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EFFECT OF NUTRITIONAL FACTORS ON CRYPTOTANSHINONE AND FERRUGINOL PRODUCTION BY CELL SUSPENSION CULTURES OF *SALVIA MILTIORRHIZA*

HITOSHI MIYASAKA, MASAO NASU, TOSHIHIKO YAMAMOTO, YUKIKO SHIOMI, HIROYUKI OHNO, YUKIKO ENDO and KAISUKE YONEDA

Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565, Japan

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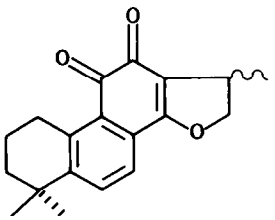
Key Word Index—*Salvia miltiorrhiza*; Labiatae; plant cell culture; nutritional factors; two-stage culture method; diterpene; cryptotanshinone; ferruginol.

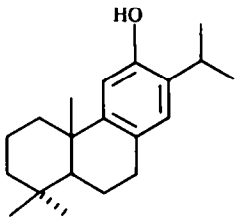
Abstract—The effects of the components of Murashige–Skoog (MS) medium on the production of the diterpenes cryptotanshinone and ferruginol in cell suspension cultures of *Salvia miltiorrhiza* were examined. Sucrose, a nitrogen source and thiamine were needed for the production of these compounds, and phosphate, $MnSO_4$ and kinetin showed slight beneficial effects. All the other components of MS medium were found to be either unnecessary or inhibitory for the production of these compounds, when tested separately. A revised medium for the production of cryptotanshinone, a clinically active principle in this plant, was designed.

INTRODUCTION

The root of *Salvia miltiorrhiza* B. is an ancient Chinese drug and recently has been used as a remedy for angina pectoris [1]. Previously we reported [2] a two-stage culture method, with a growth medium and a production medium, for the production of cryptotanshinone (**1**) and ferruginol (**2**). Since the nutritional components of culture media have been demonstrated to affect secondary metabolite production [3–5], we examined, in this study, the nutrient requirement for the production of **1** and **2** to improve the culture conditions favouring the production of **1**, a clinically active principle in this plant.



(**1**) Cryptotanshinone



(**2**) Ferruginol

RESULTS AND DISCUSSION

Examination of nutrient requirement for the production of cryptotanshinone (**1**) *and ferruginol* (**2**)

A cryptotanshinone (**1**)-producing cell line (line A5) [2] was subcultured in the liquid growth medium [2] (the stage for cell growth), MS medium with 2,4-D (2,4-dichlorophenoxyacetic acid) (0.1 ppm) and kinetin (0.1 ppm), at 20 day intervals. Cells from these stock suspension cultures in the early stationary phase of growth were inoculated to the production medium [2] (the stage for production), which was MS medium without Fe-EDTA but with kinetin (1 ppm), in all experiments. As described previously [2], cell growth was greatly suppressed in the production medium and growth index (final dry wt/initial dry wt) (GI) in this medium was 1–2 whereas GI in the growth medium was 7–9. The production of **1** and **2** (sum in the cells and medium) was expressed only as mg/l culture in all figures and tables, since cell growth in the production medium had little influence on their production. **1** and **2** produced were released from the cells into the liquid medium, but the ratio of product in cells to that in medium was not constant. To examine the effects of individual components of MS medium on the production of **1** and **2**, we cultured cells for 18 days in the production medium with omission of each component in turn.

The productivity of **1** of cell line A5 was not stable, decreasing gradually during successive subculture; therefore, we selected a cell line from cell line A5 by cell aggregate selection and named it line A501. Probably due to the use of large cell aggregates for selection, the productivity of **1** of the selected cell line (line A501) was also unstable, so we continued selection to maintain the productivity of **1**. Cell line A502 was derived from cell line

A501 and by repeated selection cell lines A503–505 were obtained and also used.

Among the macronutrients (nitrogen source, KH_2PO_4, $CaCl_2$ and $MgSO_4$), only the deficiency of the nitrogen source (NH_4NO_3 and KNO_3) was found to reduce the production of **1** and **2**. The effects of the nitrogen source on the production of these compounds were therefore examined in detail. Figure 1 shows the effects of the initial concentration of nitrogen source on the production of **1** and **2**. The initial concentration of the nitrogen source in the production medium was varied from 0 to 180 mM while the ratio of KNO_3 to NH_4NO_3 was kept equal to that in the MS medium. The production of **1** and **2** was much reduced in the nitrogen-free medium and considerably restored by adding only 6 mM of nitrogen source (one-tenth concentration of standard MS medium), indicating that active protein synthesis is required in the production medium. Table 1 shows the effect of the addition of cycloheximide, an inhibitor of protein synthesis, on the production of **1** and **2**. The total protein content increased rapidly after initiation of the culture, reaching about two times the initial level on day 3 (data not shown), and so cycloheximide (5 μg/ml) was added to the culture on day 0 or day 3. The production of **1** and **2** was much inhibited (more than 98% of **1** and 83% of **2**) by addition of cycloheximide on day 0, but only 44% of **1** and 20% of **2** were inhibited by addition of cycloheximide on day 3, when considerable amounts of protein had already been synthesized. These results indicated that active protein synthesis in the production medium was needed for the production of **1** and **2**, and the considerable decrease in production in the nitrogen-free medium may be due to the suppression of protein synthesis. The optimum concentration of nitrogen for the production of **1** was about 60 mM (the same concentration as in MS medium), whereas concentrations of between 6 and 60 mM resulted in a similar level of **2** (Fig. 1).

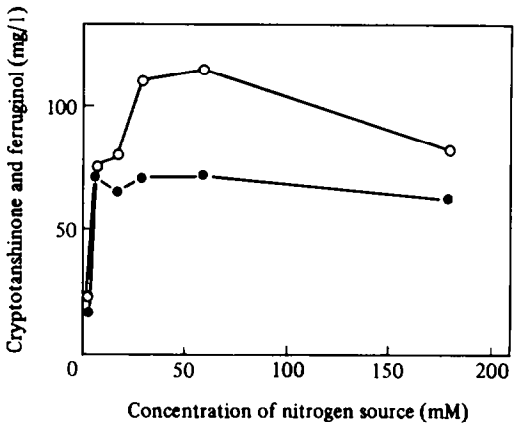


Fig. 1. Effect of the concentration of the nitrogen source on production of cryptotanshinone (○) and ferruginol (●) in cell suspension cultures of *S. miltiorrhiza* (line A502). The initial concentration of the nitrogen source in the production medium was varied from 0 to 180 mM while the ratio of KNO_3 to NH_4NO_3 was kept equal to that in MS medium. The concentration of the nitrogen source in MS medium was 60 mM (18.8 mM KNO_3 and 20.6 mM NH_4NO_3). Determinations were made 18 days after transfer of the cells to the production medium.

Table 1. Effect of cycloheximide on the production of cryptotanshinone and ferruginol in cell suspension cultures of *S. miltiorrhiza* (line A503)

Time of addition (day)	Diterpene production (% of control value*)	
	Cryptotanshinone	Ferruginol
0	Trace (< 2)	17
3	56	80

Cycloheximide (5 μg/ml) was added on day 0 or day 3. Determinations of diterpene production were carried out 18 days after transfer of the cells to the production medium (medium without Fe-EDTA but with kinetin, 1 ppm).

*Control value means the diterpene production in the culture without the addition of cycloheximide.

The standard MS medium contains 18.8 mM KNO_3 and 20.6 mM NH_4NO_3 as nitrogen source (total nitrogen: 60 mM) and the molar ratio of NO_3^- to NH_4^+ is about 2:1. To examine the influence of the form of nitrogen on the production of **1** and **2**, the cells were inoculated to the modified production medium containing KNO_3 and NH_4Cl at various molar ratios at a total nitrogen concentration of 60 mM (Table 2). The production of **1** and **2** was much reduced only when NH_4Cl was added as the sole nitrogen source. The production of **1** was considerably reduced also in the medium containing KNO_3 as the sole nitrogen source, and the appropriate ratio of KNO_3 to NH_4Cl was 1:3–3:1. The production of **2** was almost the same except in the medium with NH_4Cl as the sole nitrogen source.

The effects of KH_2PO_4, $CaCl_2$ and $MgSO_4$ on the production of **1** and **2** were examined by varying the concentration of these nutrients separately (Fig. 2a–c). The optimum concentration of KH_2PO_4 for the production of **1** and **2** was one-tenth of that in standard MS medium (Fig. 2a), while the production of these compounds were highest when $CaCl_2$ or $MgSO_4$ was completely omitted from the medium (Fig. 2b and 2c).

The effects of micronutrients (H_3BO_3, $MnSO_4$, $ZnSO_4$, KI, Na_2MoO_4, $CoCl_2$ and $CuSO_4$) on the production of **1** and **2** were examined by omitting each in turn from the production medium (Table 3). The production of **1** and **2** was slightly reduced by omission of $MnSO_4$ and was increased by omissions of the other micronutrients.

Table 2. Effects of ammonium/nitrate ratio on the production of cryptotanshinone and ferruginol in cultured cells (line A502) of *S. miltiorrhiza*

KNO_3 (mM)	NH_4Cl (mM)	Diterpene production (mg/l)	
		Cryptotanshinone	Ferruginol
Control*		121	77
0	60	32	15
15	45	120	61
30	30	109	66
45	15	103	61
60	0	47	70

Determinations were carried out 18 days after transfer of the cells to the modified production medium.

*18.8 mM KNO_3 + 20.6 mM NH_4NO_3.

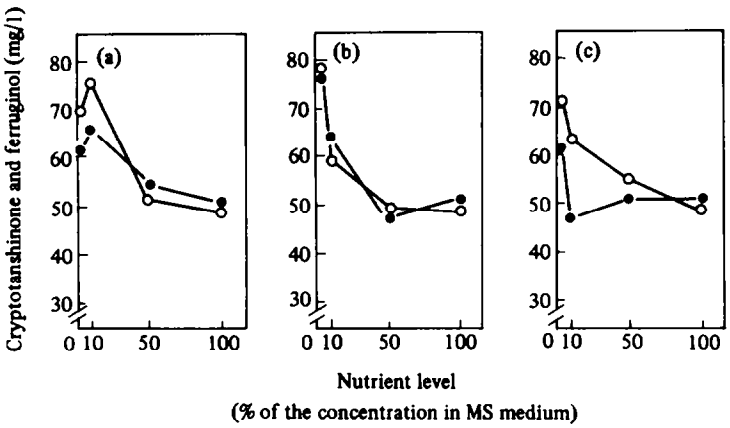


Fig. 2. Effects of the concentrations of KH_2PO_4 (a), $MgSO_4$ (b) and $CaCl_2$ (c) on the production of cryptotanshinone (○) and ferruginol (●) in cell suspension cultures of *S. miltiorrhiza* (line A503). The concentrations of KH_2PO_4, $MgSO_4$ and $CaCl_2$ in MS medium were 1.25 mM, 1.50 mM and 2.99 mM, respectively. Determinations were made 18 days after transfer of the cells to the production medium.

Table 3. Effect of omitting various micronutrients from the medium on the production of cryptotanshinone and ferruginol in cultured cells (line A5) of *S. miltiorrhiza*

Omitted micronutrients	Diterpene production (mg/l)	
	Cryptotanshinone	Ferruginol
None (control)	22	35
H_3BO_3	32	52
$MnSO_4$	21	29
$ZnSO_4$	31	47
KI	28	43
Na_2MoO_4	23	41
$CoCl_2$	32	42
$CuSO_4$	30	45

Determinations were carried out 18 days after transfer of the cells to the indicated production medium.

The effects of organic compounds (thiamine·HCl, nicotinic acid, pyridoxine·HCl, inositol and glycine) on the production of **1** and **2** were also examined by omitting each in turn (Table 4). When the organic compounds, except thiamine, were separately omitted from the production medium, the production of **1** and **2** was increased.

Table 4. Effect of omitting various organic compounds from the medium on the production of cryptotanshinone and ferruginol in cultured cells (line A5) of *S. miltiorrhiza*

Omitted organic compound	Diterpene production (mg/l)	
	Cryptotanshinone	Ferruginol
None (control)	24	30
Thiamine·HCl	3.4	6.6
Nicotinic acid	38	44
Pyridoxine·HCl	32	34
Inositol	38	44
Glycine	34	42

Determinations were carried out 18 days after transfer of the cells to the indicated production medium.

However the production of **1** and **2** was much reduced by omission of thiamine, indicating that thiamine exerts a considerable influence on their production. The production of **1** and **2** increased with an increase of thiamine concentration and 1–5 mg/l of thiamine·HCl was the optimal concentration for the production of these compounds (Table 5).

A simplified production medium for the production of cryptotanshinone (**1**)

On the basis of these results, we devised a simplified medium for production of **1** by using concentrations of the nitrogen source, KH_2PO_4 and thiamine·HCl that had been determined individually to be optimal and by omitting the unnecessary nutrients. We also examined the effects of additions of the omitted nutrients to the simplified production medium. We found that only the additions of $CaCl_2$ at one-tenth the concentration in MS medium, inositol and glycine had slight beneficial effects on the production of **1** (data not shown). Therefore we modified the final simplified production medium (SP-1

Table 5. Effect of thiamine·HCl concentration in the medium on the production of cryptotanshinone and ferruginol in cultured cells (line A5) of *S. miltiorrhiza*

Thiamine·HCl concentration (mg/l)	Diterpene production (mg/l)	
	Cryptotanshinone	Ferruginol
0	6.7	8.7
0.01	14	15
0.05	25	27
0.1 (control*)	47	50
0.5	55	86
1.0	67	90
5.0	66	105

*Control means standard concentration of thiamine·HCl in MS medium.

Determinations were carried out 18 days after transfer of the cells to the production medium.

medium) as shown in Table 6. For production of **1**, therefore, only sucrose [6], nitrogen source and thiamine were essential, and kinetin [2], KH_2PO_4, $CaCl_2$, $MnSO_4$, inositol and glycine had slight beneficial effects. Table 7 shows the production of **1** and **2** in the original production medium, MS medium without Fe-EDTA but with kinetin (1 ppm), and SP-1 medium. The concentration of thiamine·HCl in both media was 1 mg/l, the optimum concentration for production of **1**. In the SP-1 medium, the production of **1** and **2** was 126 and 77% of those in the original production medium, respectively. Thus this much limited production medium was found to be favourable for production of **1**. The cell growth was much suppressed in both media and the GI in SP-1 medium and original production medium were 1.54 ± 0.43 and 1.91 ± 0.55, respectively.

The reason for fewer nutrient requirements of *S. miltiorrhiza* cells for the production of secondary metabolites is probably that the cells do not produce primary metabolites, since cell growth is significantly suppressed. The stimulative effects of the omissions or limitations of many nutrients on production of **1** and **2** are probably due to the suppression of some metabolic pathways, causing a greater carbohydrate supply for the biosyntheses of **1** and **2**. The omissions or limitations of some nutrients have also been reported to have stimulative effects on production of secondary metabolites by plant cell cultures [3, 7–9]. Lindsey suggested [8] that both cell division and the syntheses of primary metabolites are limited by limitation of the supply of nutrients and that this results in increases in the availability of precursors (e.g. amino acids and carbohydrates) for secondary metabolic activity. The use of a simplified production medium, which contained only 8% sucrose, was reported for the production of alkaloids and polyphenols in cell suspension cultures of *Catharanthus roseus* by Knobloch and Berlin [3]. A production medium containing only 4% sucrose was also favourable for the biotransformation of L-tyrosine to L-DOPA by immobilized cultured cells of *Mucuna pruriens* [9]. The results presented here further indicate that the formation of secondary metabolites can be stimulated by reducing the number of nutrients.

Table 6. Composition of the simplified (SP-1) production medium

NH_4NO_3	1650 mg/l
KNO_3	1900 mg/l
$CaCl_2 \cdot 2H_2O$	44 mg/l
KH_2PO_4	17 mg/l
$MnSO_4 \cdot 4H_2O$	22.3 mg/l
Inositol	100 mg/l
Thiamine·HCl	1 mg/l
Glycine	2 mg/l
Sucrose	3%
Kinetin	1 ppm
pH	5.7

Table 7. A comparison of diterpene production by *S. miltiorrhiza* cells (line A505) in original production medium and SP-1 medium

	Diterpene production (mg/l)	
	Cryptotanshinone	Ferruginol
Original production medium	87.4 ± 1.44	69.3 ± 1.77
SP-1 medium	110 ± 4.86	53.2 ± 2.72

Determinations were carried out 18 days after transfer of the cells to the indicated medium. Values are means ± s.e. for three cultures. The concentration of thiamine·HCl in both media was 1 mg/l, the optimum concentration for the production of cryptotanshinone. The productions of cryptotanshinone and ferruginol in SP-1 medium were higher ($P = 0.05$–0.01) and lower ($P < 0.01$) than those in original production medium, respectively.

EXPERIMENTAL

Plant material and method of culture. Cultures of cell line A5 selected as described in the previous paper [2] and cultures of cell lines A501–505 obtained by repeated selection were used for experiments. All stock suspension cultures were grown on a rotary shaker (100 rev/min) at 25° in the dark in 500 ml flasks containing 150 ml of growth medium, MS medium with 2,4-D (0.1 ppm) and kinetin (0.1 ppm). For experiments, cells from 20-day-old stock suspension culture were collected on nylon cloth (20 mesh), washed 3 × with sterile H_2O and inoculated to 100 ml flasks containing 20 ml of production medium. The size of inoculum in the production medium was 1.5–2.5 g/l.

Selection method. Cells from 20-day-old stock suspension culture were collected on a nylon cloth (20 mesh), washed with sterile H_2O and inoculated to the production medium, MS medium without Fe-EDTA but with kinetin (1 ppm). After culture for 6 days, about 10 redder aggregates were selected, transferred together to the growth medium and subcultured as a selected cell line.

Quantitative determinations of cryptotanshinone (**1**) *and ferruginol* (**2**). The contents of **1** and **2** in cells and culture medium were determined as described elsewhere [2].

REFERENCES

1. Chen, Z.-X. (1981) *Kagaku No Ryoiki* **35**, 494.
2. Miyasaka, H., Nasu, M., Yamamoto, T., Endo, Y. and Yoneda, K. (1986) *Phytochemistry* **25**, 637.
3. Knobloch, K.-H. and Berlin, J. (1980) *Z. Naturforsch.* **35**, 551.
4. Fujita, Y., Hara, Y., Suga, C. and Morimoto, T. (1981) *Plant Cell Rep.* **1**, 61.
5. De Eknamkul, W. and Ellis, B. E. (1985) *Plant Cell Rep.* **4**, 46.
6. Miyasaka, H., Nasu, M., Yamamoto, T. and Yoneda, K. (1985) *Phytochemistry* **24**, 1931.
7. Chandler, S. F. and Dodds, J. H. (1983) *Plant Cell Rep.* **2**, 205.
8. Lindsey, K. (1985) *Planta* **165**, 126.
9. Wichers, H. J., Malingré, T. M. and Huizing, H. J. (1983) *Planta* **158**, 482.