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Growth and rosmarinic acid production in cell suspension cultures of *Salvia officinalis* L.

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Abstract

Rosmarinic acid (RA) is a natural antioxidant produced by cell suspension cultures of sage (*Salvia officinalis* L.). The growth and production of RA by these cells can be modified by the type of culture medium. Production can be increased 10-fold to attain 6.4 $g.l^{-1}$ under optimal conditions. Investigation of kinetics showed that a change in the medium caused shifts in peaks of growth and production, and modifications of the cell metabolism. RA production can be correlated with growth or begins only when growth has stopped.

Introduction

Rosmarinic acid (RA) is a natural antioxidant found especially in Labiatae and several Boraginaceae. The production of this metabolite has been the subject of numerous studies on cell suspensions of *Anchusa officinalis* L. (De-Eknamkul and Ellis 1985 a-b) and *Coleus blumei* Benth. (Zenk et al. 1977). Plants belonging to the genus *Salvia* contain RA, whose concentration varies according to the species (Adzet et al. 1988). However, to the best of our knowledge the only work performed on sage cell suspensions resulted in low RA production (Whitaker et al. 1984)(no more than 1.4% of the dry matter). We used a cell suspension initiated from medicinal sage and kept on an inorganic medium Murashige and Skoog (1962) (MS) supplemented with growth hormones and sucrose. The cells were transferred to media of different compositions for one cycle to enhance RA production. The effect of increasing the sucrose content was tested, as was the addition of different concentrations of phenylalanine to the medium. Heller's medium (1953) with 5% sucrose was also used. The growth and production kinetics were observed in each experiment.

Material and methods

Photomixotrophic suspensions of *Salvia officinalis* L. were kept on a Murashige and Skoog medium (1962) (MS) containing 3% sucrose, 0.5 $mg.l^{-1}$ 2,4-D, 0.5 $mg.l^{-1}$ kinetin and Nitsch et al. (1968) vitamins. The pH was adjusted to 5.3 before autoclaving.
The cultures were kept in Erlenmeyer flasks on a rotary agitator in continuous light (24 to 38 $\mu M.m^{-2}.s^{-1}$ at 23°C). Every 14 days, 30 ml of suspension was subcultured to 80 ml of new medium in 250 ml flasks. Aliquots were taken every 5 days, vacuum filtered, frozen in liquid nitrogen and freeze-dried for 2 days. The dry weight was then determined. Growth was expressed as the percentage of increase in mass in relation to the initial mass. 100% growth was determined by the suspension with the highest growth factor.

Three 20-minute extractions were performed using dry samples in 70% ethanol (V/V). The resulting extracts were evaporated under reflux to dryness and then resuspended in pure methanol. The samples were analysed by HPLC at 328 nm on a C-18 DB column (SUPELCO, USA). The flow rate was 1 ml/min. The eluent was a mixture of acetic acid, methanol and water. Acetic acid was used at 5% and the water-methanol gradient varied from 85:10 to 0:95 (by volume). Analysis time was 25 min.
Each figure is the average of 3 parallel flasks. Maintance medium figures are the average of separate flasks.

Results and discussion

The effect of increase in sucrose concentration

Sucrose is the preferential carbon source for the culture of cell suspensions. The sucrose concentration greatly influences the production of metabolites of the phenylpropanoid pathway (Ibrahim 1987). Growth in terms of increase in biomass (percentage of growth) and production of RA in two media containing 3 and 5% sucrose were followed. RA was intracellular in our cultures (Hippolyte 1990).
The results presented in Figure 1 show that the increase in dry matter in the two tests is not significantly different. The sampling frequency does not make it possible to know whether the modification of the peaks observed in the curve is real or whether maximum growth occurred between day 15 and day 20. However, production of RA was strongly modified. 3.5 $g.l^{-1}$ RA was obtained in the 5% sucrose medium; this corresponds to 19% dry matter (results not shown). The RA concentration was thus increased by 27-fold in relation to the initial concentration. It increased only by 6.5 fold on the standard medium containing 3% sucrose.

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Another important change was the mode of production. Indeed, production and growth began on the 3% sucrose medium at the start of the culture (or after a latency period of less than 5 days), whereas the culture had two stages on the 5% medium: production began when growth had stopped. After 15 days, RA concentration was 0.43 $g.l^{-1}$ on the 3 % sucrose medium against 0.23 $g.l^{-1}$ on the 5 % sucrose medium. Mean values were different ($P < 0.05$). Modification of the culture medium of *Anchusa officinalis* cell suspensions led to changes in growth kinetics but these were limited to the linear phase of growth (De-Eknamkul and Ellis 1984).

It would seem from our results that the increased sucrose concentration affected the secondary metabolism whereas the primary metabolism seems to be hardly affected. In the literature, varied optimal sucrose concentrations are used for the production of rosmarinic acid. Indeed, the optimal concentrations for *Coleus blumei* vary between 5% (Ulbrich et al. 1985) and 7% (Zenk et *al.* 1977). The best production in cell suspensions of *Anchusa officinalis* was observed with 3% sucrose (De-Eknamkul and Ellis 1985 a).

Effect of the addition of precursors

Phenylalanine is one of the biosynthetic precursors of RA (Ellis and Towers 1970). Different concentrations (0.1, 0.25, 0.5 and 0.75 $g.l^{-1}$) were added to the basal medium. It can be seen in Figure 2 that the dry matter increased with the amino acid concentration. Maximum growth is obtained after 10 days against 20 days in the control.

The RA production time was also shortened (Figure 3) to only 5 days on the medium containing 0.75 $g.l^{-1}$ of the precursor. Maximum values were hardly affected by 0.1 and 0.25 $g.l^{-1}$ of phenylalanine (0.68 and 0.69 $g.l^{-1}$ respectively). The figures are 1.09 and 1.18 $g.l^{-1}$ for the 0.5 and 0.75 $g.l^{-1}$ concentration respectively. The addition of phenylalanine thus improves growth and productivity in the standard medium. Nevertheless, the amount of RA obtained with the most favourable amino acid concentration was smaller than that observed on the standard medium with an increased sucrose content. Therefore phenylalanine was added at the same concentrations as in the sucrose-enriched medium.

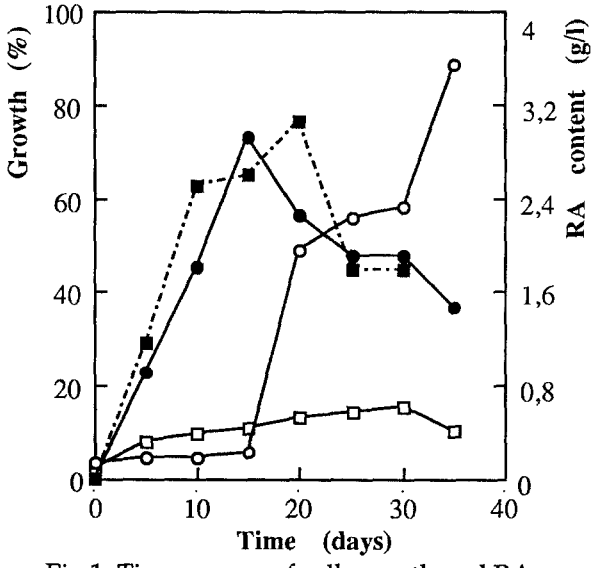

Fig.1 Time-course of cell growth and RA production of *Salvia officinalis* cell cultures on basal MS media containing 3% sucrose: ----■---- Growth; —□— RA content and 5% sucrose: —●— Growth; —○— RA content

Figure 4 shows that the dry matter content was hardly affected by these new conditions. However, RA production is strongly enhanced in the medium containing 0.1 $g.l^{-1}$ of phenylalanine (Fig. 5), reaching 6.4 $g.l^{-1}$, representing 31% of the dry matter (result not shown). This effect was not observed with higher concentrations of the precursor.

The results of the addition of phenylalanine to the standard medium (3 % sucrose) agree with those of Margna (1977), i.e. that the avaibility of the precursors limits the production of secondary metabolites. Indeed, increased concentrations of phenylalanine are followed by increased RA production (Figure 3). The primary metabolism is also active, since the maximum growth is greater, and attained earlier, with an increase in precursor concentration.

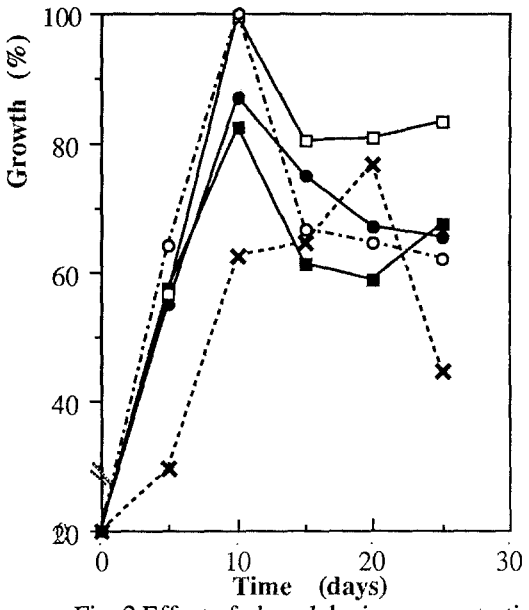

Fig. 2 Effect of phenylalanine concentration (g/l) on cell growth of *Salvia officinalis* cell culture on basal MS medium containing 3% sucrose: ----×---- Control —■— 0.1 —●— 0.25 —□— 0.5 ----○---- 0.75

The results are different for the sucrose-enriched media (Figure 5). Production only increased with a concentration of 0.1 $g.l^{-1}$ of phenylalanine, and reached ten times that of the standard culture conditions. An increase of the same magnitude was observed by Merillon et al. (1986) after the addition of secologanine to cell suspensions of *Catharanthus roseus* for the production of ajmalicine.

A synergistic effect appears between phenylalanine and sucrose with 0.1 $g.l^{-1}$ of the former . The phenomenon does not occur at higher concentrations of phenylalanine and the precursor has hardly any effect in comparison with sucrose. We cannot explain these findings but they show that the addition of factors which enhance RA production have a considerable effect on yield, although the optimal concentrations differ from those required when each factor is used alone. Similar results were reported by De-Eknamkul and Ellis (1985 a), who found that combining the optimal concentrations of inorganic substances did not result in maximum yield. It is also reported in the literature that the effects of phenylalanine differ according to the medium used. When Razzaque and Ellis (1977) added this precursor to the standard culture medium, the production of RA decreased by 35% in *Coleus blumei* cell suspensions. However, when it was added to *Coleus blumei* cultures in the medium with limited sucrose, RA production increased by 100% (Zenk et al. 1977).

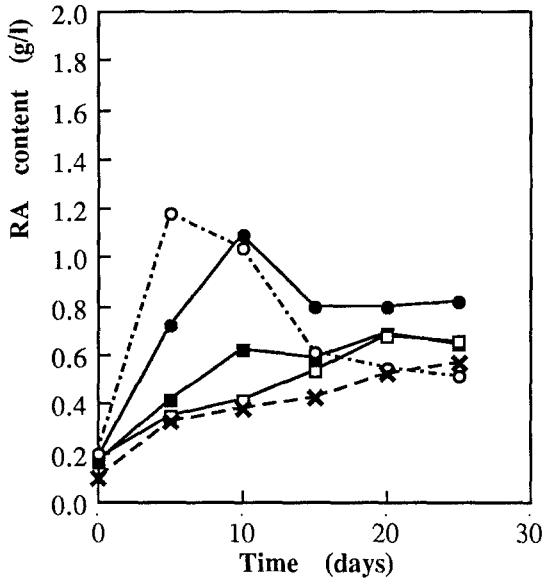

Fig.3 Effect of phenylalanine concentration (g/l) on RA production of *Salvia officinalis* cell cultures on basal MS medium containing 3 % sucrose : Control; 0.1; 0.25; 0.5; 0.75.

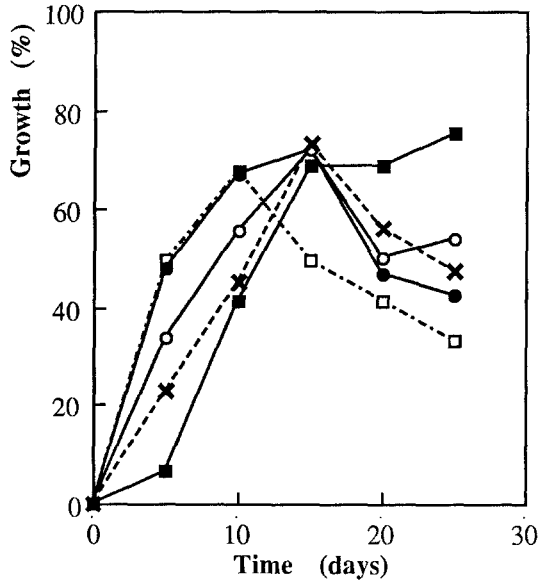

Fig.4 Effect of phenylalanine concentration (g/l) on cell growth of *Salvia officinalis* cell cultures on basal MS medium containing 5 % sucrose : Control; 0.1; 0.25; 0.5; 0.75.

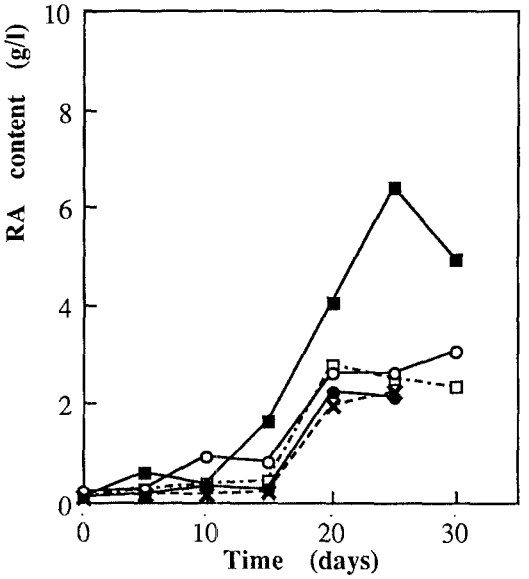

Fig.5 Effect of phenylalanine concentration (g/l) on RA production of *Salvia officinalis* cell cultures on basal MS medium containing 5 % sucrose : Control; 0.1; 0.25; 0.5; 0.75.

Effect of a medium with low osmolarity

RA production in *Coleus blumei* suspensions increased strongly in a medium with low osmolarity (Ulbrich et al. 1985). The osmolarity of Heller's medium enriched with 5 % sucrose is 185 mOsmol. We subcultured cells on to this medium supplemented with the same amount of hormone and vitamins as the maintenance medium which is 181 mOsmol . RA production was considerably high in this medium, reaching 6.4 $g.l^{-1}$ and 36% of the dry matter (results not shown). Production started at the beginning of the culture.

MS medium enriched with 5 % sucrose is hypertonic (238 mOsmol). One could think that transferring cells to such a medium creates an osmotic chock.

If osmotic pressure regulation is considered from an energy point of view at the cellular level, it is much more "expensive" with MS medium than on Heller's medium.

The metabolic modifications - especially with regards to energy - in the strongly hypertonic MS medium may disturb the enzymatic functioning of the secondary metabolism, but the very different mineral composition of these two media must have an influence too.

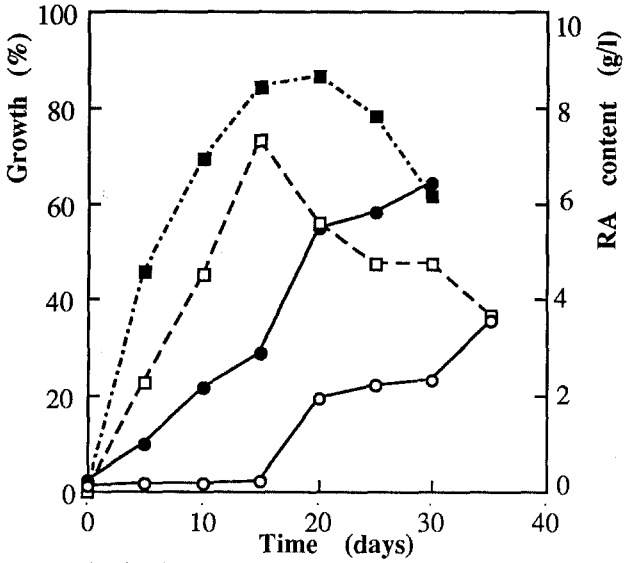

Fig.6 Time-course of cell growth and RA production of *Salvia officinalis* cell cultures on Heller's (HE) and MS media containing 5% sucrose, 0.5 mg/l 2,4-d and 0.5 mg/l kinetin :

Heller Growth — Heller RA
MS Growth — MS RA

Conclusion

Modification of the culture medium for one cycle resulted in an increase in growth of about 30% and, above all, productivity. In the most favourable cases (MS medium containing 5% sucrose, 0.1 $g.l^{-1}$ of phenylalanine and the hormones of the standard medium or Heller's medium with 5 % sucrose) productivity attained was 6.4 $g.l^{-1}$.

In addition to the extremely interesting quantitative results, the monitoring of growth and productivity kinetics revealed considerable modification in cell functioning. Firstly, the production cycle is considerably shortened when phenylalanine is added to the standard medium; it can be reduced to 5 days with a 0.75 $g.l^{-1}$ concentration of precursor.

Secondly, production in some media increases from the first sampling onwards. This is the case of the standard MS medium and Heller's medium containing 5% sucrose. In contrast, production starts after the halting of growth in MS medium containing 5% sucrose. The osmotic pressure of the medium may be involved in this phenomena. The first two media display similar osmolarity (181 and 185 mOsmol respectively). The second medium was considerably more concentrated (238 mOsmol). The importance of osmotic pressure for RA production has also been noted in cell suspensions of *Anchusa officinalis* L (Su and Humphrey 1990).
However, if osmotic pressure could influence the production pattern of the cultures, RA maximal concentrations are dependent on the media constituents.

These are the first results to show such important quantitative and qualitative changes in RA production. To the best of our knowledge, the largest RA production figures reported are those of Ulbrich et al. (1985) on cell cultures of *Coleus blumei* Benth., with 5.6 $g.l^{-1}$ (21.4% of the dry matter).

The same cell strain was used in all the experiments reported here, then the modifications in production patterns (production correlated with growth or consecutive to growth) were the result of the composition of the culture medium.
The optimisation tests described concern the improvment of the culture medium, but it would be beneficial to investigate the influence of other parameters, and in particular the gaseous atmosphere, light, aeration and pH of the culture medium.

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