mg ml^{-1}); (*B*) rosmarinic acid (% of the dry weight); (*C*) sugar in the medium (%), measured as refractive index; (*D*) phosphate in the medium (mM).

not come to an end during the culture period of 14 days.

The sugar added as sucrose is consumed by the cells at about the same linear rate in media with different sucrose concentrations (Fig. 1C). This eventually leads to a total depletion of sugar from the medium, dependent on the initial amount added. Phosphate on the other hand is taken up by the cells at a rate that is independent of the initial sucrose concentration (Fig. 1D). The complete consumption of phosphate at days 4 to 5 coincides with the onset of net RA accumulation. Nitrate, on the other side, is never totally depleted from the CB-media (data not shown). The rise in medium levels of phosphate, nitrate, osmolality and conductivity towards the

end of the culture period reflects cell lysis and the concomitant release of cell contents into the medium.

In cultures grown in CB2- and CB4-media over 14 days the sucrose concentration rapidly declines whereas glucose and fructose appear in the media (Fig. 2B + C). This suggests that the sucrose is cleaved into glucose and fructose by the cultured cells of *Coleus blumei*. The relatively lower amount of glucose in comparison to fructose, may indicate preferential uptake and metabolism of glucose by the cells. By days 5 (CB2) and 8 (CB4) all three sugars are depleted from the media.

The maintenance of a 2% sugar concentration ($CB2S_N$) over the whole culture period finally resulted in a 60% higher dry weight of the cell cultures compared to CB4. Nevertheless, the RA content related to the dry weight was about the same as in CB4-medium (8.5%), although due to the higher dry weight, the RA content per flask was double that in CB4-medium. Other parameters like pH, phosphate and nitrate concentration and conductivity in the medium were similar to those in CB4-medium. However, unlike the CB4-pattern, they did not show the rise at the end of the culture period, indicating that the cells remained viable for a longer time. Despite the continuous feeding of sucrose in this experiment, only glucose and fructose could be found

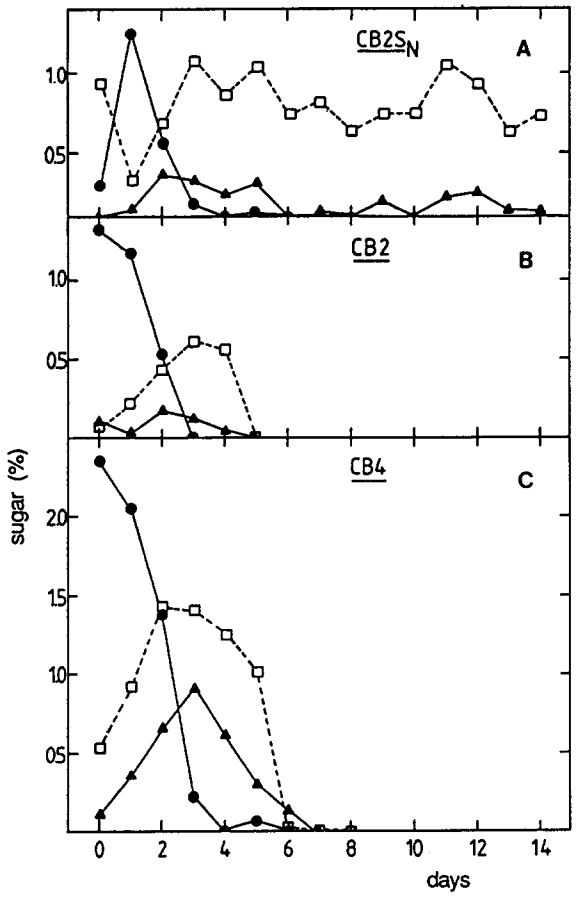

Fig. 2. Concentrations of sucrose (●), glucose (▲) and fructose (□) in the medium during a culture cycle of 14 days in CB2- (*B*) and CB4-medium (*C*), and in medium (*A*) in which a sugar concentration of 2% (measured as refractive index) was maintained by daily feeding of sucrose ($CB2S_N$).

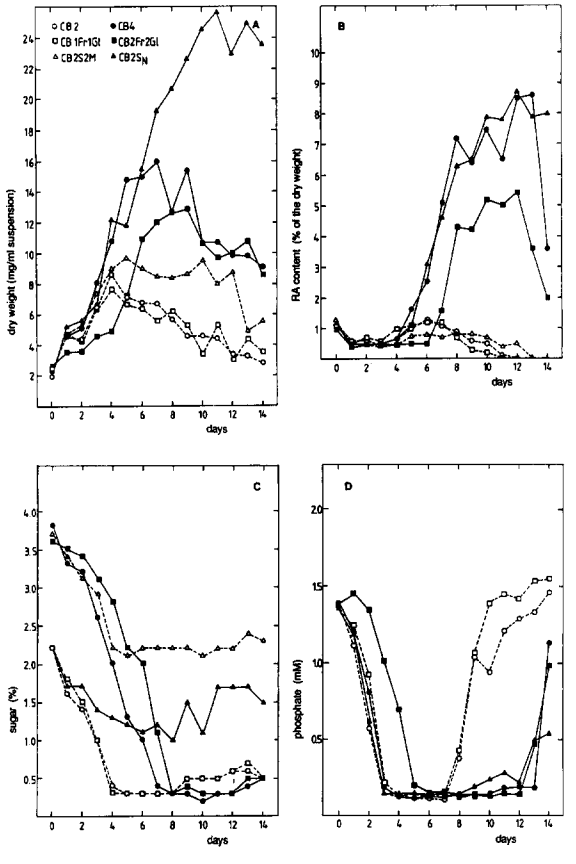

Fig. 3. Characterization of a suspension culture of *Coleus blumei* in CB-media with 2% sucrose (○, CB2), 4% sucrose (●, CB4), 1% fructose plus 1% glucose (□, CB1Fr1Gl), 2% fructose plus 2% glucose (■, CB2Fr2Gl), 2% sucrose plus 2% mannitol (△, CB2S2M) and a medium where the sugar concentration was held at 2% (measured as refractive index) by daily feeding of sucrose (▲, $CB2S_N$). (*A*) Dry weight ($mg\,ml^{-1}$); (*B*) rosmarinic acid (% of the dry weight); (*C*) sugar in the medium (%), measured as refractive index; (*D*) phosphate in the medium (mM).

in the medium (24 h after addition of sucrose) from day 4 on (Fig. 2A). The total sucrose consumption in the supplemented cultures was 3 g per flask compared to 1 g in CB4-cultures.

To examine the possible osmotic effect of a medium with 4% sugar, suspension cultures of *Coleus blumei* were inoculated into media containing 2% sucrose plus 2% mannitol (CB2S2M). 2% mannitol alone could not sustain growth and viability of the cell cultures (data not shown). Cultures in medium with 2% sucrose plus 2% mannitol behaved essentially as cultures in CB2-medium, but showed a reduced fresh and dry weight (Fig. 3A) and RA accumulation (Fig. 3B). At the end of the culture period, the values for nitrate, osmolality and conductivity in the medium showed a delayed increase compared to CB2 and CB4 indicating a slightly delayed cell death and lysis in CB2S2M medium. Phosphate concentrations in media containing mannitol could not be measured correctly by the method of Gomorri (1942). In fresh and previously inoculated media, the absorption values of the photometrical test remained low irrespective of the added phosphate.

Influence of glucose and/or fructose on growth and RA accumulation in suspension cultures of Coleus blumei

Cell cultures of *Coleus blumei* in CB-medium with 2% or 4% glucose or fructose show a strongly reduced growth measured as fresh or dry weight (Fig. 4A) compared to media with 2% or 4% sucrose. This reduction of growth is more prominent in medium with fructose than in medium with glucose. The reduced and delayed growth is accompanied by a slower uptake of nutrients, e.g. sugar (Fig. 4C) and phosphate (Fig. 4D) from the medium. RA accumulation in cells grown in media with 2% glucose or fructose is negligible as is the case in CB2-medium. Nearly the same time course of RA accumulation can be observed in media with 4% sucrose or glucose, whereas in medium with 4% fructose the synthesis of RA is delayed and reduced more than 50% (Fig. 4B). Due to the reduced growth, media with neither glucose nor fructose yield an equivalent RA-accumulation to the sucrose-containing CB-media.

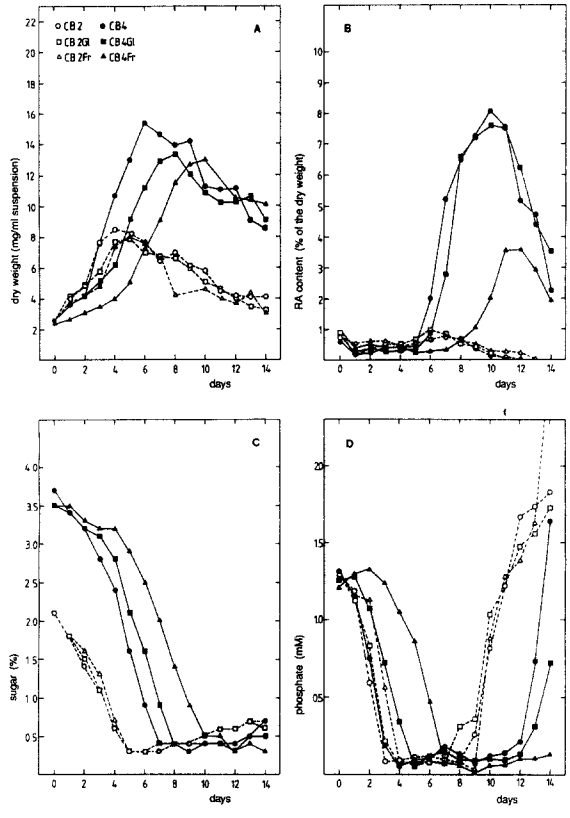

Fig. 4. Characterization of a *Coleus blumei* culture in CB-media with 2% sucrose (○, CB2), 4% sucrose (●, CB4), 2% glucose (□, CB2Gl), 4% glucose (■, CB4Gl), 2% fructose (△, CB2Fr) and 4% fructose (▲, CB4Fr). (*A*) Dry weight (mg ml^{-1}); (*B*) rosmarinic acid (% of the dry weight); (*C*) sugar in the medium (%), measured as refractive index; (*D*) phosphate in the medium (mM).

Since sucrose is cleaved into equimolar amounts of glucose and fructose by the cell cultures of *Coleus blumei*, we also tested media with 1% fructose plus 1% glucose (CB1Fr1Gl) or 2% fructose plus 2% glucose (CB2Fr2Gl) for their effects on growth and RA production of the cell cultures. Fresh and dry weight accumulation (Fig. 3A) are reduced in these media compared to media with 2% or 4% sucrose. Again, as in media with glucose or fructose alone, growth is not only reduced but also delayed. RA accumulation in medium with only 2% total sugar content is again very low. In medium with 2% fructose plus 2% glucose synthesis of RA is markedly reduced and reaches only about 55% of the amount in CB4-medium (Fig. 3B). Here again the uptake of carbohydrates and phosphate

(Fig. 3C, D) is slower than in CB-media with sucrose.

Comparing growth and RA accumulation of cell cultures of *Coleus blumei* as a function of the carbohydrate source added to the medium, sucrose has the optimal effect on both parameters. Glucose sustains equal RA accumulation on a cell dry weight basis, but supports only reduced growth of the cell cultures, while fructose is only able to sustain a modest level of growth and RA synthesis.

Discussion and conclusions

With an accumulation of up to 21% of the cell dry weight as rosmarinic acid, cell suspension cultures of *Coleus blumei* range among the highest producing plant cell cultures with respect to secondary product formation (Yokoyama & Yanagi 1991). Additionally, these cell cultures of *Coleus blumei* are interesting, since the secondary product formation can be strongly influenced by the sugar content of the medium (Zenk et al. 1977; Petersen & Alfermann 1988; Petersen 1991). This is not the case for all RA accumulating cell cultures. Heterotrophic cultures of *Anchusa officinalis* and another strain of *Coleus blumei* do not show such a dependence of RA synthesis on the medium sugar content (De Eknamkul & Ellis 1988). Highest accumulation of RA in these cultures is reached with 3% sucrose in the medium. Glucose, fructose and equimolar mixtures of these two sugars have the same effect on RA accumulation in heterotrophic *Anchusa officinalis* cultures as sucrose. On the other hand, in photomixotrophic suspension cultures of *Salvia officinalis* the sugar content of the culture medium has a strong promoting effect on the RA accumulation (Hippolyte et al. 1990, 1992).

In the experiments reported in this paper we investigated the effect of different sucrose concentrations as well as different sugars and sugar combinations on the production of RA in our cell cultures of *Coleus blumei*. These *Coleus* cultures are slightly different to those of Zenk et al. (1977) and Razzaque & Ellis (1977) with respect to the time course of growth and RA accumulation. Our strain synthesizes RA at the end of the growth phase whereas the other cultures have been reported to synthesize RA parallel to their growth. Increasing sucrose concentrations from 1 to 6% result in stimulated growth and RA accumulation. RA synthesis starts at day 4 to 5 of the culture period irrespective of the sucrose concentration, but very low sucrose levels (1%) can not sustain secondary product formation. The onset of RA synthesis coincides with the depletion of phosphate from the medium. However, at high phosphate concentrations in the medium, other nutrients may become growth-limiting as well. In this case, RA accumulation starts, but remains repressed (Gertlowski & Petersen, unpublished results). The impact of intra- and extracellular phosphate concentrations on the production of secondary compounds has been investigated in numerous systems. In most cases, phosphate inversely influences growth and secondary product formation. In *Nicotiana tabacum*, phosphate is completely taken up by the cells within 24 h and stored in the vacuole (Wray et al. 1983). While the cytoplasmic phosphate concentration remains constant, the phosphate in the vacuole is successively mobilized. High intracellular phosphate levels repressed the production of cinnamoyl putrescine in *Nicotiana tabacum* cells, whereas the maintenance of a continuously low level of intracellular phosphate promoted secondary product formation (Schiel et al. 1984). In comparison to these results, phosphate is taken up rather slowly by cells of *Coleus blumei*. This might indicate a lower degree of intracellular storage of phosphate and a real depletion of this nutrient as soon as the medium phosphate is exhausted.

The sucrose added to the culture medium is cleaved into glucose and fructose by cell cultures of *Coleus blumei*. Afterwards glucose is consumed faster than fructose. However, equimolar amounts of glucose and fructose, 1% or 2% of each, as well as 2% or 4% glucose or fructose separately do not have the same effect on the accumulation of RA as sucrose. The differences are more prominent in media with 4% total sugar concentration. Glucose alone sustains

about the same RA accumulation in the cells as sucrose, but dry weight accumulation is lower. A mixture of glucose and fructose results in lower growth and RA content and the lowest yield can be observed in medium with fructose alone. This is in contrast to results of Ulbrich et al. (1985) who also tested the effect of different sugars on growth and RA accumulation of suspension cultures of *Coleus blumei*. They reported higher RA yields in medium with fructose than with glucose. However, also in this experiment sucrose sustained the highest RA production.

The effect of high sucrose concentrations on the accumulation of RA is not an osmotic effect, since *Coleus* cultures in medium with 2% sucrose plus 2% mannitol behave similarly to cultures in CB2-medium. It is also not essential for the cultures to have a high sugar concentration at the beginning of the culture period. A sucrose level which is maintained at 2% results in a 60% higher dry weight accumulation and a RA production per flask which is double the amount of cultures in CB4-medium. The RA content related to the dry weight of the cells is at about 8.5%, which is comparable to CB4. However, the productivity of the supplemented cultures is lower (23 mg RA/g sucrose) than that in CB4-medium (30 mg RA/g sucrose).

In conclusion, our experiments show that RA accumulation in our cell culture strain of *Coleus blumei* is dependent on the amount of carbohydrates remaining in the culture medium when growth of the culture becomes limited by the depletion of other nutrients, e.g. phosphate. The more carbon is left in the medium the higher is the amount of RA produced. Of course this correlation is restricted by the inhibitory effect of the high osmotic pressure of media with high sugar concentrations. However, the kind of sugar added to the culture medium is important as well. The highest dry weight and RA accumulation can be observed with sucrose in the medium followed by glucose and fructose as carbon source. In normal CB-media, phosphate is depleted from the medium first, at day 4 to 5 of the culture period. Therefore, phosphate might be the nutrient becoming growth limiting and responsible for the onset of RA synthesis. The effect of macronutrients other than the carbon source, however, has still to be investigated in more detail.

Acknowledgement

The financial support of the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

References

De Eknamkul W & Ellis BE (1988) Rosmarinic acid: Production in plant cell cultures. In: Bajaj YPS (Ed) Biotechnology in Agriculture and Forestry. Medicinal and Aromatic Plants I, Vol 4 (pp 310–329). Springer Verlag, Berlin, Heidelberg

Gamborg OL, Miller RA & Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151–158

Gomorri G (1942) A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Lab. Clin. Med. 27: 955

Häusler E, Petersen M & Alfermann AW (1991) Hydroxyphenylpyruvate reductase from cell suspension cultures of *Coleus blumei* Benth. Z. Naturforsch 46c: 371–376

Hippolyte I, Marin B, Baccou JC & Jonard R (1990) Influence du milieu d'entretien et de la concentration en saccharose sur la production d'acide rosmarinique par des suspensions cellulaires de Sauge (*Salvia officinalis* L.). C.R. Acad. Sci. Paris, Série III, 313: 365–371

Hippolyte I, Marin B, Baccou JC & Jonard R (1992) Growth and rosmarinic acid production in cell suspension cultures of *Salvia officinalis* L. Plant Cell Rep. 11: 109–112

Petersen MS (1991) Characterization of rosmarinic acid synthase from cell cultures of *Coleus blumei*. Phytochemistry 30: 2877–2881

Petersen M & Alfermann AW (1988) Two new enzymes of rosmarinic acid biosynthesis from cell cultures of *Coleus blumei*: Hydroxyphenylpyruvate reductase and rosmarinic acid synthase. Z. Naturforsch. 43c: 501–504

Petersen M, Häusler E, Karwatzki B & Meinhard J (1993) Proposed biosynthetic pathway for rosmarinic acid in cell suspension cultures of *Coleus blumei*. Planta 189: 10–14

Razzaque A & Ellis BE (1977) Rosmarinic acid production in *Coleus* cell cultures. Planta 137: 287–291

Schiel O, Jarchow-Redecker K, Piehl GW, Lehmann J & Berlin J (1984) Increased formation of cinnamoyl putrescines by fedbatch fermentation of cell suspension cultures of *Nicotiana tabacum*. Plant Cell Rep. 3: 18–20

Ulbrich B, Wiesner W & Arens H (1985) Large-scale production of rosmarinic acid from plant cell cultures of *Coleus blumei* Benth. In: Neumann KH, Barz W & Reinhard E (Eds) Primary and Secondary Metabolism of Plant Cell Cultures (pp 293–303). Springer Verlag, Berlin, Heidelberg

Wray V, Schiel O & Berlin J (1983) High field phosphorous-31 nuclear magnetic resonance investigation of the phosphate metabolites in cell suspension cultures of *Nicotiana tabacum*. Z. Pflanzenphysiol. 112: 215–220

Yokoyama M & Yanagi M (1991) High-level production of arbutin by biotransformation. In: Komamine A, Misawa M & DiCosmo F (Eds) Plant Cell Culture in Japan (pp 79–91). CMC Co., Ltd., Tokyo

Zenk MH, El-Shagi H & Ulbrich B (1977) Production of rosmarinic acid by cell-suspension cultures of *Coleus blumei*. Naturwissenschaften 64: 585–586