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PRODUCTION OF FERRUGINOL BY CELL SUSPENSION CULTURES OF *SALVIA MILTIORRHIZA*

HITOSHI MIYASAKA, MASAO NASU, TOSHIHIKO YAMAMOTO and KAISUKE YONEDA

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

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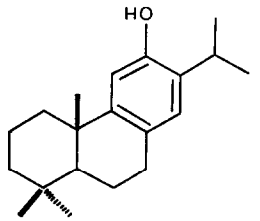
Key Word Index—*Salvia miltiorrhiza*; Labiatae; plant cell culture; auxin; diterpene; ferruginol.

Abstract—The time-course production of ferruginol, a diterpene, was examined during growth of *Salvia miltiorrhiza* in cell culture. Ferruginol was produced in the lag and stationary phases of growth and so production of this secondary metabolite was inversely related to active cell division. The effects of auxins and light on ferruginol production were also examined.

INTRODUCTION

There have been few studies of diterpenes as secondary metabolites in plant tissue cultures, despite considerable investigations on other mevalonates [1]. Previously we reported [2] that undifferentiated cultured cells of *Salvia miltiorrhiza* produced a diterpene, ferruginol (**1**), in larger quantity than the intact plant did and we also obtained a cell line that produced a large amount of another diterpene, cryptotanshinone. These diterpene are found only in the roots of intact plants [3]. In this study we examined the time-course of production of ferruginol and the effects of auxins and light on cell growth and ferruginol production.



1 Ferruginol

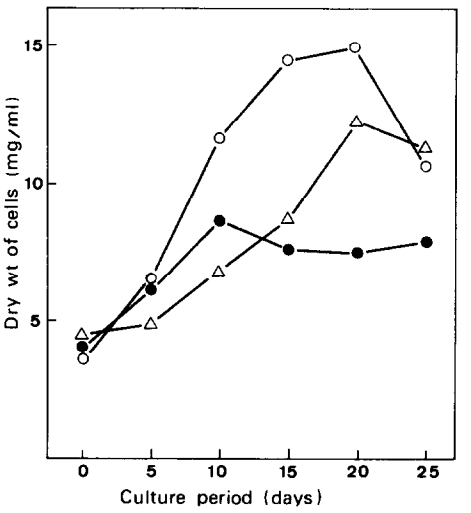


Fig. 1. Effect of 2,4-D concentration on growth of *S. miltiorrhiza* cells in suspension culture in medium containing kinetin (0.1 ppm). 2,4-D: (●) 0 ppm; (○) 0.1 ppm; (△) 1 ppm. Values are means from two experiments.

RESULTS AND DISCUSSION

As described previously [2], of six established cell lines of *Salvia miltiorrhiza*, only one produced large amounts of both cryptotanshinone and ferruginol. The cell line used in this work produced only ferruginol as a predominant secondary metabolite and red pigments, tanshinones, only in trace amounts. Suspension culture was initiated from an 11-month-old callus and cells were subcultured for 5–10 months. All experiments were made on suspension cultures.

The effects of 2,4-dichlorophenoxyacetic acid (2,4-D) on cell growth are shown in Fig. 1. In medium with 2,4-D (0.1 or 1 ppm) dry weight of cells increased till about day 20, whereas in the medium without 2,4-D (but with 0.1 ppm kinetin), it increased till about day 10 and was much less. Moreover cells in the stationary phase in medium without 2,4-D did not grow further when transferred to fresh medium without 2,4-D. These results indicate that the cells require 2,4-D for growth in culture; the slight growth in the first passage in auxin-free medium may have been supported by 2,4-D carried in from the stock culture.

Table 1 shows the relation between the 2,4-D concentration and the ferruginol content of cells harvested 5 and 20 days after inoculation. Production of ferruginol was observed only in 2,4-D-free medium and 2,4-D at low concentration of 0.1 ppm inhibited its production. This result is in contrast with Kutney's finding [1] that the production of triptolide and tripdiolide, which are abietane-type diterpenes like ferruginol, was not inhibited by 2,4-D in the tissue cultures of *Tripterygium wilfordii*.

Table 1. Effect of 2,4-D on ferruginol production in 5- and 20-day-old suspension cultures of *Salvia miltiorrhiza*

2,4-D (ppm)	Culture period (day)	Ferruginol content (μg/ml)
0	5	5.9
	20	68.4
0.1	5	Trace (>0.1)
	20	Trace (>0.1)
1.0	5	Trace (>0.1)
	20	Trace (>0.1)

Values are means for two experiments.

Kinetic studies of the rate of metabolite formation and cell growth are essential to obtain a basic understanding of secondary metabolism on plant tissue culture [4]. The time-course of ferruginol production in 2,4-D-free medium is shown in Fig. 2. In addition to dry weight of cells and the ferruginol content, the mitotic index, which is a parameter of the frequency of cell division, was also followed. The amount of ferruginol increased rapidly and linearly during the lag phase (0–5 days), and then during the following logarithmic phase (5–10 days) ferruginol production was almost suppressed. With approach to the stationary phase (day 10), the amount of ferruginol started to increase linearly again. According to Tabata [4], growth and production of secondary metabolites by cultures of plant cells can be classified into three main types: (1) Production proceeding almost in parallel with cell growth. (2) Production delayed until cell growth declines or ceases. (3) Production showing a biphasic curve with a lag behind the growth curve. In addition, Noguchi recently reported a fourth type; namely production of germichrysone by a suspension culture of *Cassia torosa* was induced in the lag and logarithmic phases by two different mechanisms [5]. The time-course of production of ferruginol was clearly different from any of these four types. A plot of the ferruginol content (% of dry weight) against the dry weight of cells gave two maxima during the lag and stationary phases, respectively. Thus production of ferruginol was inversely related with active cell division. The reason for this inverse relationship is not yet clear, because metabolism is very different in the lag and stationary phases [6, 7]. But, since ferruginol is probably mainly synthesized from sucrose in the medium, it is reasonable to assume that its production is suppressed in the logarithmic phase as a result of active carbohydrate consumption for biosynthesis of primary metabolites (e.g. cell walls and lipids).

The functional difference between natural auxin IAA and synthetic auxin 2,4-D in the regulation of growth and ferruginol production was examined (Fig. 3). To eliminate the effect of 2,4-D carried over from the stock culture, we transferred the cells to auxin-free medium (Fig. 3a) and after 11 days, when the cells were in the early stationary phase, collected them and transferred them to auxin-free medium or medium with 2,4-D (0.1 ppm) or IAA (1 ppm). Preliminary tests on the effect of IAA concentration on cell growth in the first passage from the stock culture

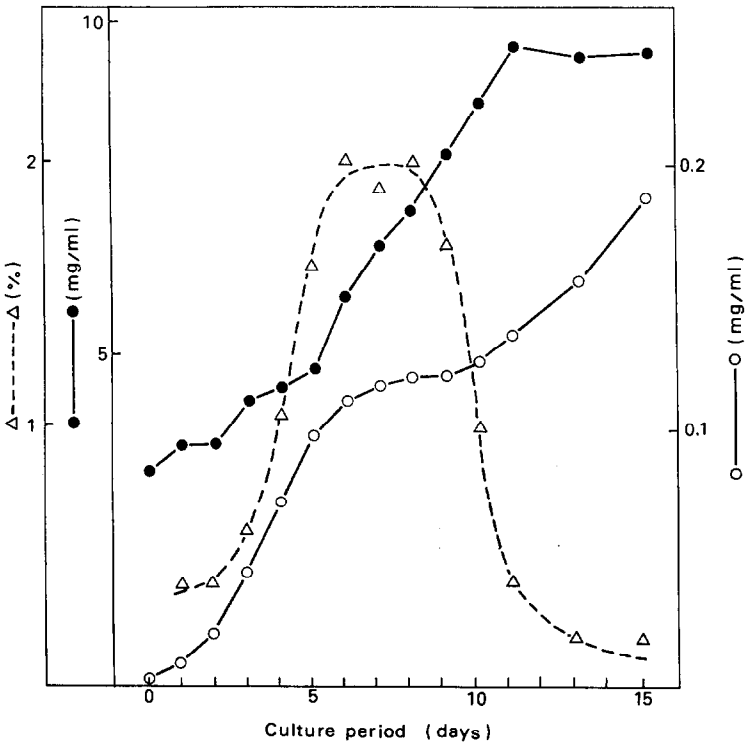


Fig. 2. Changes in dry weight (●), ferruginol content (○) and mitotic index (△) of *S. miltiorrhiza* suspension culture in 2,4-D-free medium.

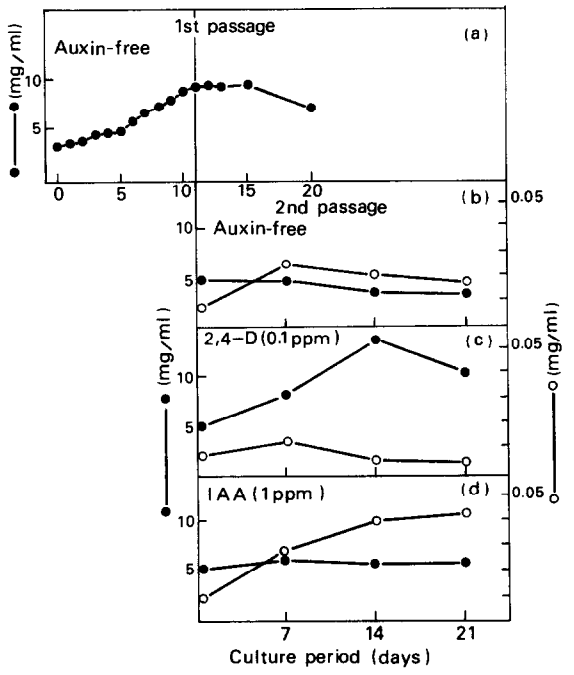


Fig. 3. Changes in dry weight (●) and ferruginol content (○) of suspension cultures of *S. miltiorrhiza*. In the early stationary phase of the first passage in auxin-free medium, part of the culture was again inoculated into medium containing either (b) no auxin, (c) 2,4-D (0.1 ppm) or (d) IAA (1 ppm).

showed that 1 ppm was an appropriate concentration. In auxin-free medium the dry weight of cells did not increase (Fig. 3b) and the cells soon turned black. Moreover, when these cultures were examined microscopically on day 14 or 21, much cell debris was seen and many cells appeared to be dead. The ferruginol content of the culture also decreased gradually from day 7, probably as a result of death of the cells from this time. In medium with 2,4-D (0.1 ppm) the dry weight of cells increased but the ferruginol content did not (Fig. 3c). Unlike 2,4-D, IAA did not promote cell growth (Fig. 3d), but the appearance of the culture was different from that in auxin-free medium and even on day 21 no cell decomposition or discoloration was observed. IAA also differed from 2,4-D in its effect on ferruginol production. The ferruginol content increased continuously after the initiation of the second passage to about twice the initial level within 3 weeks (Fig. 3d). The production of secondary metabolites derived from mevalonate was also reported to be stimulated by substitution of IAA for 2,4-D [8]. The mechanism of this effect is unknown.

Table 2 shows the cell growth and the ferruginol content of cultures grown under white light and in the dark. Light had little influence on cell growth, but the ferruginol content of cells grown in the light was about 56% of cells grown in the dark. This inhibitory effect of light on ferruginol production is interesting since ferruginol is found only in the roots of the intact plant.

Table 2. Effect of light on ferruginol production in 14-day-old suspension cultures of *S. miltiorrhiza*

	Dry wt (mg/ml)	Ferruginol content (μg/mg dry wt)
In darkness	51.7±0.53	10.2±1.7
*In light	47.4±1.8	5.7±1.0

Values are means ± s.e. for three cultures. 2,4-D-free medium with 0.1 ppm kinetin was used.

*Cultures were illuminated continuously with white light (900 lx) throughout the culture period.

EXPERIMENTAL

Plant material and method of culture. Cultures of a cell line derived from a seedling of *Salvia miltiorrhiza* and maintained as described in the previous paper [2] were used. All callus cultures were grown in 25° in the dark on solid Murashige–Skoog's (MS) medium [9] containing 2,4-D (1 ppm), kinetin (0.1 ppm) and agar (0.6%), and subcultures were made about once each 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 using 15% inocula once a month. Fluorescent lamps were used for irradiating cultures with white light (900 lx). For experiments, cells from the stock suspension culture in the early stationary phase were collected on filter paper (Toyo filter paper No. 1; Toyo Kagaku Sangyo Co., Osaka) with suction, washed three times with test medium, and then suspended in the test medium. For determination of dry weight, cells were collected on pre-weighed tetrafluoroethylene polymer filter (10 μm pore size: Polyfrone paper PF-2, Toyo Kagaku Sangyo Co., Osaka), washed with distilled water and dried at 80° for 2 hr.

Quantitative determination of ferruginol. The dried cells were extracted with $CHCl_3$ in a Soxhlet for 3 hr, and the ferruginol content of the extract was determined by GLC as described previously [2].

Determination of mitotic index. Cells were collected, washed and fixed in EtOH–HOAc (3:1) for 1 hr. Then they were stained with a soln prepared by dissolving 1 g of orcein in 100 ml 45% HOAc. The mitotic index was determined by examination of a total of about 1000 nuclei and expressed as the percentage of nuclei undergoing mitosis.

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