Natural Product Sciences
2(2) : 90 ~ 95 (1996)

The Effect of Light on the Production of Reserpine in Cultured *Rauwolfia serpentina* Cells

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Abstract – When reserpine-producing cell strains of *Rauwolfia serpentina* were transferred from the dark to the light irradiation, the production of reserpine was extremely enhanced whereas the cell growth was suppressed. In an incubation period of 20 days, the most effective culture condition for reserpine production was the combination of 8 days of dark culture and following 12 days of light culture. The time courses of both cell growth and reserpine production were measured *in vitro* in order to clarify the effect of wave length range of light on the biosynthesis of reserpine. Although the growth of cultured cells which had been incubated under continuous red, yellow, and green lights, respectively, was similar to that of the cultured cells subcultured in the dark. The cells cultured under red light irradiation produced less reserpine than dark-grown cultures. Both blue and near-ultraviolet light inhibited the growth of cultured cells. The production of reserpine was strikingly enhanced by blue light, but was strongly inhibited by near-ultraviolet light.

Key words – indole alkaloids, reserpine, 3,4,5-trimethoxybenzoic acid, cultured cells, biosynthesis, light irradiation, *Rauwolfia serpentina* Benth.

Introduction

In many cases, secondary metabolite production characteristic of the intact plant is possible by its cell cultures, and also many studies on regulation of secondary metabolism as well as industrial application have been reported. Detailed studies on the production of pharmaceutical compounds such as indole alkaloids (Ohta *et al.* 1979, Kurz *et al.* 1980, Lindsey *et al.* 1983, Kohl *et al.* 1983), tropane alkaloids (Hashimoto *et al.* 1982), steroids (Chandler *et al.* 1983, Furuya *et al.* 1983), and quinones (Ikeda *et al.* 1977, Fujita *et al.* 1987) by plant cell cultures have been reported. In spite of intensive research, many plant cell cultures produce such compounds in only small amounts.

We previously reported the production of reserpine in dark-grown cell suspension cultures of *Rauwolfia serpentina* by metabolic regulation (Yamamoto *et al.* 1986) and the selection of high reserpine-producing cell strains from dark-grown cell aggregates by the fluorescence (Yamamoto *et al.* 1987). The reserpine content (0.03~0.1% dry weight) of these cell suspension cultures, however, was lower than that of intact plant. Therefore, various factors which influence the productivity of reserpine should be plurally elucidated in our *Rauwolfia serpentina* suspension cultures. Light irradiation is one of the important factors for the biosynthesis of secondary metabolites of cultured cells and the cell growth. There are some reports on the biosynthesis of secondary metabolites by different wavelength of light in various plant tissue cultures. Knobloch *et al.* (1982) reported that cell cultures of *Catharanthus roseus* accumulated anthocyanins and the in-

dole alkaloid serpentine under fluorecent light. It has been reported that blue light stimulates anthocyanin production in populus cell cultures (Matsumoto *et al.* 1973) and 5-aminolevulinic acid in tobacco callus cultures (Kamiya *et al.* 1983), but inhibits the naphthoquinone pigments formation in *Lithospermum erythrorhizon* callus cultures (Mizukami *et al.* 1978). In the present study further experiments were undertaken to examine the effects of light irradiation on the reserpine production in cell suspension cultures of *Rauwolfia serpentina.*

Experimental

Cultured cells – Callus cultures induced from the stem of *Rauwolfia serpentina* have been maintained for more than 13 years in the dark at 25 °C on modified Linsmaier-Skoog (Linsmaier *et al.* 1965) medium supplemented with 1 μM 2,4-D and 1 μM kinetin and containing KNO_3 instead of NH_4NO_3 as the nitrogen source. Thereafter, the strains selected were cultured in the original growth medium. These cell suspension cultures were yellowish white, and very fine in texture. Moreover, the cells had lost the potential for redifferentiation. These reserpine-producing cell strains were used as a model strain for ourexperiments.

Culture conditions – (1) About 3.0 g fresh weight of cell suspension cultures were inoculated into a 300 ml Erlenmeyer flasks containing 75 ml of liquid medium and incubated in the dark and/or under the light irradiation at 25 °C. The light-grown cultures were continuosly irradiated with white fluorescent tubes of 40 W/m^2 (TOSHIBA FLR -40S) at an intensity of 1500 lux.

(2) About 1.0 g fresh weight of cell suspension cultures were inoculated into a 100 ml Erlenmeyer flasks containing 25 ml of the same liquid mediumand incubated not only in the dark but also under the five monochromatic lights, red, yellow, green, blue, and near-ultraviolet light at 25 °C. Each monochromatic light was supplied with color fluorescent tubes (MATSUSHITARed; FL-20S · R-F, Yellow; FL-20S · Y-F, Green; FL-20S · G-F, Blue; FL-20S · B-F and TOSHIBA Nearultraviolet; GL 20).

All culture flasks used in the experiments of (1) or (2) were shaken at 100 rpm on a rotary shaker.

Measurement of cell growth in suspension culture – The cell suspension cultures were collected on a 62 μm nylon sieve under suction for measurement of fresh weight. Dry weight was determined using freeze-dried cells.

Extraction and quantitative analysis of reserpine – Freeze-dried cells (50 mg) were extracted twice with 5 ml of MeOH for 3 hrs at 50 °C using a shaker, after which the material was centrifuged at 3000 rpm for 10 min. The combined MeOH extracts were dried completely under vacuum. The dried residue was dissolved in 3 ml of $CHCl_3$ and shaken vigorously for about 15 sec. The $CHCl_3$ extracts were separated by centrifugation at 3000 rpm for 3 min, after which the residue was re-extracted with 2 ml of $CHCl_3$. The combined $CHCl_3$ extracts were dried thoroughly under vacuum. The dried residue dissolved in the same solvent used for HPLC; n-hexane-ethanol-acetic acid-triethyl amine (87-23-0.3-0.4). This solution was centrifuged at 3000 rpm for 5 min, and the supernatant was subjected to separation by HPLC. The HPLC analysis was carried out by normal phase partition chromatograph (Shimazu LC-6A). Separation was achieved using SILICA-150 (ToyoSoda Co.,Ltd) in a 4.6~250 mm stainless steel column at a flow rate of 1.2 ml/min and the effluent was monitored at 254 nm. Reserpine and othercompounds, e.g., ajmaline, rescinnamine and 3,4,5-trimethoxybenzoic acid in the $CHCl_3$ extracts were identified with authentic samples.

Results and discussion

Difference of reserpine production be-

tween light-and dark-grown cultures – In previous studies, we reported (Yamamoto *et al.* 1986) that reserpine-pro-ducing cell strains were selected from cultured *Rauwolfia serpentina* cells under-stress induced by altering both the nitrogen source and the hormones of the medium, and these dark-grown cell cultures maintain the potential of reserpine production as long as they are regularly subcultured. In order to examine the influence of light on reserpine production, the behavior of reserpine-producing cell strains was compared between in the dark and under continuous irradiation with white fluorescent tubes (1500 lux), at 25 °C. Time courses of cell growth and reserpine content in these cell suspension cultures were compared. The cell growth and reserpine contents in both light and dark-grown cell suspension cultures were measured at intervals of 2-4 days during the 32 days of incubation period. The results obtained are shown in Fig.1. Judging from the time courses of cell dry weight, the growth of light and dark-grown cultures reached a maximum at day 12 and at day 15, respectively, after which the both declined. Regarding cell fresh weight, the light-grown cultures continued to grow until day 18, at which the ratio of its fresh weight to inoculum weight was apporoximately 10, where as the maximum growth in dark-grown cultures was reached at day 24, at which its ratio reached approximately 14. The content of reserpine in the lightgrowncells was the greatest at day 21, and that of dark-grown cells was the largest at day 28. The reserpine content in light-grown cultures was more than three times of that in dark-grown cultures. We previously reported (Yamamotoet *et al.* 1986) that although reserpine was produced by the culture while in the growth phase, its concentration reached a maximum at the last stationary phase of the growth cycle. These results suggest that the light irradiation not only shortens the life cycle of

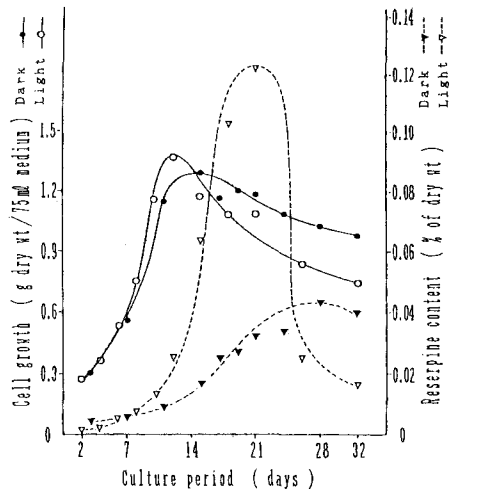

Fig. 1. Time courses of cell growth(—○—: dark, —●—: light) and reserpine content(--▼--: dark, --▽--: light) in *Rauwolfia serpentina* suspension cultures in the dark and under continuous irradiation with white fluorescent lamp.

cultured cells but also restricts the cell growth at the stationary phase. Fig. 2 shows the typical high performance liquid chromatograms obtained by 254 nm UV detection of the $CHCl_3$ extracts of light and dark-grown cultures. Although the components of the $CHCl_3$ extracts were similar, the content of reserpine produced by light-grown cultures was higher than that of reserpine produced by dark-grown cultures. At the same time, accumulation of reserpine was influenced by the amount of its biosynthetic precursor 3,4,5-trimethoxybenzoic acid, because reserpine is an ester alkaloid yielding reserpic acid, 3,4,5-trimethoxybenzoic acid and MeOH on hydrolysis. Fig. 3 shows the biosynthesis of reserpine.

Influence of the period of light irradiation on cell growth and reserpine production – In the present study further experiments were carried out to investigate the influence of light irradiation on cell growth and reserpine production in cell suspension cultures. In the first experiment, it was found that cell growth was inhibited under the continuous light irradiation for a

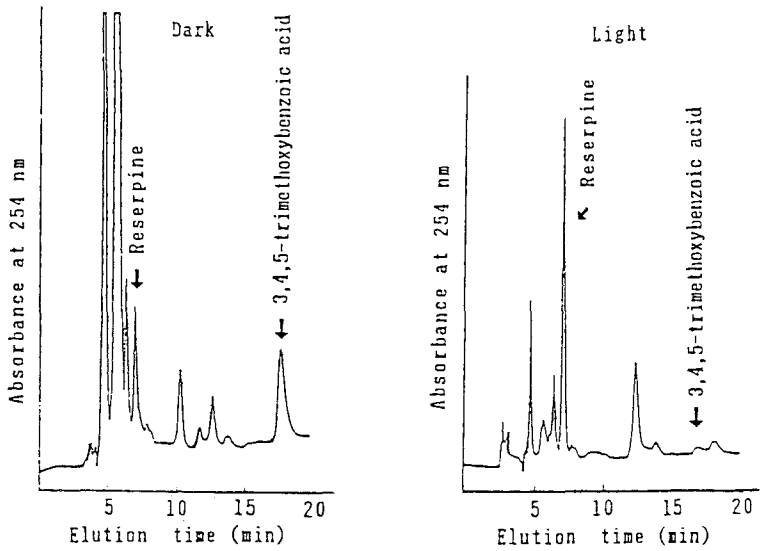

Fig. 2. High performance liquid chromatograms of the $CHCl_3$ extracts of dark-grown cultures (left) and light-grown cultures (right) of *Rauwolfia serpentina*.

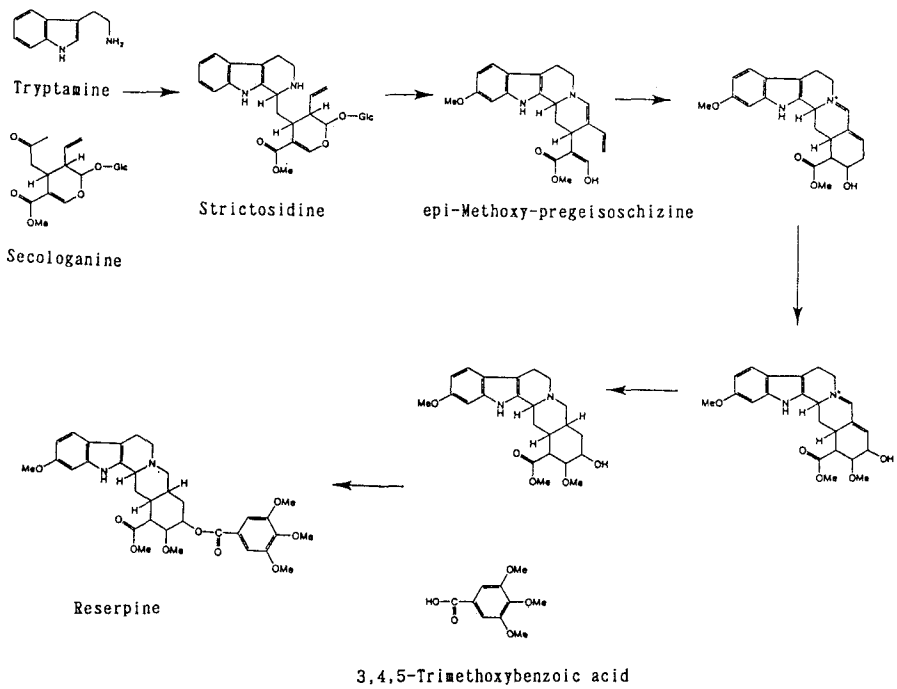

Fig. 3. Biosynthesis of reserpine.

culture period of 4 weeks. Therefore, the cell cultures were transferred step by step under the light irradiation from the dark as described in Fig. 4. After incubation of 20 days, each of cell suspension cultures was harvested, wei-ghed and extracted with MeOH. The extracts were treated as described above and the reserpine contents were measured by HPLC. Results are plotted in Fig. 4. With increasing the period of light irradiatin, the cell growth decreased and the fine cell suspension cultures which had been yellowish

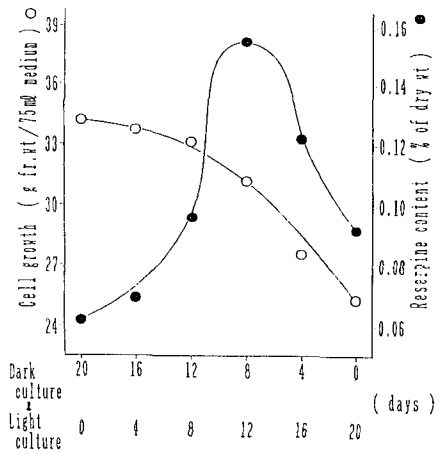

Fig. 4. Effects of the duration of dark culture and following light culture on cell growth (○) and reserpine content (●) in *Rauwolfia serpentina* suspension cultures.

white in the dark turned yellowish brown in color. The reserpine contents reached the maximum at 8 days of dark culture and following 12 days of light culture, after which there was a large reduction with increasing the period of irradiation. In order to check the potential of reserpine reproduction, each of these cell strains tested was repeatedly subcultured under the same conditions. The strains which had been precultured in the dark during 12 to 20 days began to produce similar amounts of reserpine to that produced in the former incubation.

Influence of monochromatic light irradiation on cell growth and reserpine production – Our results showed that the production of reserpine in cultured *Rauwolfia serpentina* cells was enhanced by light irradiation with white fluorescent tubes. It is interesting to investigate what wave length range of light might affect the cell growth and reserpine production. Time courses of cell growth and reserpine production in the cell suspensions cultured under the continuous irradiation of monochromatic light, red, yellow, green, blue, and near-ultra violet lights were monitored. Fig. 5 shows the cell growth (mg dry weight/flask) and the reserpine content (% of dry weight) in cell suspen-

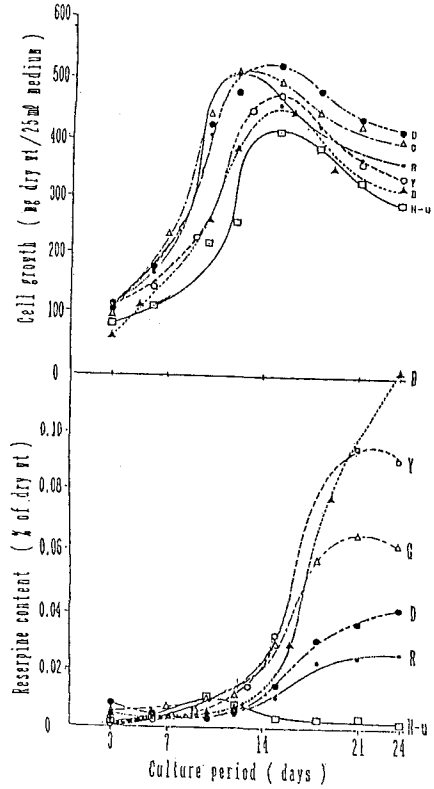

Fig. 5. Effects of irradiation with light of different wave bands (Red:— · —, Yellow: --○--, Green: —△—, Blue: ···▲···, Near-ultraviolet: —□— and Dark: --●--) on cell growth and reserpine content in *Rauwolfia serpentina* suspension cultures.

sion cul-tures. Although each growth of cell suspensions cultured under both red and green light irradiation was similar to that of dark-grown cultures, the production of reserpine was significantly differ among them. Blue light was the most effective for reserpine production, but near-ultraviolet light extremely restricted growth as well as reserpine production. The reserpin econtent under blue light irradiation was about three times of that producedin the dark. Only cell suspensions cultured under near-ultraviolet light irradiation began to form aggregates after 8-12 days, and then turned brown. Other cell cultures were yellowish white, and very fine in texture. The fact that the contents of reserpine in cultured *Rauwofia serpentina* suspension cells differ with mono-

chromatic light seems to be specific for the reserpine biosynthesis. Possible regulation of useful secondary metabolites by changing the monochromatic light or the period of irradiation instead of altering the composition of the medium might be available for the process of industrial production.

Acknowlegement

I wish to thank professors Y. Yamada (Nara Institute of Science and Technology, Japan) and T. Yasuda (Kobe University, Kobe, Japan) for valuable and discussion and advice, and to professor A. Kobayashi (Osaka University, Osaka, Japan) for language editing of the manuscript.

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(Accepted August 13, 1996)