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REGULATION OF FERRUGINOL AND CRYPTOTANSHINONE BIOSYNTHESIS IN CELL SUSPENSION CULTURES OF *SALVIA MILTIORRHIZA*

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

Abstract—Ferruginol was produced only in the lag phase in habituated cell suspension cultures of *Salvia miltiorrhiza*, while it was produced in both lag and stationary phases by 2,4-dichlorophenoxyacetic acid-requiring cultures. On addition of sucrose to habituated cultures, in the stationary phase, production of ferruginol was restored, indicating that habituated cultures still retained the capacity to produce ferruginol. A two-stage culture method, with normal medium and then medium without Fe-EDTA, for ferruginol production was established. This culture method was also found to be suitable for production of cryptotanshinone, a clinically active principle in this plant.

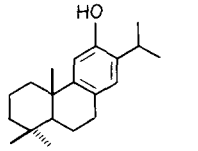
INTRODUCTION

Previously we reported [1] that, of six established cell lines (A–F) of *Salvia miltiorrhiza* B., only one (line A) produced large amounts of both cryptotanshinone (**2**) and ferruginol (**1**), while the others (B–F) produced only **1** in appreciable amount. However, the production of **2** by cell line A was not stable and decreased gradually in successive subcultures. To obtain basic information on secondary metabolism in *S. miltiorrhiza* cells in culture, we examined the time-course of production of **1** and the effects of auxins and light on **1** production and on growth [2]. In this study, we investigated **1** production in habituated cultures. Furthermore, we developed a two-stage culture method for the more efficient production of **1**.

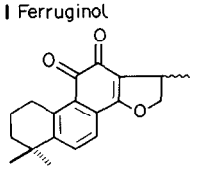
RESULTS AND DISCUSSION

Growth of, and ferruginol (**1**) *production in, habituated cell cultures*

We reported previously [2], that cell cultures of *S. miltiorrhiza* require 2,4-D for growth and that the production of **1** is induced in the lag and stationary phases of suspension cultures, when cell division is not active. However, further subculture of these suspension cultures, designated as line B, resulted in loss of their requirement for 2,4-D. Figure 1 shows the growth and ferruginol production of the suspension cultures subcultured for 20 and 33 months after initiation of the callus. The cells required 2,4-D for growth after subculture for 20 months (Fig. 1a); these cells are called normal cells in this paper. On the other hand, after subculture for 33 months, growth of the cells was the same with (0.1 ppm) or without 2,4-D (Fig. 1b). Ferruginol (**1**) was produced only in 2,4-D-free medium by both normal and habituated cells (Fig. 1a and b) and, as described previously [2], **1** was produced in lag (0–4 days) and stationary (day 7) phases by normal cells (Fig. 1a). In the habituated culture (Fig. 1b), the content of **1** increased in the lag phase (0–4 days) and decreased in the logarithmic phase (4–12 days). However, in the stationary phase (day 12), in which normal cells produced **1** actively, only a slight increase was observed (12–15 days) and production of **1** ceased completely by day 15. The reason for the absence of ferruginol production in the stationary phase of habituated culture was most likely due to depletion of some nutrients needed for its production in this phase, since habituated cells grow for a longer period of time in 2,4-D-free medium than normal cells do.



1 Ferruginol



2 Cryptotanshinone

To examine the influence of nutrient deficiency on production of **1**, we grew cells for 5 days in medium without major nutrients (NH_4NO_3, KNO_3, $CaCl_2$, $MgSO_4$ and KH_2PO_4), minor nutrients (H_3BO_3, $MnSO_4$, $ZnSO_4$, KI, Na_2MoO_4, $CuSO_4$ and $CoCl_2$), Fe-EDTA, organic compounds or sucrose, and compared the contents of **1** of these cells with those of control cells grown in complete medium (Table 1). The production of **1** was suppressed completely only by omission of sucrose, indicating that sucrose in the medium probably exerts a strong influence on production of **1**. Figure 2 shows changes in the dry weight of cells, the content of **1** and the

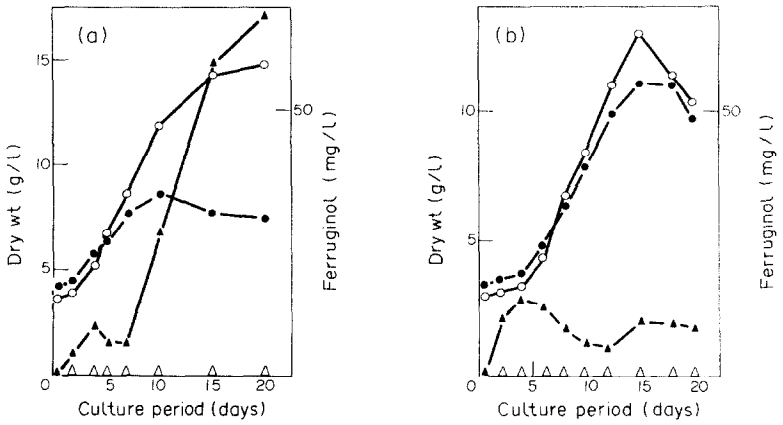


Fig. 1. Effect of 2,4-D concentration on changes in dry weight (●, ○) and ferruginol content (▲, △) of *S. miltiorrhiza* suspension cultures (cell line B) after subculture for 20 months (a) or 33 months (b). The cells were inoculated into the medium with kinetin (0.1 ppm). 2,4-D: ● and ▲, 0 ppm; ○ and △, 0.1 ppm.

Table 1. Effect of omitting various nutrients from MS medium on the ferruginol content of cultured cells of *S. miltiorrhiza*

Omitted component(s)	Dry wt (g/l)	Ferruginol content (mg/l)
None (control)	16.6	19
Macronutrients	13.5	4
Micronutrients	16.1	11
Fe-EDTA	13.2	19
Organic compounds	14.4	18
Sucrose	9.0	trace (1>)

Determinations were carried out 5 days after transfer of the cells to the indicated medium. Initial dry weight of cells was 11.1 g/l.

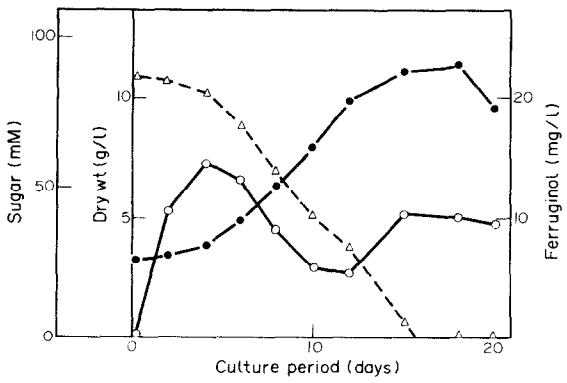


Fig. 2. Changes in dry weight (●), ferruginol content (○) and sugar (sucrose and glucose) concentration (△) of habituated *S. miltiorrhiza* suspension cultures (cell line B) in 2,4-D-free medium with kinetin (0.1 ppm).

sugar concentration (sucrose and glucose) in habituated suspension cultures in 2,4-D-free medium with kinetin (0.1 ppm). After initiation of the culture, sucrose in the medium was rapidly hydrolysed and only glucose was detected on day 2. The medium was almost completely depleted of glucose by day 15, when production of **1** ceased. These results indicated that the absence of ferruginol production in the stationary phase of habituated cultures is probably due to depletion of the medium of sugar.

On the basis of these results, we examined the effect of adding sucrose to habituated cultures in the early stationary phase (Fig. 3). When we added 3% sucrose to habituated cultures on day 12, when 65% of the initial

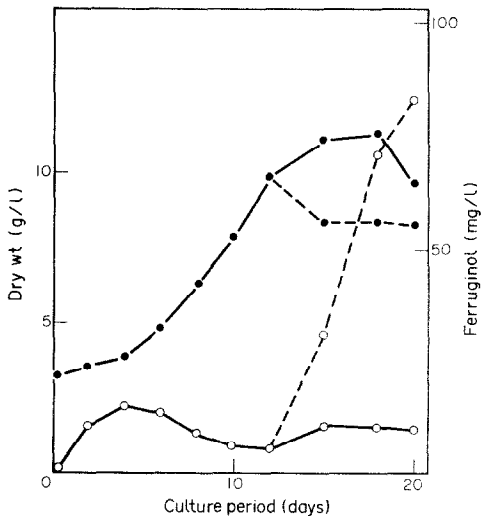


Fig. 3. Changes in dry weight (●) and ferruginol content (○) of habituated *S. miltiorrhiza* suspension cultures (cell line B) in 2,4-D-free medium with kinetin (0.1 ppm). 3% sucrose was added to half of the cultures on day 12 when 65% of the initial sucrose had been consumed (broken lines); the remaining cultures received no further supplement (continuous lines).

sucrose had been consumed, the content of **1** increased rapidly, reaching about 8.7 times the control level on day 20. After addition of sucrose, the dry weight of the cells decreased slightly (12–15 days). The reason for this decrease is unknown, but is probably caused by the sudden onset of ferruginol production. Furuya *et al.* reported that the capacity of *Panax ginseng* for saponin production is significantly lower in the habituated callus than in the auxin-requiring callus [3]. This is in contrast to our findings on *S. miltiorrhiza.*

Establishment of a two-stage culture method

As production of **1** is induced while cell division is not active, it seemed possible that cells would produce **1** continuously if their growth was suppressed by using limited medium. We examined this possibility by testing the effects of omission of various nutrients from the medium on cell growth and production of **1** in cell line B. We found that on omission of Fe-EDTA, cell growth was almost completely suppressed and continuous production of **1** occurred. Figure 4 shows the growth and ferruginol content of habituated cells (cell line B) grown in media with and without Fe-EDTA. In normal medium, cells grew well and production of **1** was active only in the lag phase, whereas in medium without Fe-EDTA cell growth was almost completely suppressed and **1** was produced continuously throughout the culture period. Thus a two-stage culture method was established; namely, cells were firstly grown in normal medium with 2,4-D (0.1 ppm) and kinetin (0.1 ppm), and then in the medium without Fe-EDTA but with kinetin (0.1 ppm) and no auxin for production of **1**. By this method, we eliminated the need to add sucrose to habituated cultures in the early stationary phase to restore production of **1**. Medium without Fe but with EDTA gave the same result as that without Fe-EDTA, so suppression of growth was due to limitation of Fe.

Next we examined the application of this two-stage culture method to production of the red pigment, cryptotanshinone (**2**). Previously we established a cell line (line A) producing **2** [1], but during subsequent subculture, its productivity decreased gradually. So we selected a cell line producing **2** from cell line A by cell aggregate selection and named it line A5. Cells of line A5 were subcultured in liquid medium with 2,4-D (0.1 ppm) and kinetin (0.1 ppm) at 20 day intervals (the stage for cell growth). Figure 5 shows the growth and production of **1** and **2** in suspension culture by line A5 in medium without Fe-EDTA but with 1 ppm of kinetin (the stage for production). A preliminary test showed that 1 ppm of kinetin was an appropriate concentration for production of **2**. Unlike cell line B, cell line A5 released secondary metabolites into the liquid medium and so we also determined the contents of **1** and **2** in the medium. Under these conditions cell growth was almost completely suppressed in this cell line and continuous production of **1** and **2** were observed. The amounts produced (**1**: 730 mg/l; 460 mg/l in the cells and 270 mg/l in the medium. **2**: 190 mg/l; 70 mg/l in the cells and 120 mg/l in the medium) on day 16 were 13.7% and 3.6% of the dry weight of cells respectively and were much higher than those (1.1% and 0.7%, respectively) found in the dried roots of the intact plant. Hence, this two-stage culture method was also suitable for production of **2**. Use of a two-stage culture method has been reported to be effective for production of some secondary metabolites [4–6]. Zenk designed an 'alkaloid production medium' which consisted of modified basal medium with desirable plant hormones and precursors for alkaloid biosynthesis [4]. The production of shikonin derivatives in suspension culture of *Lithospermum erythrorhizon* was also reported to be much increased by a two-stage culture method [5], and medium without phosphate was found to be effective for production of some alkaloids and phenolics [6]. The present medium for production of **1** and **2** by suspension cultures of *S. miltiorrhiza* is unique in that Fe-EDTA was omitted for production of secondary metabolites. Since cell growth was significantly suppressed in the production medium, this culture method may be applicable for the production of **1** and **2** by immobilized cells. Studies on immobilized cultured cells of *S. miltiorrhiza* will be reported in separate papers.

EXPERIMENTAL

Plant material and method of culture. Cultures of two cell lines (lines A and B) derived from seedlings of *S. miltiorrhiza* as described in the previous paper [1] were used. All callus cultures were grown at 25° in the dark on solid Murashige–Skoog (MS) medium [7] containing 2,4-D (1 ppm), kinetin (0.1 ppm) and agar (0.6%), and subcultures were made once a month. Stock suspension cultures, initiated by transferring callus tissues to 150 ml of liquid medium in 500 ml flasks, were maintained on a rotary shaker (100 rev/min) at 25° in the dark by regular subculture of 15% inocula once a month. After subculture for 27 months, the concn of 2,4-D in both solid and liquid media was

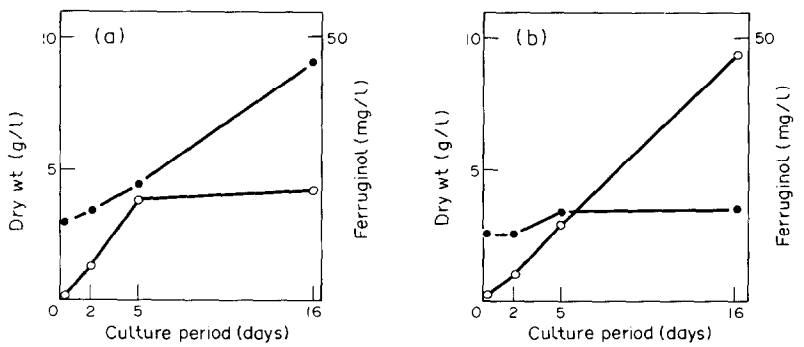


Fig. 4. Changes in dry weight (●) and ferruginol content (○) of habituated *S. miltiorrhiza* cells (cell line B) in suspension culture in medium with (a) or without (b) Fe-EDTA. Kinetin (0.1 ppm) was added to each medium.

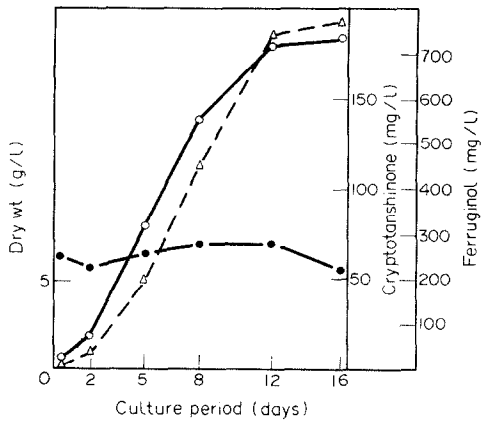


Fig. 5. Changes in dry weight (●) and ferruginol (○) and cryptotanshinone (△) contents of *S. miltiorrhiza* cells (cell line A5) in suspension culture in medium without Fe-EDTA and auxin but with kinetin (1 ppm).

reduced to 0.1 ppm. The dry weight of cells was measured as described previously [2].

Selection method. A callus of cell line A in agar medium with 2,4-D (0.1 ppm) and kinetin (0.1 ppm) was inoculated into 2,4-D-free medium with kinetin (0.1 ppm). After culture for 1 month, some of the redder parts of the callus were transferred to agar medium with 2,4-D (0.1 ppm) and kinetin (0.1 ppm) and subcultured.

Quantitative determinations of ferruginol (**1**), *cryptotanshinone* (**2**) *and sugars.* The contents of **1** and **2** in cells were determined as described elsewhere [1]. The culture medium was extracted with EtOAc and the contents of **1** and **2** were determined by GC as described previously [1]. Sucrose and glucose in the medium was measured with a food 1555 analysis kit (Boehringer–Mannheim GmbH).

REFERENCES

1. Nakanishi, T., Miyasaka, H., Nasu, M., Hashimoto, H. and Yoneda, K. (1983) *Phytochemistry* **22**, 721.
2. Miyasaka, H., Nasu, M., Yamamoto, T. and Yoneda, K. (1985) *Phytochemistry* **24**, 1931.
3. Furuya, T., Yoshikawa, T., Ishii, T. and Kajii, K. (1983) *Planta Med.* **47**, 183.
4. Zenk, M. H., El-Shagi, H., Arens, H., Stöckigt, J., Weiler, E. W. and Deus, B. (1977) in *Plant Tissue Culture and Its Biotechnological Application* (Barz, W., Reinhard, E. and Zenk, M. H., eds) p. 27. Springer, Berlin.
5. Fujita, Y., Tabata, M., Nishi, A. and Yamada, Y. (1982) in *Plant Tissue Culture* 1982 (Fujiwara, A., ed.) p. 399. Maruzen, Tokyo.
6. Sasse, F., Heckenberg, U. and Berlin, J. (1982) *Plant Physiol.* **69**, 400.
7. Murashige, T. and Skoog, F. (1962) *Physiol. Plant.* **15**, 473.