Comparative Study of the Effects of Various Culture Conditions on Cell Growth and Gagamine Synthesis in Suspension Culture of *Cynanchum wilfordii* (MAXIM.) HEMSLEY

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Gagamine, a steroidal alkaloid isolated from the roots of *Cynanchum wilfordii*, exhibited potent inhibitory effects on aldehyde oxidase activity and lipid peroxidation. To determine whether it would be possible to mass produce this active component, which would be useful for animal tests, we tried to synthesize it using *in vitro* cell culture methods with various growth conditions. In a previous study it was found that calli were easily induced from the stem of this medicinal plant and cultivated effectively on MS medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) 2 mg/l. In this work we attempted to determine the effects of various culture conditions on cell growth and gagamine synthesis in suspension culture. Gagamine production was increased markedly when cell growth proceeded to the death phase. Cell growth was more effective with 5% (v/v) sucrose, in the light (at 38 $\mu\text{E}/\text{m}^2 \cdot \text{s}$), on medium containing 2,4-D 2 mg/l, with 2.5 g/10 ml medium as the initial cell concentration. The concentration of gagamine was optimal with 3% sucrose, in darkness on medium 2,4-D 1 mg/l, with 2.5 g/10 ml medium as an initial cell concentration. However, the highest growth rate was 0.18 d^{-1} , when the gagamine concentration was seven- and three-fold (at 140 $\mu\text{g}/\text{ml}$) that of the plant stem and 10 ml of medium respectively, on the 50 ml of medium in suspension culture.

Key words *Cynanchum wilfordii*; gagamine; biosynthesis; suspension culture

Recently, the demand for and range of use of the secondary metabolites of plants, which are more available than synthetic compounds, has been gradually increased, in medical treatment. Plant tissue culture is useful method for the production of secondary metabolites and it is possible to control productivity by changing the culture conditions, for example, of light, pH, carbon source, amino acid, precursor, polarity, plant growth regulator *etc.*^{1–5)}

Cynanchum wilfordii HEMSLEY (Asclepiadaceae) is a perennial herb of which the benefits were previously described.^{6–8)} In Korea, its roots, which are known as *backhasuo*, have been used in folk medicine for the prevention and treatment of various geriatric diseases including vascular disorders, diabetes mellitus, ischemia-induced diseases, and aging progression.⁶⁾

The roots of this plant have been reported to contain some polyoxypregnane glycosides.^{8–12)} In addition, recently one steroidal alkaloid, gagamine, was isolated for the first time from this plant.¹³⁾ Gagamine has also been isolated from other species in the *Cynanchum* genus^{14,15)} and evaluated for its potent inhibitory effects on the activity of aldehyde oxidase, an oxygen radical generating enzyme, and hepatic lipid peroxidation in an *in vitro* assay.¹³⁾ The structure of this active compound (Fig. 1) contains a pregnane skeleton with two ester bonds bound with cinnamic acid at C-12 and nicotinic acid at C-20. Thus the introduction of an ester group at

the corresponding carbon positions in the skeleton could not easily be achieved using any chemical approach. However, cell cultures often produce secondary metabolites in significant quantities¹⁶⁾ which would be useful for animal tests and are regarded as genetically more stable. In our previous work, we reported the establishment and cultivation of stem cultures of *C. wilfordii* and presented the results designed to optimize growth and gagamine content in these cultures.¹⁷⁾ In this report, we describe the comparative results of the effects of various culture conditions, such as 2,4-dichlorophenoxyacetic acid (2,4-D), known as one of the most effective plant growth regulators¹⁷⁾; different sucrose and various initial cell concentrations; light and dark conditions; and different temperatures on cell growth and gagamine production in suspension culture of *C. wilfordii*.

MATERIALS AND METHODS

Plant Materials *C. wilfordii* seeds were collected from the Agricultural Technology Center of Young-Ju City, Kyoung-Sang-Buck-Do, Korea and authenticated by Professor B. S. Kang, Professor of Taxonomy of Medicinal Plants, College of Oriental Medicine, Dongguk University, Kyongju, Korea. A voucher specimen of this plant material was deposited in the Herbarium of the Department of Biology, Dongguk University. The seeds were germinated on pots filled with vermiculite. Gagamine was isolated according to the previous method¹⁵⁾ and used for HPLC analysis as a standard material. Plant growth regulators and medium reagents were purchased from Sigma Co. All other chemicals were of the highest grade available.

Callus Induction The explant tissue was obtained by removing the plantlets (*ca.* 15 cm in length; 20 d old) and cutting discs of approximately 5 mm in diameter and 1 cm long

Fig. 1. Chemical Structure of Gagamine

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from the stem. The stems were surface sterilized with 70% ethanol for 3 min and then in a solution of 5% sodium hypochlorite for 15 min. After rinsing three times with sterile distilled water for 10 min each. The explants were cultured on Murashige and Skoog (MS) medium,¹⁸⁾ which was prepared by incorporating 3% (v/v) sucrose, 2,4-D 2 mg/l, and 4.4% phytigel. The pH of the medium was adjusted to 5.8 using 1 N NaOH or HCl, and then autoclaved at 120 °C at a pressure of 1.2 kg/cm² for 20 min. All cultures were incubated at 25 ± 1 °C in the dark.

Cell Suspension Culture The proliferated calli from the second subculture were used to initiate the cell suspension. When sufficient callus was available, approximately 0.9 g fresh weight of calli was placed in a 100-ml Erlenmeyer flask containing 10 ml of the liquid MS medium supplemented with 3% (v/v) sucrose and 2,4-D 1 mg/l. Three replicates were employed for each treatment. Flasks were incubated in a growth chamber at 25 ± 1 °C on a rotary shaker at 250 rpm, with a photon flux density of 38[μE/m²·s], with a 16-h light/8-h dark photo period.

Cell Growth To measure the growth rate during the culture time, the weight of the sample was measured every 2 d. After measuring the weight of an empty centrifuge tube, the cell suspension of the sample was poured into the tube and centrifuged for 10 min at 2800 rpm. The supernatant was removed completely and the fresh weight of callus was measured.

The maximum specific growth rate ($\mu_{\max}$) was calculated using the following equation:

$$\mu_{\max} = \frac{\ln \frac{x_2}{x_1}}{t_2 - t_1}$$

where t and x are the cultivation time and the concentration of callus, respectively, and x_1 and x_2 are the concentration of callus for cultivation time (t_1 , t_2) in the log phase.

Culture Conditions To compare the effects of various culture conditions on cell growth and gagamine synthesis in the cell suspension culture, the experiments were performed with 2,4-D 0.1, 0.5, 1.0 and 2.0 mg/l, cool white fluorescent light, and dark conditions with 8- to 16-photo periods under 38[μE/m²·s], with initial cell concentrations of 0.6, 0.9, 1.7, and 2.5 g fresh weight of callus using an initial sucrose concentration of 3% or 5% with cultured temperatures at 25 °C or 31 °C.

Statistical Analysis The mean and standard deviation of the mean as well as standard errors were calculated for the cell weight of callus and gagamine concentration.

RESULTS AND DISCUSSION

Relation between Cell Growth and Gagamine Production As shown in Fig. 2, there was little growth of *C. wilfordii* (MAXIM.) HEMSLEY until the 15th culture day. The exponential phase started on the 15th day after inoculation and the cells proceeded to the stationary phase from the 22nd day. During 7-d cultivation in the exponential phase, the cell concentration increased three-fold. The amount of gagamine synthesis increased twice, slightly in the initial phase and markedly higher when cell growth proceeded to the death phase.

These results confirmed the relationship between cell growth and synthesis of gagamine. The synthesis of gagamine abruptly increased after the stationary phase even if cell growth stopped. Therefore the concentration of gagamine was extremely high after 5–7 d during the maxi-

Fig. 2. Changes in Cell Growth and Gagamine Synthesis

C. wilfordii was cultivated at 25 °C, 2000 lx, 130 rpm. Data are expressed as means of three samples ± standard deviation.

Fig. 3. (A) Effect of Concentrations of Plant Growth Regulator on Cell Growth in Suspension Culture and (B) Effect of Concentration of Plant Growth Regulator on Gagamine Concentration in Suspension Culture

C. wilfordii was cultivated at 25 °C, 2000 lx, 130 rpm in the dark. Data are expressed as means of three samples ± standard deviation.

Fig. 4. Effect of Light on Cell Growth and Gagaminine Concentration in Suspension Culture

C. wilfordii was cultivated at 25 °C, 130 rpm. Data are expressed as means of three samples $\pm$ standard deviation.

imum cell concentration. It appeared that cells died in this period, and therefore gagaminine synthesis occurred in the cell death phase. Similarly, small increases in gagaminine occurred in the calli, which were transferred from solid to liquid medium and adapted for new medium.

Effects of 2,4-Dichlorophenoxyacetic Acid The growth rates and synthesis of gagaminine were affected by 2,4-D. The acid accelerated the dedifferentiation of plant and then induced callus. The maximum growth rates were 0.037, 0.032, 0.040, and 0.068/d, when the concentrations of 2,4-D were 0.1, 0.5, 1.0, and 2.0 mg/l, respectively. The cell growth was the highest with 2,4-D 2 mg/l (Fig. 3A). On the contrary, the synthesis of gagaminine was the greatest with 2,4-D 1 mg/l (Fig. 3B). However, when the 2,4-D concentration was 1 mg/l, cell growth was initially faster than at other concentrations, but abruptly decreased after 17 d of cultivation.

Effects of Light Cell growth was higher in the light than in dark conditions (Fig. 4A). The maximum growth rates in light and dark conditions were 0.040 and 0.021/d with 2,4-D 1 mg/l and 0.068 and 0.053/d with 2,4-D 2 mg/l, respectively (Fig. 4B). However, the synthesis of gagaminine was greater in dark than in light conditions. The results thus correlate with callus cultivation of *C. wilfordii* in solid medium.¹⁷⁾

Fig. 5. (A) Effect of Various Initial Cell Concentrations on Cell Growth in Suspension Culture and (B) Effect of Various Initial Cell Concentrations on Gagaminine Synthesis in Suspension Culture

C. wilfordii was cultivated at 25 °C, 2000 lx, 130 rpm. Data are expressed as means of three samples $\pm$ standard deviation.

Fig. 6. (A) Effect of Sucrose Concentrations on Cell Growth in Suspension Culture and (B) Effect of Sucrose Concentration on Gagamnine Synthesis in Suspension Culture

C. wilfordii was cultivated at 25 °C, 2000 lx, 130 rpm. Data are expressed as means of three samples $\pm$ standard deviation.

Effects of Inoculate Concentration The cells were cultured in the 10 ml of MS liquid medium containing 2.0 mg/l of 2,4-D to observe cell growth and synthesis of gagamnine with various inoculate concentrations. The maximum growth rates ($\mu_{\max}$) were 0.037, 0.068, 0.12, and 0.13/d, when the initial cell concentrations were 0.6, 0.9, 1.7 and 2.5 g, respectively, fresh weight of callus (Fig. 5A). With higher inoculate concentration, the cell growth rate was greater. However, the growth rates only varied slightly between the initial weights of 1.7 g and 2.5 g, while the synthesis of gagamnine was greater when 0.6 g fresh weight of callus was inoculated (Fig. 5B). Generally the inoculate concentration is 20–30% of the medium in plant cell cultivation. It appeared, however, that the growth rate of *C. wilfordii* was excellent specifically with a 20% initial concentration.

Effects of Initial Sucrose Concentration Sucrose as the carbon source is an essential component in the plant cell culture. It appeared that 5% sucrose was superior to 3% for cell growth with the maximum growth rates of 0.068 and 0.039/d, respectively (Fig. 6A). But gagamnine synthesis was the reverse of cell growth rate so that gagamnine contents were higher in 3% initial sucrose than in 5% (Fig. 6B). However, many researchers reported that sucrose concentration usual-

Fig. 7. (A) Effect of Temperature on Cell Growth in Suspension Culture and (B) Effect of Temperature on Gagamnine Synthesis in Suspension Culture

C. wilfordii was cultivated at 25 °C, 2000 lx, 130 rpm. Data are expressed as means of three samples $\pm$ standard deviation.

ly improves during the production of secondary metabolites.^{2,7,19} Thus it appeared that sucrose functions to enhance osmosis activation in the medium, rather than directly influence of high sucrose concentration.²² But in case of *C. wilfordii* cultivation, there was higher growth rate and gagamnine synthesis at lower sucrose concentration. Thus it appears that carbon concentration has no influence on gagamnine biosynthesis.

Effects of Culture Temperature The cells were cultured at the usual temperature of 25 °C and at 31 °C, at which the growth rate is slightly higher as reported by some researchers.²³ The cell growth at 31 °C was greater than at 25 °C and the maximum growth rates were 0.068 and 0.085/d at 25 °C and 31 °C, respectively (Fig. 7A). The gagamnine production from cells cultured at 31 °C was slightly less than that at 25 °C, and thus the temperature had no significant effect on it (Fig. 7B). Matsumoto *et al.* reported that the optimal growth temperature for cultivation of the tobacco callus was 25 °C.²² However, in the *Peganum* callus cultivation, it was reported that the optimum temperature for the cell growth was 30 °C and for alkaloid biosynthesis 25 °C.²³ Those researchers also reported that the productivity of sec-

ondary metabolites was decreased at higher temperature rather than under optimal conditions.²¹⁾ However, it was thought that the culture temperature had no effect on cell growth and gagamnine synthesis in cell cultures of *C. wilfordii*. Also, it appeared that white suspension materials were secreted at 5 d of cultivation and the cells were subjected to much more stress than under established culture conditions.

The evidence shows that the relation between cell growth and biosynthesis of gagamnine was not proportional in terms of medium composition and culture conditions. The synthesis of gagamnine increased after reaching maximum growth in the period when the cells were starting to die. Culture conditions also influenced cell growth, especially light

rather than dark conditions, higher inoculate concentrations, and high 2,4-D content. However, these conditions negatively affected the biosynthesis of gagamnine. It is thought that the biosynthesis of gagamnine occurred associated with its strong antioxidant function during cell aging or cell extinction. Therefore the two-stage culture is necessary to increase gagamnine production during cell cultivation after callus induction.

There were slightly higher cell growth and production of gagamnine in large-scale (50 ml of medium) (Figs. 8A, B). Large-scale culture is more effective for cell growth due to the fact that callus was cultured in aggregate form rather than as single cells in suspension culture. Moreover, these results may have possible industrial applications.

Acknowledgments This work was financially supported by the Dongguk University Research Fund.

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Fig. 8. (A) Changes in Cell Growth of *C. wilfordii* (MAXIM.) HEMSLEY in Suspension Culture and (B) Changes in Gagamnine Synthesis in Suspension Culture

C. wilfordii was cultivated at 25 °C, 2000 lx, 50 rpm. Data are expressed as means of three samples $\pm$ standard deviation.