# Flavonoid accumulation in *Scutellaria baicalensis* Georgii *in vitro* cultures upon treatment with sodium cinnamate

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SUMMARY

**Flavonoid accumulation in *Scutellaria baicalensis* Georgii *in vitro* cultures upon treatment with sodium cinnamate**

The production of secondary metabolites by plant cell cultures has been suggested as a feasible technology that attracted considerable industrial and academic interest in the past three decades. However, many secondary pathways are not well expressed in plant cell cultures. Optimization of culture medium, elicitation, genetic engineering and the supply of biosynthetic precursors are among the strategies adopted to increase secondary metabolite production *in vitro*. In this study, the effects of five potential biosynthetic precursors on the production of baicalin and baicalein in *Scutellaria baicalensis* Georgii suspension and callus cultures were measured. The results of this work show that flavonoid biosynthesis was most stimulated by the addition of sodium cinnamate in the concentration of 5 $mg.l^{-1}$ and by cinnamic acid (1 $mg.l^{-1}$). Feeding with L-phenylalanine, malonic acid and sodium malonate did not induce changes in the amounts of flavonoids, and the biomass production was not affected.
**Key words:** *Scutellaria* – skullcap – precursor – sodium cinnamate – cinnamic acid – *in vitro* cultures

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SOUHRN

**Nárůst obsahu flavonoidů v *in vitro* kulturách *Scutellaria baicalensis* Georgii po přidání skořičnanu sodného**

Za jednu z možností, jak získávat sekundární metabolity rostlin, je považována produkce pomocí *in vitro* kultur. V těchto kulturách však bývá biosyntéza těchto látek často nedostatečná, nebo je potlačena úplně. Ke zvýšení produkce sekundárních metabolitů se používá řada metod, jako jsou například optimalizace kultivačního média, elicitace, genetická manipulace, a také přidávání prekurzorů do média. V této studii byl zkoumán vliv pěti prekurzorů na množství baicalinu a baicaleinu v suspenzních a kalusových kulturách šišáku bajkalského (*Scutellaria baicalensis* Georgii). Výsledky ukazují, že zejména skořican sodný v koncentraci 5 $mg.l^{-1}$ a kyselina skořicová (1 $mg.l^{-1}$) významně zvyšují obsah obou flavonoidů. Přídavek L-fenylalaninu, kyseliny malonové a malonanu sodného nemělo vliv na množství flavonoidů ani na růst kultur.
**Klíčová slova:** *Scutellaria* – šišák – prekurzor – skořican sodný – kyselina skořicová – *in vitro* kultury *Má*

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### *Introduction*

The Skullcap (*Scutellaria baicalensis* Georgii) is a perennial plant from East Asia and its dried roots are a very old and well known drug in traditional Chinese medicine. The skullcap contains a variety of flavonoids which are receiving scientific attention as modifiers of inflammatory processes, as well as for antihypertensive, antiviral, antibacterial and antitumor properties [1]. The most commonly studied flavonoids in *Scutellaria baicalensis* Georgii are baicalin, its aglycon baicalein, wogonin and wogonoside [2].

*In vitro* cultures are promising potential alternative sources for the production of high-value secondary metabolites of industrial importance. The advantages of this technology over the conventional agricultural production are as follows:

- It is independent of geographical and seasonal variations and various environmental factors.
- It offers a defined production system, which ensures the continuous supply of products, uniform quality and yield.
- It is possible to produce novel compounds that are not normally found in the parent plant.
- It is independent of political interference.
- Efficient downstream recovery and product.
- Rapidity of production.
- In addition, plant cell can perform stereo- and regiospecific biotransformations for the production of novel compounds from cheap precursors [3].

In this context, the production of bioactive substances by plant tissue and organ cultures is an important and promising aspect of modern biotechnology.

Unfortunately, many secondary pathways are not well expressed in plant cell cultures. Optimization of culture medium, elicitation, genetic engineering, as well as the supply of biosynthetic precursors are among the strategies adopted to increase flavonoid production *in vitro*.

In this study, the authors report the effects of five potential biosynthetic precursors on the production of baicalin and baicalein in *Scutellaria baicalensis* Georgii suspension and callus cultures: L-phenylalanine, cinnamic acid and its sodium salt, malonic acid and its sodium salt.

## EXPERIMENTAL

### *Material and methods*

#### *Materials*

Precursors and chemicals for cultivation and analyses were purchased from Lachema (Brno, Czech Rep.) and Sigma (St. Luis, USA), baicalein and baicalin standards from Sigma-Aldrich Chemie (Steinheim, Germany), and chromatographic solvents of HPLC quality from Fluka (Buchs, Germany) and Merck (Darmstadt, Germany).

#### *Plant cell cultures*

Surfaced sterilized seeds of *Scutellaria baicalensis* Georgii were germinated on Murashige and Skoog agar medium (MS) [4], supplemented by coconut milk. One week old seedlings were used for inoculation to MS medium with 0.01 $g.l^{-1}$ α-naphtaleneacetic acid (callus cultures). Cultures were subcultured at a four-week interval on the same medium in Erlenmeyer flasks. Suspension cultures were derived from calluses and subcultured at a two-week interval on a rotary shaker (110 rpm).

Callus and suspension cultures were cultivated in a culture chamber at 25 °C and white light of the intensity of 3500 lux in a 16-hour photoperiod was used.

#### *Feeding of precursors and sample adjustment*

To improve baicalin and baicalein production in plant cell cultures, some known precursors of flavonoid biosynthesis (L-phenylalanine, cinnamic acid, sodium cinnamate, malonic acid and sodium malonate) were added [5].

Always one of the potential precursors was fed into two-week old suspension cultures as well as to four-week old callus cultures in the concentrations of 1 $mg.l^{-1}$, 5 $mg.l^{-1}$ and 10 $mg.l^{1}$ achieved in medium. In the control cultures, the volume of sterile distilled water equal to the volume of solvent for precursor was added aseptically to the culture medium.

After 20 and 40 hours since the precursor was added, cultures were harvested, dried, powdered, and extracted with 80 % aqueous methanol solution (2 × 10 ml) for 30 min (at the temperature of 80 °C). Chlorophyll and lipids were eliminated by liquid–liquid extraction with petroleum ether. Samples were analyzed by HPLC after filtration.

Bulked aqueous culture medium was reduced in volume to 5 ml, brought to a final volume of 20 ml with methanol, and then filtered and analyzed by HPLC.

For dry weight determination, *S. baicalensis* suspension cultures were filtered and dried in an oven at 60 °C for 24 h and their weight (g) was recorded.

#### *Quantitative analysis of baicalein and baicalin*

HPLC analyses were carried out with a Unicam Crystal System (Unicam Analytical Systems, Cambridge, UK), fitted with a LiChrospher RP-18 250 × 4 (5 μm) chromatographic column (Merck, Darmstadt, Germany) and spectrometric detection at 277 nm. Elution of compounds was achieved at a flow rate of 1.2 $ml.min^{-1}$ using the gradient from 50 % aqueous methanol to 75 % aq. methanol in 15 minutes. The mobile phase included 0.15 % phosphoric acid as the buffer. Peak areas were integrated, compared with standards and concentrations of individual compounds were calculated with a Unicam Crystal 4880 Data Station.

## RESULTS AND DISCUSSION

The concept of precursor feeding is based upon the idea that any compound which is an intermediate, in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product.

In this study, five potential precursors were used to enhance the baicalin and baicalein yields: L-phenylalanine, cinnamic acid and its sodium salt, malonic acid and its sodium salt. An aqueous solution of each of these precursors was added to grown plant cell cultures and incubated for 20 and 40 hours. The amounts of the precursors added to the cultures are mentioned in the Material and Methods section.

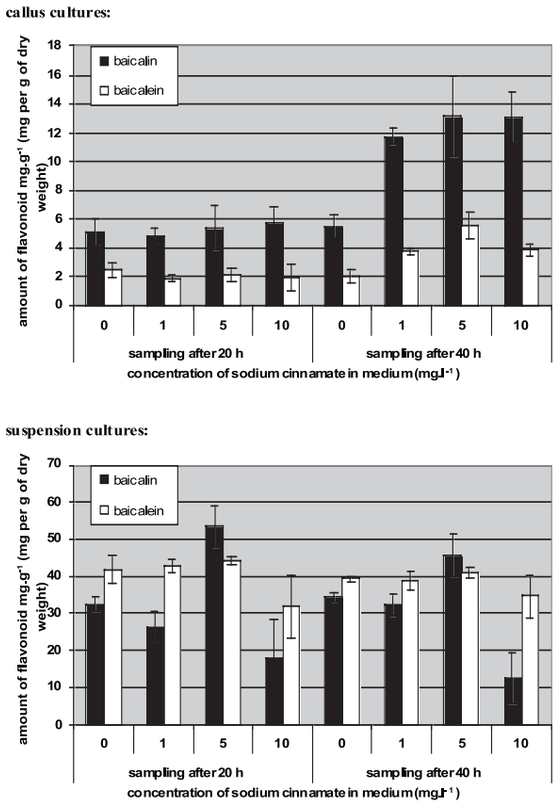


*Fig. 1. The effect of sodium cinnamate on in vitro cultures after 20 and 40 hours*

The effects of sodium cinnamate (Fig. 1) were the most significant and different according to the type of cultures concerned. In the case of callus cultures, the production of baicalin and baicalein after 40 hours was increased more than twofold in comparison with the control. This positive effect was found in all concentrations of the precursor. There was no remarkable enhancement in baicalin or baicalein amounts after 20 hours.

In contrast to callus cultures, higher quantities of flavonoids were already detected after 20 hours in suspension cultures. Also a toxic effect of sodium cinnamate in

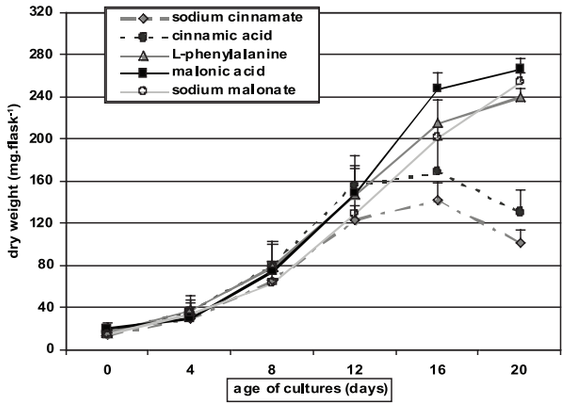


*Fig. 2. The cell growth of suspension cultures (precursors were added after 14 days of cultivation, in a concentration of 10 mg.l-1)*

*Tab. 1. The amount of flavonoids in culture medium - control suspension cultures and suspension cultures after sodium cinnamate (10 mg.l-1) treatment*

| Culture | time | baicalin (mg.l-1) | baicalein (mg.l-1) |
|---|---|---|---|
| control | 20 h | <0.01* | <0.01* |
| | 40 h | <0.01* | <0.01* |
| with sodium cinnamate | 20 h | 1.23 | 0.14 |
| | 40 h | 1.74 | 0.16 |

*the amount of flavonoid was under the detection limit (0.01 mg.l-1)

the concentration 10 mg.l-1 was manifested by increasing the number of dead cells and by elution of baicalein and baicalin to the culture medium, where these compounds are not normally present (Tab. 1). Consequently, the inhibiting effect of feeding with this precursor on cell growth was determined (Fig. 2). Therefore the results indicate that the optimal concentration of sodium cinnamate for the maximum flavonoid content and cell growth is 5 mg.l-1.

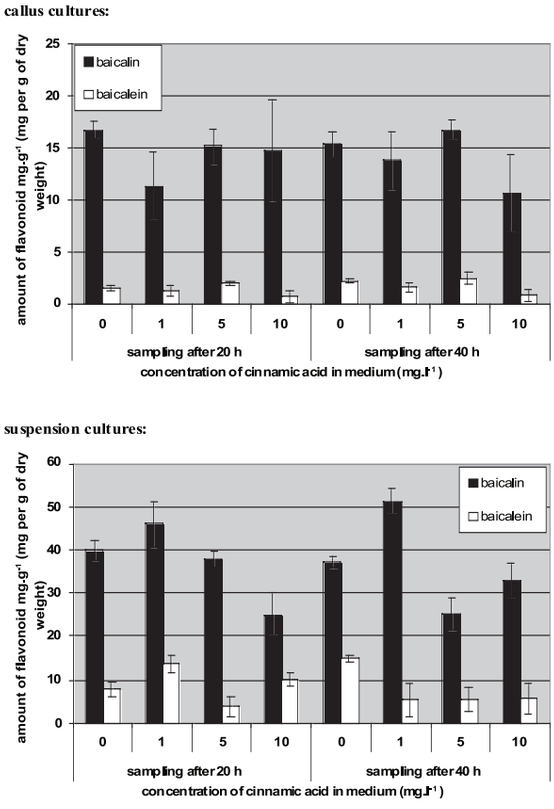


*Fig. 3. The effect of cinnamic acid on in vitro cultures after 20 and 40 hours.*

Figure 3 shows the effect of cinnamic acid on the production of baicalin and baicalein in skullcap callus and suspension cultures. The increment in flavonoid production was less marked after the treatment with cinnamic

acid than with sodium cinnamate and the enhancement was observed only when the concentration of cinnamic acid was 1 $mg.l^{-1}$. In the concentration of 10 $mg.l^{-1}$, the toxic effect of cinnamic acid was found as shown in Figure 2.

Feeding of malonic acid, sodium malonate and L-phenylalanine did not induce changes in the amounts of flavonoids, and the biomass production was not affected.

It was consistently observed that flavonoid biosynthesis fluctuated from subculture to subculture and even from flask to flask in the same subculture. This type of variability of plant cell cultures has been well-documented on cultures of others species as well [6].

Although precursor feeding is an obvious and popular approach to increase secondary metabolite production in plant cell cultures, there are no reports concerning an enhancement of baicalin and baicalein yield in *Scutellaria baicalensis* cultures by treatment of biogenic precursors.

Attempts to induce or increase the production of plant secondary metabolites by supplying a precursor or intermediate compounds were effective in many cases. For example, the addition of phenylalanine as a precursor resulted in an improvement in rosmarinic acid yield in *Coleus blumei* cell cultures [7]. Addition of phenylalanine to *Salvia officinalis* suspension cultures stimulated the production of rosmarinic acid and decreased the production time as well [8]. Phenylalanine is also the precursor of the N-benzoylphenylisoserine side chain of taxol, and supplementation of *Taxus cuspidata* cultures with phenylalanine resulted in increased yields of taxol [9]. $^{13}C$-labelled cinnamic acid was incorporated into phenylphenalenones in root cultures of *Anigozanthos preissii* [10].

Results of this study extend the list of successful precursor feedings to flavonoid production in *S. baicalensis* and show a potential enhancement strategy for further productivity increases.

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