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Studies on Factors Affecting the Establishment of *Gentiana davidii* var. *formosana* (Hayata) T. N. Ho Cell Suspension Cultures

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ABSTRACT

Gentiana davidii var. *formosana* (Hayata) T. N. Ho (Gentianaceae), commonly known as long-dan in Chinese, is a perennial herb indigenous to Taiwan. It is distributed throughout the island, ranging from low to high elevations. The roots, which contain bitter-tasting secoiridoid glucosides, are used in traditional Chinese medicine. It is mainly used in the treatment of gastrointestinal tract diseases. Continuous collection of plant material from natural habitat has led to the depletion of *Gentiana* population. The purpose of this study was to establish the cell suspension cultures of *Gentiana*, which could be used for large-scale production of active principles such as gentiopicroside and swertiamarin. Callus was initiated by culturing stem explants of *G. davidii* var. *formosana* on Murashige and Skoog's (1962) basal medium supplemented with 0.2 mg/L 6-furfurylaminopurine (kinetin) and 1.0 mg/L α-naphthaleneacetic acid (NAA). Fast-growing suspension cell cultures were established by subculturing the callus in MS basal medium (pH 4.2-5.2) supplemented with 0.2 mg/L kinetin and 3% sucrose. The cultures were incubated on an orbital shaker (80-100 rev/min) at 25 ± 1˚C and low light intensity (2.33 $\mu E \cdot m^{-2} \cdot s^{-1}$).

Key words: *Gentiana davidii* var. *formosana*, cell suspension culture, gentiopicroside, swertiamarin

INTRODUCTION

Long-dan (*Gentiana* spp.) is listed as one of the medicinally important plant species by the Shen-Nong-Ben-Tsou-Ching. The Kai-Bao-Ben-Tsou of the Song Dynasty described long-dan as "leaves similar to *Solanum nigrum*, tastes bitter as bile". Long-dan is also known as ling-yo, ku-tang or tang-tsou. Categorized as a cold-based herb, *Gentiana* species were recorded to have a soothing effect on joint soreness and pain, shock and hysteria, and the ability to heal old injuries, stabilize internal organs and kill germs. Regular consumption of long-dan has been believed to enhance memory, prevent obesity and aging, and protect the liver by removing toxic metabolites[1,2]. Recent pharmacological studies have confirmed the anti-inflammatory, anti-allergic, and hepatoprotective properties of the *Gentiana* species[1-3]. *Gentiana davidii* var. *formosana* (Hayata) T. N. Ho (herein after referred to as *G. davidii*) is a perennial herb distributed in the central mountains of Taiwan at 1000 to 3500 meters above sea level[4]. The entire dried herb, collected from the wild habitat, is used as a crude drug in traditional Chinese medicine in Taiwan. However, the plant is fully protected by law and collection of plants from the wild is illegal. Development of a rapid *in vitro* propagation method for rare medicinal herbs of Taiwan for commercial cultivation and/or methods of production of active principles by cell suspension culture would help in the conservation of germplasm. Recently we have optimized a method of mass propagation of *G. davidii* through tissue culture[5]. Gentiopicroside and swertiamarin are the two important secoiridoid glucosides found in Gentianaceae. Gentiopicroside is found to be capable of suppressing chemically and immunologically induced hepatic injuries[6]. Cell suspension culture has been successfully used in the production of important secondary metabolites in many plant species[7]. The aim of this investigation is to optimize the conditions for the establishment of cell suspension cultures of *G. davidii* for the production of gentiopicroside and swertiamarin, the two pharmacologically important compounds.

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MATERIALS AND METHODS

I. *Plant Material*

Flowering plants of *G. davidii* var. *formosana* (Hayata) T. N. Ho were collected from the shores of Tien-lake in the Ta-Shuei Mountain forest-park of Taichung county, Taiwan. Stem explants were used for the establishment of callus cultures. The explants were surface disinfected as described earlier[5]. The stem explants (1-1.5 cm in length) were cultured on MS (Murashige and Skoog's, 1962)[8] basal medium supplemented with 1 mg/L NAA and 0.2 mg/L kintein for callus induction (Figure 1) as described[9]. The cultures were incubated at 25 ± 1˚C in dark for 45 days. The callus (0.5 g) was cut into small pieces and cultured in 20-mL liquid MS medium supplemented with 1 mg/L NAA and 0.2 mg/L kintein in 125-mL Erlenmeyer flask for the establishment of primary cell suspension cultures. The cultures were placed on an

Figure 1. Stem-derived callus of *G. davidii* var. *formosana*. The stem segments were cultured on medium containing MS basic salt supplemented with 3% sucrose, 0.9% Difco agar, 1 mg/L NAA and 0.2 mg/L kintein for 45 days.

orbital shaker (Model SK-302A, Sun Kaun Instruments Co., Taichung, Taiwan) with a rotary motion of 100 rev/min and incubated at 25 ± 1˚C under cool white fluorescent light (Philips, Holland) at 2.33 $\mu E \cdot m^{-2} \cdot s^{-1}$. The established primary cell suspension cultures were routinely subcultured in the same medium (5-mL inoculum/20-mL fresh medium) at 14-day intervals.

II. *Preparation of Medium*

The medium consisted of Murashige and Skoog's (MS)[8] basal salts and vitamins, 3% sucrose, and growth regulators α-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-furfurylaminopurine (kinetin). The pH of all media was adjusted to 5.2 ± 0.1 with 1N NaOH or HCl before autoclaving at 121˚C, 105 kPa for 15 min.

III. *Method of Measuring Growth of G. davidii Cell Suspension Cultures*

From the primary cell suspension culture, 5-mL culture [with 1-mL Packed Cell Volume (PCV)] was subcultured in 20-mL medium in the side-arm Erlenmeyer flask (total volume at 25-mL) (Figure 2). Packed Cell Volume (PCV) of the cell suspension in the medium was measured every four days starting from the day of culture. Prior to measuring the packed cell volume, suspension cells were allowed to settle at the bottom of the side arm of the flask for about 20 minutes. A growth curve was drawn in order to compare the differences between processes. All treatments were repeated five times. Standard deviation was used for statistical analysis.

IV. *Extraction and Quantitative Analysis of Gentiopicroside and Swertiamarin*

Cell suspension cultures were freeze-dried in a lyophilizer (FTS Systems™, NY, USA). The freeze-dried (3 g) cell suspension cultures were extracted 3 times at room temperature with methanol (10 mL) under 20 min sonication (Branson Ultrasonic Cleaner, Branson Cleaning Equipment Co., Shelton, CT, USA) to ensure the complete extraction of the secoiridoid glucosides. The extract was filtered through a Advantec No. 1 filter paper (Toyo Roshio Kaisha Ltd., Japan) and the methanol was evaporated *in vacuo* to dryness. The residue of the combined extracts was re-dissolved in 3-mL methanol, filtered through a membrane filter (0.45 μm pore size, Nalgene®, New York, USA) and 10 μL of the solution was subjected to HPLC thrice. Analysis was performed on a

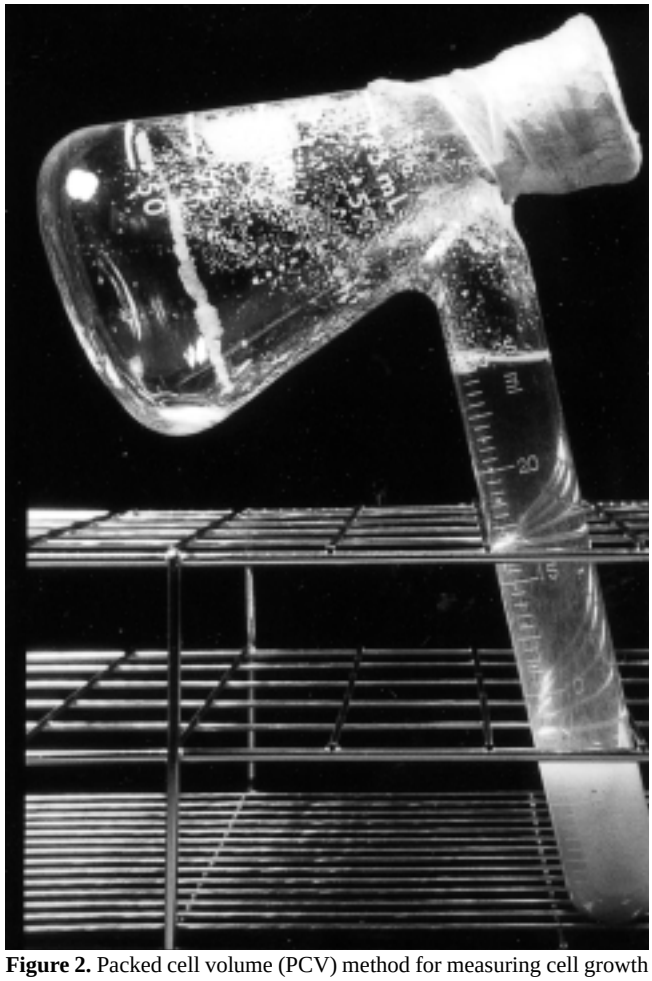

Figure 2. Packed cell volume (PCV) method for measuring cell growth of *G. davidii* var. *formosana*.

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Waters high-performance liquid chromatograph (Waters™, Milford, Massachusetts, USA), which was connected to a Intersil ODS-3-5 μm, 4.6 mm $\times$ 250 mm HPLC column (GL Sciences Inc., Shinjuku, Tokyo, Japan) fitted with a Guard-Pak™ precolumn (Waters). The HPLC system consisted of a Waters™ 600 controller, Waters™ 717 plus autosampler, a 996 photo diode array variable wave length detector and Millennium^{32} software. The mobile phase (methanol:water = 30:70) was pumped at a flow rate of 1.0 mL per min. The cycle time of analysis was about 45 min. Gentiopicroside and swertiamarin (10 mg each) were individually dissolved in methanol, diluted stepwise (0.33, 0.17, 0.08, and 0.04 mg/mL), and 10 μL portions were injected into the HPLC thrice. The calibration plots were obtained by measuring their respective peak areas. The regression equations for gentiopicroside and swertiamarin were:

gentiopicroside: Y=13321856X-23046.1 r=0.999359;
swertiamarin: Y=13289126X-8877.95 r=0.999918.

RESULTS AND DISCUSSION

A cell suspension culture system is useful in obtaining fast-growing and uniform cells. Such cells are suitable for studying biochemical aspects of secondary metabolism. Fast-growing callus is ideal for the establishment of cell suspension cultures. Callus induced from stem segments of *G. davidii* on MS basal medium supplemented with 1 mg/L NAA and 0.2 mg/L kinetin (Figure 1) was used in the present investigation. Cell suspension cultures were established using the stem-derived callus. The callus was transferred in a 125-mL Erlenmeyer flask containing 20-mL of the same callus induction medium for establishing cell suspension cultures. We selected the same callus induction medium for establishing cell suspension cultures based on earlier observations in *Foeniculum vulgare* by Hunanult *et al.* (1988)[10]. Various factors affecting the growth of cell suspension cultures such as growth regulators, shaker speed, pH of the culture medium and light intensity were studied.

I. *Effect of Growth Regulators on Growth of Cell Suspension Cultures Established from Stem-derived Callus of G. davidii*

(I) *Effect of Auxins*

Auxin is generally considered as the key growth regulator in sustaining even cell distribution and growth of suspension cells. In our studies with *G. davidii*, initial growth of cell suspension cultures was slow when the cells were cultured in media supplemented with different concentrations (0, 0.5, 1, 2, or 4 mg/L) of 2,4-D, NAA and IAA. The growth of suspension cells increased rapidly after 20 days of culture. Maximum PCV (22 ± 1.2 mL) was detected after 28 days of culture when MS medium contained either 0.2 mg/L kinetin or without growth regulators (Figure 3). Cell growth was better than the media containing auxins, and the auxin stimulus were not required for the growth of suspension cells. MS medium with low level of kinetin (0.2 mg/L) alone was found to be sufficient to maintain fast growing suspension cells. However, Chen *et al.* (1994) reported that suspension cells of *Angelica dahurica* var. *formosana* grew well under different concentrations of auxin[11]. Narayanaswamy (1977)[12] reported that in *Nicotiana bigelovii*, callus induced on auxin (2,4-D) containing medium could proliferate, upon subculture, on medium without any growth regulators. Suspension cells of *G. davidii* could be of similar nature to that of *N. bigelovii*.

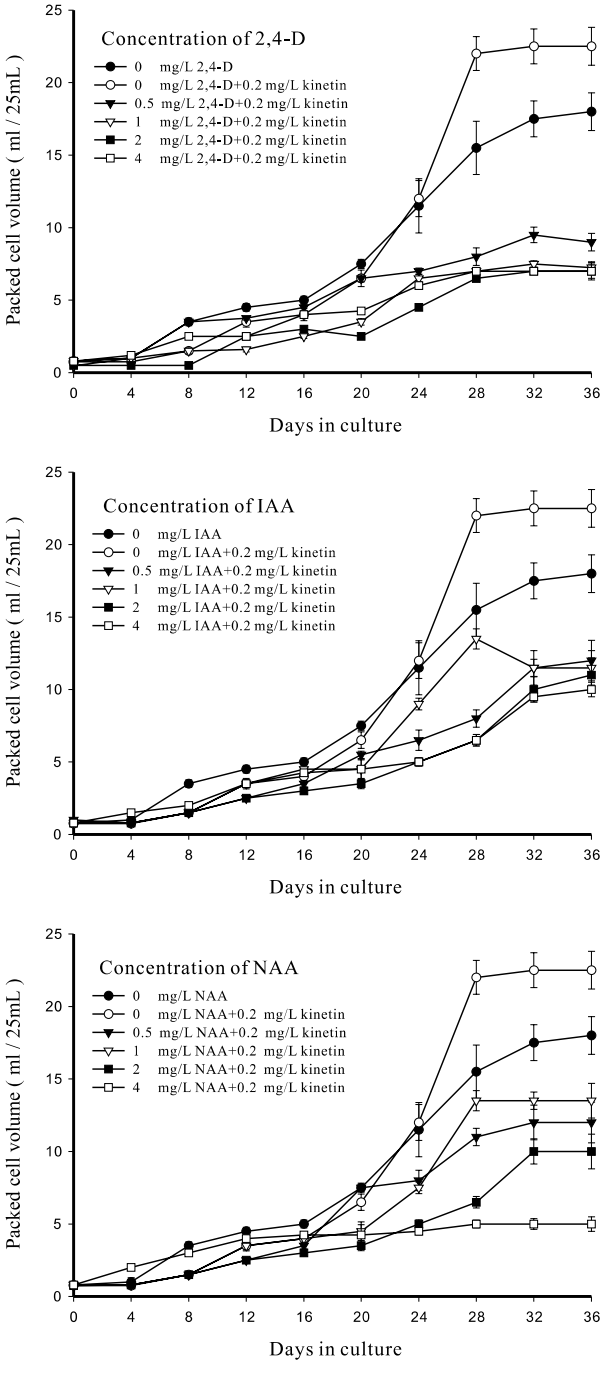

Figure 3. The effect of various auxin concentrations on the growth of cell suspension culture of *G. davidii* var. *formosana*. The basal medium was MS salts supplemented with 3% sucrose, pH 5.2, initial PCV 1 mL and incubated under low light intensity (2.33 $\mu E \cdot m^{-2} \cdot s^{-1}$) and 100 rev/min shaker speed.

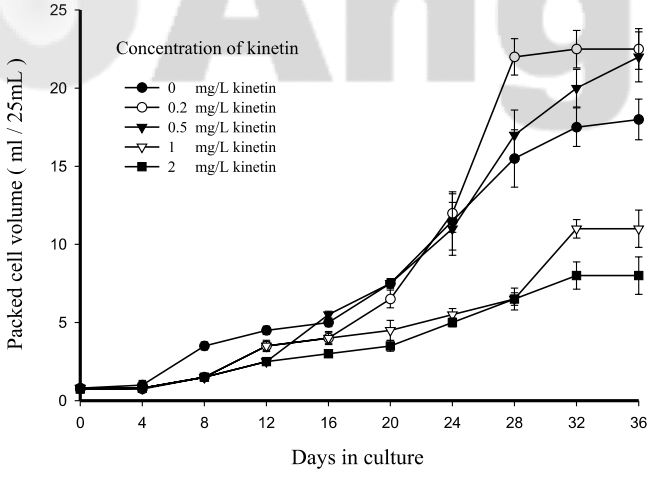

Figure 4. The effect of various kinetin concentrations on the growth of cell suspension culture of *G. davidii* var. *formosana*. The basal medium was MS salts supplemented with 3% sucrose, pH 5.2, initial PCV 1 mL and incubated under low light intensity (2.33 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 100 rev/min shaker speed.

(II) *Effect of Kinetin Concentration*

To study the effect of kinetin concentration in the medium on cell proliferation, the suspension cells were cultured in MS basal medium with 0, 0.2, 0.5, 1 and 2 mg/L kinetin for 36 days. Figure 4 shows the effect of kinetin concentration on PCV. The results revealed that maximum PCV (20-23 mL) was observed when the kinetin concentration in the medium was either 0.2 or 0.5 mg/L (Figure 4). When the concentration of kinetin in the medium was increased (1 or 2 mg/L), it not only affected cell growth but also induced browning of cells (Figure 4).

II. *Effect of Culture Conditions*

(I) *Shaker Speed*

Suspension cells were cultured in MS medium with 0.2 mg/L kinetin and incubated under 60, 80, 100 and 120 rev/min shaker speed. Results indicated that during the initial period of culture, the shaker speed did not have much influence on the growth of cell suspension cultures. However, after 16 days of culture, cell growth was faster under 80 and 100 rev/min shaker speed than 60 or 120 rev/min (Figure 5). Cells increased in size and turned brown when the cultures were incubated under 60 rev/min. Under 120 rev/min, the cell size was smaller than cells incubated under 80-100 rpm, but the color was grayish-white and the rate of cell growth was slower.

(II) *Light Intensity*

Suspension cells cultured in MS basal medium with 0.2 mg/L kinetin were incubated under 0, 2.33 and 23.33 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity for 36 days. Results showed that the cells grew faster when incubated under 2.33 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity and maximum PCV (21 ± 1.5 mL) was observed after 28 days of culture. Cultures incubated under either high light intensity or darkness showed a slower growth rate (Figure 6). The photosynthesis carried out by most plant material *in vitro* is relatively low and cultures are mainly dependent on an external supply of sucrose[13]. Reinert (1958) reported that light intensity would influence the growth of carrot suspension cells: low light intensity (3.5 $\mu E \cdot m^{-2} \cdot s^{-1}$) could stimulate the growth, while higher light intensity (35 $\mu E \cdot m^{-2} \cdot s^{-1}$) would inhibit the growth[14]. Nigra *et al*. (1989) reported that low light intensity stimulated the callus growth of *Solanum eleagnifolium*; however, higher light intensity was beneficial to the production of solasodine[15]. Effect of light on cell growth and secondary metabolite production was reported in *Cornus officinalis* by Yazaki and Okuda (1989)[16].

(III) *Initial pH of Medium*

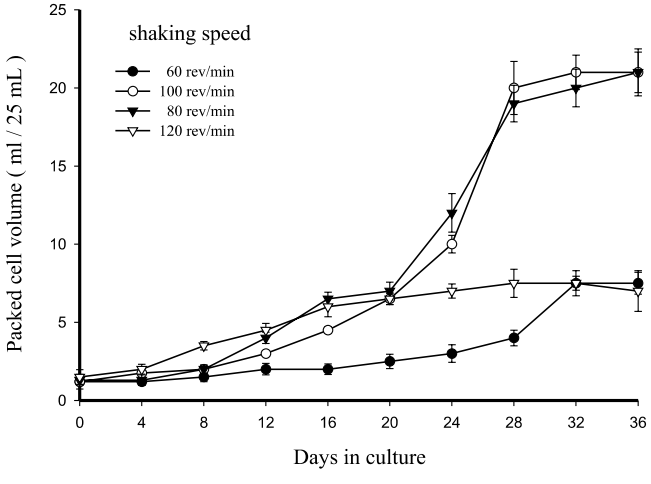

Figure 5. The effect of shaker speed on the growth of cell suspension culture of *G. davidii* var. *formosana*. The basal medium was MS salts supplemented with 0.2 mg/L kinetin, 3% sucrose, pH 5.2, initial PCV 1 mL and incubated under low light intensity (2.33 $\mu E \cdot m^{-2} \cdot s^{-1}$).

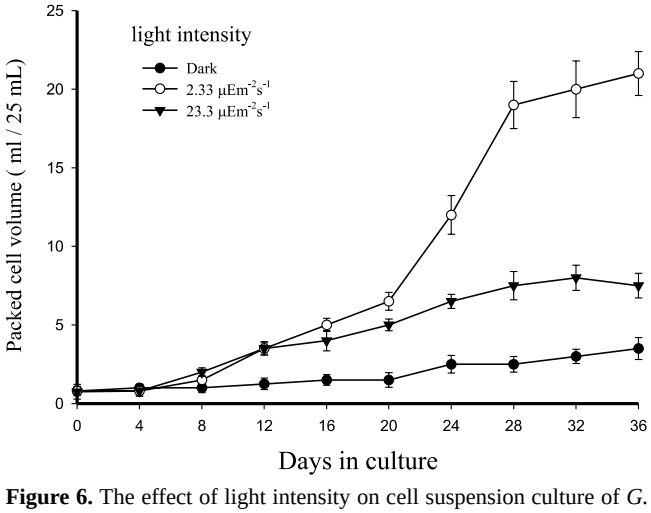

Figure 6. The effect of light intensity on cell suspension culture of *G. davidii* var. *formosana*. The basal medium was MS salts supplemented with 0.2 mg/L kinetin, 3% sucrose, pH 5.2, initial PCV 1 mL and incubated at 80 rev/min shaking speed.

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Culture mediu m pH is an important factor in plant morphogenesis and production of secondary metabolites. Figure 7 shows the effect of medium pH (4.2, 5.2, 6.2 and 7.2) on growth of suspension cells after 36 days of culture. Optimum growth of suspension cells was observed in medium with pH 4.2 and 5.2. Higher pH (6.2 and 7.2) resulted in not only browning of cell suspension but also affected the cell growth. By contrast, optimum growth of suspension cells of *Angelica dahurica* var. *formosana* and rice was observed in medium with pH 7 and pH 6 respectively[11,17].

III. *Effect of Culture Duration on Production of Gentiopicroside and Swertiamarin*

Suspension cells cultured in MS medium containing 0.2 mg/L kinetin, 3% sucrose, with pH at 5.2 under 2.33 μE·m^{-2}·s^{-1} and 80 rev/min were harvested every four days to measure the content of gentiopicroside and swertiamarin. We found that the maximum content of gentiopicroside (1.6 ± 0.01 mg/g dry wt. of suspension cells) was observed after 24 days of culture; while the highest content of swertiamarin (2.24 ± 0.26 mg/g dry wt.) was obtained after 12 days of culture (Figure 8).

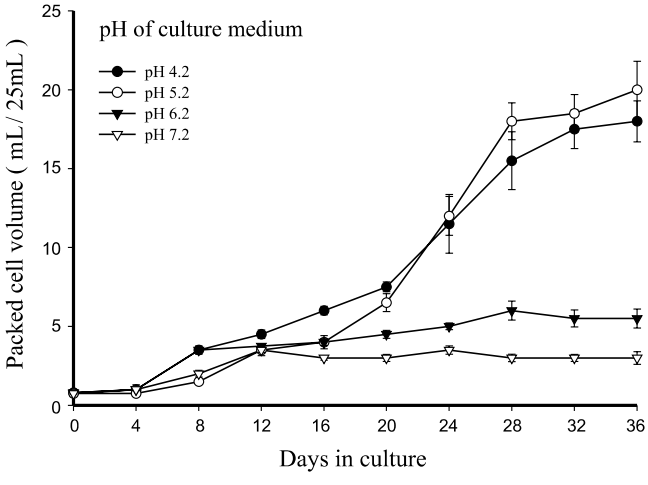

Figure 7. The effect of initial pH of culture medium on cell suspension culture of *G. davidii* var. *formosana*. The basal medium was MS salts supplemented with 0.2 mg/L kinetin, 3% sucrose, initial PCV 1 mL and incubated under low light intensity (2.33 μE·m^{-2}·s^{-1}) and 80 rev/min shaker speed.

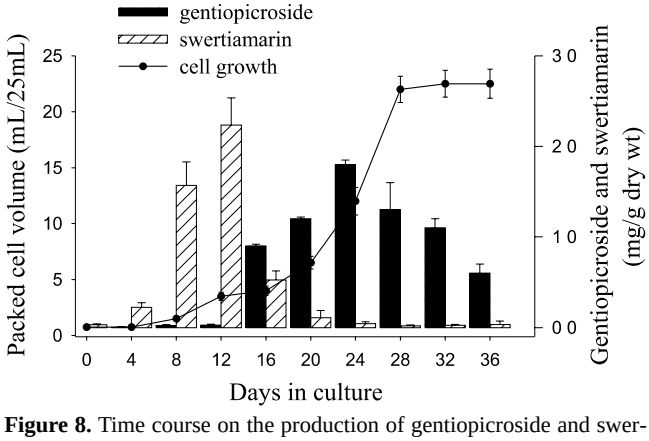

Figure 8. Time course on the production of gentiopicroside and swertiamarin and the suspension cell growth of *G. davidii* var. *formosana*. The basal medium was MS salts supplemented with 0.2 mg/L kinetin, 3% sucrose, pH 5.2, initial PCV 1 mL and incubated under low light intensity (2.33 μE·m^{-2}·s^{-1}) at 80 rev/min shaker speed.

Figure 9. The cell suspension culture of *G. davidii* var. *formosana*. The suspension cell was cultured in a medium containing MS basic salts supplemented with 0.2 mg/L kinetin, 3% sucrose, pH 4.2-5.2 and incubated under low light intensity (2.33 μE·m^{-2}·s^{-1}) at 80 rev/min shaker speed.

CONCLUSIONS

In summary, our study showed that evenly distributed, fast-growing suspension cells could be obtained from stem-derived callus of *G. davidii*. Optimal cell growth was obtained when the callus was cultured in 25-mL liquid MS basal medium supplemented with 0.2 mg/L kinetin, 3% sucrose, pH between 4.2-5.2, incubated in light 2.33 μE·m^{-2}·s^{-1} at 25 ± 1°C, under 80-100 rev/min shaker speed (Figure 9) and subcultured every five weeks. The maximum content of the two active principles, swertiamarin and gentiopicroside, in cell suspension were obtained after 12 and 24 days of culture respectively. Using this standardized protocol, it may be possible to study the effect of precursor feeding on the content of active principles.

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臺灣龍膽細胞懸浮培養之建立及培養條件之探討

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摘　要

龍膽（Gentianae Radix）首載於神農本草經，列為上品，為常用之苦味健胃消炎藥。近年來由於龍膽藥材市場需求量遠超出其自然繁殖量，使得野生資源瀕臨枯竭。省產優質生藥的開發，藉由細胞懸浮培養技術，進行藥用植物二次代謝物之生產，除具保育作用外，亦有助於臺灣製藥工業競爭力之提升，本研究乃進行臺灣龍膽 [*Gentiana davidii* var. *formosana* (Hayata) T. N. Ho] 細胞懸浮培養之建立。試驗結果顯示利用含有0.2 mg/L kinetin 及1.0 mg/L NAA之MS (Murashige & Skoog, 1962）基本鹽類培養基培養臺灣龍膽莖段所誘導之癒合組織，經繼代培養在MS基本鹽類添加0.2 mg/L kinetin、3% sucrose及pH 4.2~5.2之液體培養基，於2.33 $\mu E \cdot m^{-2} \cdot s^{-1}$光照、25 ± 1˚C恆溫及 80~100 rev/min轉速下培養，可得一分散均勻，生長良好之懸浮細胞。本研究所建立之臺灣龍膽懸浮培養細胞，將有助於其二次代謝物龍膽皂啟（gentiopicroside）及當藥皂啟（swertiamarin）之生產及高產細胞系之篩選。

關鍵詞：臺灣龍膽，懸浮培養，龍膽皂啟，當藥皂啟