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Full Length Research Paper

Induced accumulation of 20-hydroxyecdysone in cell suspension cultures of *Vitex glabrata* R.Br.

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This study describes the effects of culture medium, culture temperature, sucrose concentration and cholesterol feeding on cell growth and 20-hydroxyecdysone production in suspension cultures of *Vitex glabrata*, an important medicinal plant in Thailand. Cell growth and 20-hydroxyecdysone production were not significantly different when cells were cultivated on B5 or half-strength MS medium. However, cultivation of *V. glabrata* cell cultures at 25°C yielded 1.06- and 1.09-fold higher values of cell growth and 20-hydroxyecdysone content, respectively than those at 30°C. Sucrose at 30 and 40 g/L favors the production of 20-hydroxyecdysone in suspension cultures of *V. glabrata*. Feeding of cholesterol at 5 mg/L, as precursor for biosynthesis of 20-hydroxyecdysone, yielded 1.11-fold higher accumulation of 20-hydroxyecdysone than the control cells. Increasing of cholesterol to 10 mg/L resulted in decreased production of 20-hydroxyecdysone.

Key words: *Vitex glabrata*, 20-hydroxyecdysone, suspension culture, cholesterol.

INTRODUCTION

Vitex glabrata R.Br., commonly known in Thailand as Kai Nao Tree, is an important medicinal plant belonging to the Verbenaceae family. This plant species produces some specific sterols such as 7-dehydrocholesterol as well as ecdysteroids such as alpha-ecdysone and 20-hydroxyecdysone (Werawattanametin, 1986). Among these compounds, 20-hydroxyecdysone is particularly attractive since it has been used in a wide range of applications, example, as a growth stimulating agent for shrimp culture (Chaiwatcharakool, 1986), insecticides (Dhadialla and Tzertzinis, 1998), an anabolic steroid in sport and bodybuilding, and tonic supplement for male and female reproductive system (Ahmad et al., 2000; Bathori, 2002; Dinan and Lafont, 2006). *V. glabrata* accumulates high levels of 20-hydroxyecdysone in the stem bark. However, this plant grows quite slowly and the stem bark suitable for direct extraction takes years to grow. Therefore, the strategy of plant cell culture offers great potential for 20-hydroxyecdysone production. Plant cell culture has several advantages over the traditional cultivation, example, the control of the production conditions, weather independency and continuous production (Rao and Ravishankar, 2002; Zabala et al., 2010).

There have been only a few papers concerning the *in vitro* cell culture of *V. glabrata* for the production of 20-hydroxyecdysone, since its first report by Thavornnithi (1990). For example, Prasertsom (1990) investigated the effect of culture medium and plant growth regulator on cell growth and 20-hydroxyecdysone production in *V. glabrata* cell cultures. Duanghaklang (2001) examined the cultivation kinetics of *V. glabrata* suspension cultures in a 2-L air-lift bioreactor. Recently, Sinlaparaya et al. (2007) used precursors to improve the production of 20-hydroxyecdysone in suspension cultures of *V. glabrata*. Obviously, there still remain many other issues to be evaluated for the cell cultures. These include identification of key factors influencing the production of 20-hydroxyecdysone in suspension cultures of *V. glabrata*

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and optimization of the cultivation conditions.

It is known that many cultivation factors could affect cell growth and metabolites production in plant cell cultures. These include culture medium, carbon source, precursors, elicitors, oxygen, pH of the medium, culture temperature and light condition (Endress, 1994; Sinlaparaya et al., 2007). Among these conditions, culture medium, carbon concentration, culture temperature and precursors have been shown to influence the cell growth and secondary metabolites production in several plant species such as *Taxus chinensis* (Choi et al., 2000), *Uncaria tomentosa* (Feria-Romero et al., 2005), *Artemisia annua* (Wang and Weathers, 2007), *Ehinacea purpurea* (Wu et al., 2007) and *Withania somnifera* (Nagella and Murthy, 2010). Therefore, these cultivation conditions were investigated in this study with the goal to enhance the production of 20-hydroxyecdysone in suspension cultures of *V. glabrata*.

MATERIALS AND METHODS

Callus of *V. glabrata*, initially induced from stem and maintained in this laboratory for more than 10 years by subculturing every 2 weeks, was used in this study. It was cultured on solidified growth medium, which is the half-strength Murashige and Skoogs's (MS) medium (Murashige and Skoog, 1962), supplemented with 2.0 mg/L 6-benzylaminopurine (BAP), 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/L sucrose and 8.0 g/L agar. The cultures were incubated at 25 ± 2°C under 2000 lux intensity of light and 16/8 h light/dark photoperiod.

Establishment of cell suspension cultures

For cell suspension cultures, 20 g of callus (fresh weight basis) were aseptically transferred to 500 ml Erlenmeyer flasks containing 200 ml of half-strength MS liquid medium supplemented with 2.0 mg/L BAP, 1.0 mg/L 2,4-D and 30 g/L sucrose. The flasks were incubated in a rotary shaker at 120 rpm under 2000 lux intensity of light and 16/8 h light/dark photoperiod at 25 ± 2°C. Cells from 7-day-old suspension culture were used for further experiments.

Optimization of cultivation conditions

The 7-day-old suspension cultures were aseptically transferred to 500 ml Erlenmeyer flasks containing 200 ml of half-strength MS liquid medium supplemented with 2.0 mg/L BAP, 1.0 mg/L 2,4-D and 30 g/L sucrose. The cultures were kept under continuous agitation at 120 rpm in a rotary shaker and incubated at 25 ± 2°C, with a 16/8 h light/dark photoperiod. The effect of culture media; B5 (Gamborg et al., 1968) and half-strength MS, on the accumulation of biomass and 20-hydroxyecdysone production in suspension cultures of *V. glabrata* was assessed. To determine the optimal temperature for cell growth and 20-hydroxyecdysone production, different levels of temperature (25 ± 2 and 30 ± 2°C) were tested in separate experiments. The effect of different sucrose concentrations on cell growth and 20-hydroxyecdysone production were investigated by addition of sucrose into the culture medium at 20, 30 and 40 g/L. The effect of different concentrations of cholesterol (0, 5 and 10 mg/L), a precursor in the biosynthesis of 20-hydroxyecdysone was also examined for the biomass accumulation and 20-hydroxyecdysone production. During cultivation, cells were harvested every 2 days and analyzed for biomass and 20-hydroxyecdysone content by using HPLC. The data shown represents the mean of three replicates and ± standard deviation (SD) values are presented as error bars.

Determination of cell growth

Cell growth was monitored by measuring the increase in the cell dry weight (DW) of the cultures. After harvesting the cultures, media and cells were separated by filtration through filter paper under vacuum. The cell mass on the filter was rinsed twice with double distilled water and dry weight was determined after drying the cell at 60°C in a hot air oven until a constant weight was obtained.

20-Hydroxyecdysone extraction and HPLC analysis

20-Hydroxyecdysone was extracted from dried cells as described by Duanghaklang (2001). A 0.3 g mass of dried cells was extracted with 95% ethanol (180 ml) in a soxhlet apparatus for 6 h. The ethanol extract was evaporated by rotary evaporator at 60°C. The resulting residue was dissolved in 3 ml methanol and vortexed with 2 ml hexane twice. The methanol extracts were evaporated at 60°C in a hot air oven and the residue was dissolved in 2 ml distilled water. The resulting solution was filtered through Sep-pak C18 cartridge. Highly polar material was separated from the retained 20-hydroxyecdysone fraction by elution with 10 ml distilled water. 20-Hydroxyecdysone was eluted from the cartridge with 20% (v/v) methanol-water (10 ml) and 80% (v/v) methanol-water (10 ml), respectively. The eluted extract was collected and dried at room temperature and dissolved in methanol for HPLC analysis.

20-Hydroxyecdysone was analyzed by HPLC using ODS-3 C18 column with detection at 254 nm. The elution was performed with isocratic gradient of 14% acetonitrile in 2% acetic acid at the flow rate of 1.0 ml/min. The injection volume of each sample was 20 µl and the quantity of 20-hydroecdysone was calculated as described by Sinlaparaya et al. (2007). The 20-hydroxyecdysone standard was purchased from Sigma-Aldrich Co., Ltd.

RESULTS AND DISCUSSION

Effect of culture media on cell growth and 20-hydroxyecdysone production

Level of secondary metabolites produced by *in vitro* cultures can vary dramatically depending on the composition of culture medium (Rao and Ravishankar, 2002). In the present study, the effects of culture media like B5 and half-strength MS on cell growth and 20-hydroxyecdysone production in *V. glabrata* suspension cultures were assessed and the results are illustrated in Figure 1. The maximum accumulation of biomass and 20-hydroxyecdysone content derived from cells cultivated in half-strength MS medium were slightly lower than that of cells cultivated in B5 medium. The lower cell growth and 20-hydroxyecdysone production observed under half-strength MS medium could be explained by the fact that in this medium, all the nutrients added were half the concentration as compared to MS medium, thus, the cells could have exhausted the medium faster, causing the lower biomass and 20-hydroxyecdysone production. Our results are similar to those of Zabala et al. (2010) who

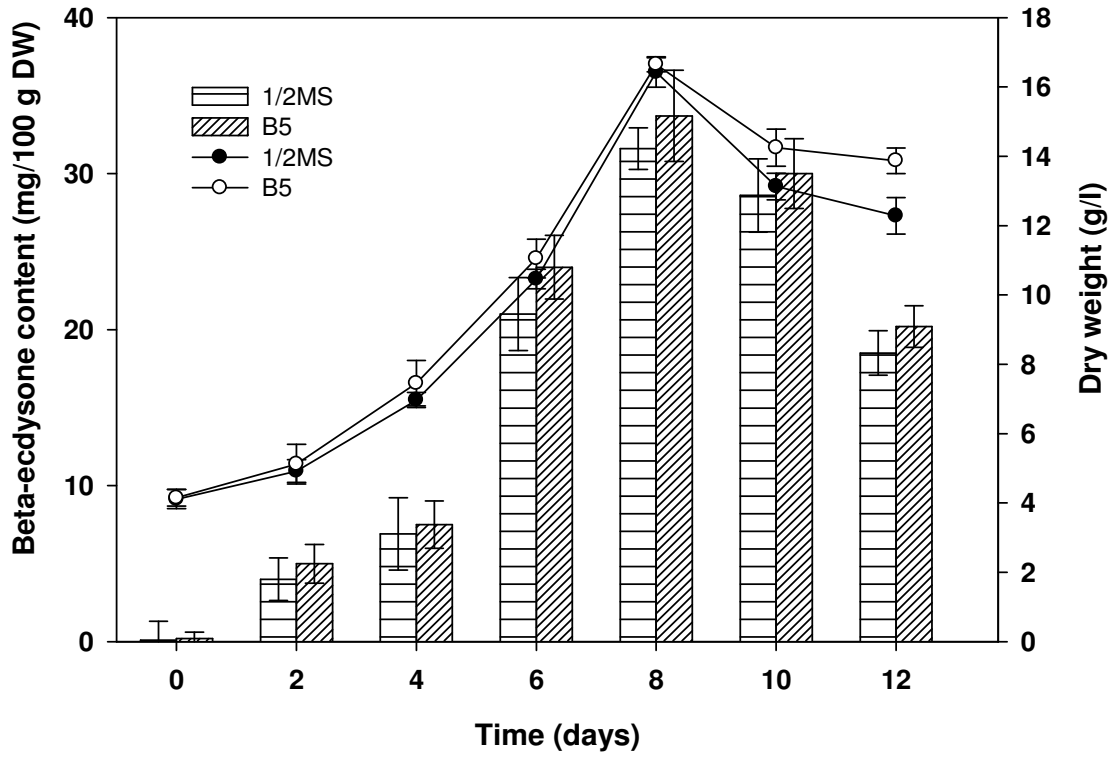

Figure 1. Effect of culture medium on cell growth and 20-hydroxyecdysone production in *V. glabrata* suspension cultures. Cells were grown in B5 or half-strength MS medium and incubated in a rotary shaker at 120 rpm and 25 ± 2°C under 2000 lux intensity of light and 16/8 h light/dark photoperiod. Data represents mean values ±SD of three replicates.

observed the lower cell growth index of *Thevetia peruviana* suspension cultures when cultivated in half-strength MS medium as compared to full strength medium. In contrast to our findings, Lian et al. (2002) reported that half-strength and full strength MS medium were equally suitable for cell growth and ginsenoside production in suspension cultures of *Panax ginseng*.

It should be noted that the composition of B5 nutrients was almost 2 times higher than that of half-strength MS medium but cell growth and 20-hydroxyecdysone production were not significantly different. Therefore, from an economical point of view, the half-strength MS medium was chosen for further experiments.

Effect of culture temperature on cell growth and 20-hydroxyecdysone production

Temperature as a fundamental parameter has many effects on the nature and mechanisms of metabolic regulation, permeability, nutritional needs and the rate of intracellular reactions in plant cell cultures. Thus, changing the culture temperature may change the physiology and metabolism of cultured cells (Zhang et al., 1997). In this study, the effect of culture temperature on cell growth and 20-hydroxyecdysone production in suspension culture of *V. glabrata* was studied by incubating cells at 25 and 30°C in temperature controlled shaking incubator. As shown in Figure 2, maximum cell growth was achieved when cells were cultivated at 25°C and its dry cell weight was 16.30 g/L at day 8. When cells were cultivated at 30°C, dry cell weight was 15.31 g/L at day 8, which was 1.06-fold lower than that at 25°C. Cell growth declined sharply after day 8 under both culture temperatures. This might be due to the loss of viability of cells as shown in other plant cell species (Zhang et al., 1997; Hoopen et al., 2002).

The accumulation of 20-hydroxyecdysone in the suspension culture of *V. glabrata* was also affected by culture temperature. The highest concentration of 20-hydroxyecdysone (33.49 mg/100 g DW) was obtained when cells were cultivated at 25°C, which was 1.09-fold higher than that at 30°C. This result implies that 20-hydroxyecdysone production in *V. glabrata* suspension culture favors low temperature environment than high temperature. Our result is comparable to the production of anthocyanin in strawberry culture cells (Zhang et al., 1997), ginsenoside in hairy root cultures of *Panax ginseng* (Yu et al., 2005) and caftaric acid, chlorogenic acid and cichoric acid in suspension culture of *E. purpurea* (Wu et al., 2007), in which lower temperature yielded high concentration of the secondary metabolites.

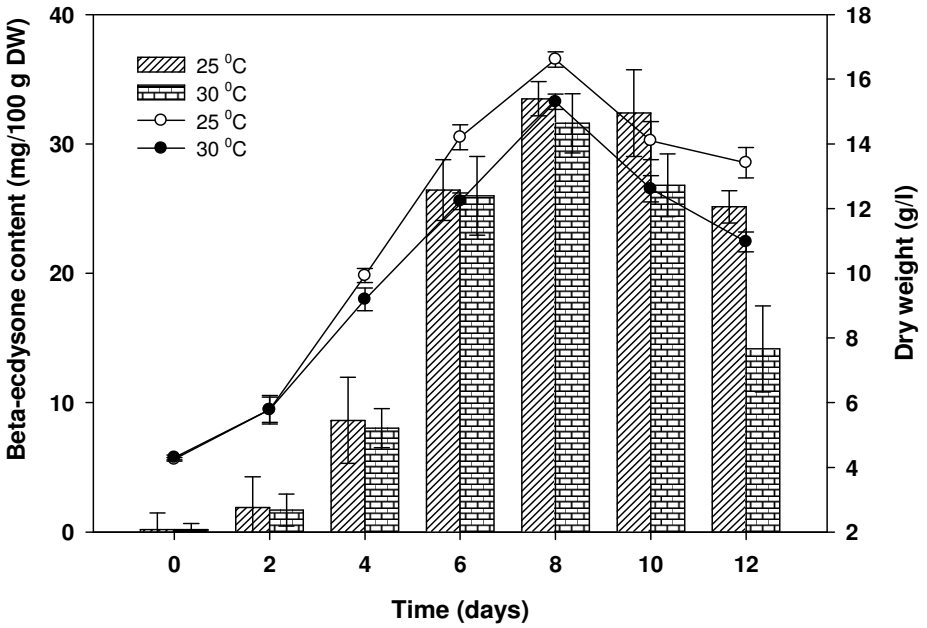

Figure 2. Effect of incubation temperature on cell growth and 20-hydroxyecdysone production in *V. glabrata* suspension cultures. Cells were grown in half-strength MS medium containing 30 g/L sucrose and incubated in a rotary shaker at 120 rpm and 25 ± 2 or 30 ± 2℃ under 2000 lux intensity of light and 16/8 h light/dark photoperiod. Data represents mean values ±SD of three replicates.

Effect of sucrose concentration on cell growth and 20-hydroxyecdysone production

Sucrose has been used as a common carbon source for plant tissue and cell cultures. It serves as the principal energy source and also as a molecule that can be sensed in plants, thereby inducing signals that affect metabolism, development, growth and secondary metabolite biosynthesis (Rao and Ravishankar, 2002; Nagella and Murthy, 2010). Generally, the growth rate and secondary metabolite production in plant are directly correlated with sugar consumption. Therefore, the effect of sucrose concentration on cell growth and 20-hydroxyecdysone production in suspension culture of *V. glabrata* was investigated in this study and the results are shown in Figure 3. The initial sucrose concentration influenced the time for reaching the maximum biomass accumulation and 20-hydroxyecdysone content. Cells grown in half-strength MS medium containing 30 g/L sucrose showed the highest biomass and 20-hydroxyecdysone concentration at day 8, while those cultivated in medium containing 20 and 40 g/L sucrose showed the highest biomass and 20-hydroxyecdysone concentration at day 10. The maximum accumulation of biomass (16.07 g/L) and 20-hydroxyecdysone content (33.57 mg/100 g DW) were achieved when cells were cultivated in half-strength MS medium containing 40 g/L sucrose, while the lowest values were obtained when cells were cultivated in the medium containing 20 g/L sucrose. There were no significant differences in the maximum value of 20-hydroxyecdysone concentration between cells cultivated in the medium containing 30 and 40 g/L sucrose, suggesting that sucrose at both 30 and 40 g/L favor the production of 20-hydroxyecdysone in *V. glabrata* suspension cultures. Our results are similar to ginsenoside production in *P. ginseng* suspension culture (Lian et al., 2002) and withanolide A production in *Withania somnifera* suspension culture (Nagella and Murthy, 2010), in which 30 g/L sucrose favored production of the secondary metabolites. In contrast to our findings, Zhong (2002) showed that 60 g/L sucrose favored the paclitaxel accumulation in cell cultures of *T. chinensis*. In addition, Shinde et al. (2009) also showed that sucrose at 50 to 70 g/L favored daidzein production in suspension cultures of *Psoralea corylifolia*.

Effect of cholesterol on cell growth and 20-hydroxyecdysone production

In the biosynthesis of secondary metabolites by plant cell cultures, precursor feeding at appropriate concentrations can promote the accumulation of secondary metabolites (Ouyang et al., 2005). Cholesterol is one of the precursors involved in the biosynthesis of 20-hydroxyecdysone. Previous studies by Sinlaparaya et al. (2007) demonstrated that cholesterol feeding at 100 and 200 mg/L inhibited cell growth and 20-hydroxyecdysone production in *V. glabrata* suspension cultures. This might be due to the feedback inhibition of the metabolite

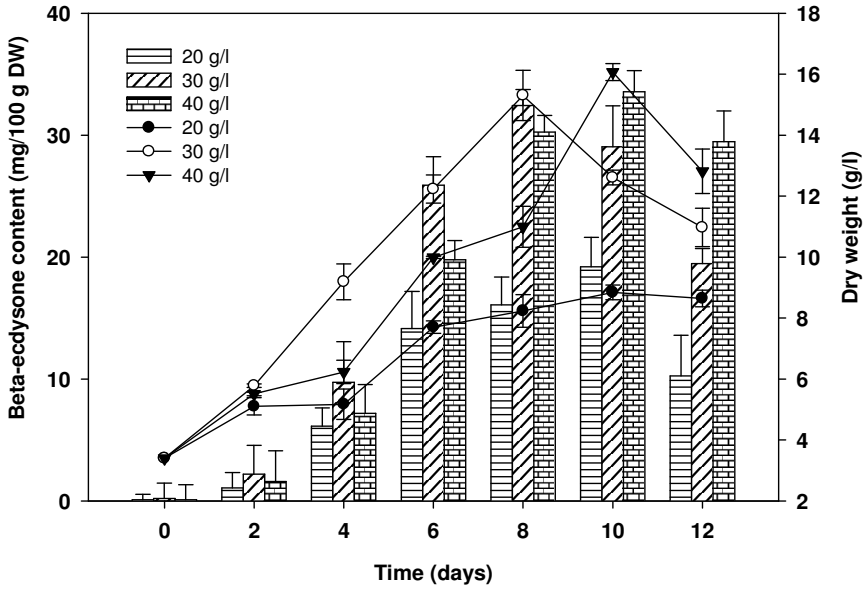

Figure 3. Effect of sugar concentration on cell growth and 20-hydroxyecdysone production in *V. glabrata* suspension cultures. Cells were grown in half-strength MS medium containing 20, 30 or 40 g/L sucrose and incubated in a rotary shaker at 120 rpm and 25 ± 2°C under 2000 lux intensity of light and 16/8 h light/dark photoperiod. Data represents mean values ±SD of three replicates.

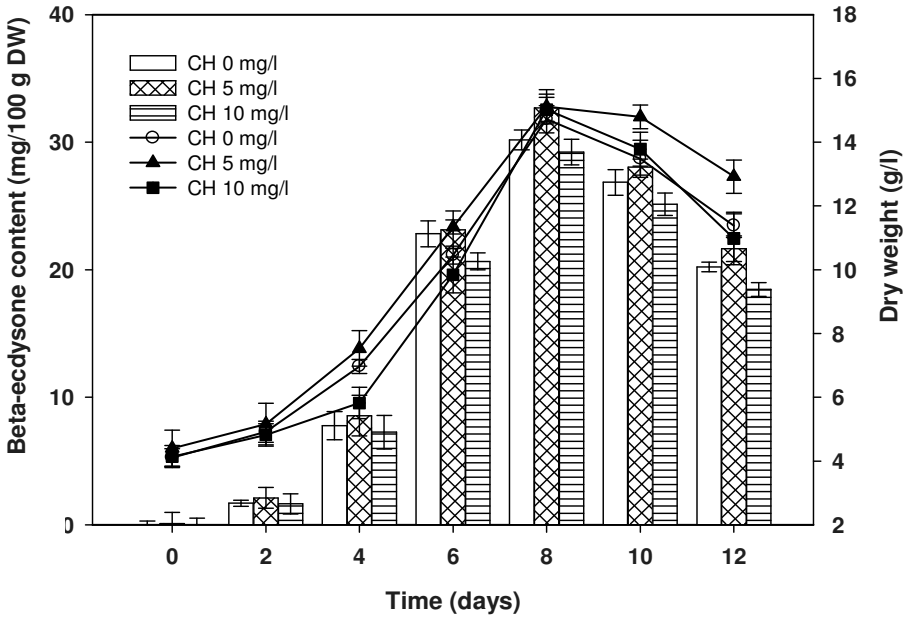

Figure 4. Effect of cholesterol on cell growth and 20-hydroxyecdysone production in *V. glabrata* suspension cultures. Cells were grown in half-strength MS medium containing 30 g/L sucrose and 5 or 10 mg/L cholesterol and incubated in a rotary shaker at 120 rpm and 25 ± 2°C under 2000 lux intensity of light and 16/8 h light/dark photoperiod. Data represents mean values ±SD of three replicates.

pathway caused by the excess amount of cholesterol (Ouyang et al., 2005). Therefore, in this study, the effect of cholesterol at low concentrations (5 and 10 mg/L) was tested and the results are shown in Figure 4. Cholesterol at 5 mg/L showed a positive effect on the cell growth and 20-hydroxyecdysone production. After 8 days cultivation,

20-hydroxyecdysone content in the medium with 5 mg/L cholesterol reached 33.45 mg/100 g DW, which was 1.11-fold higher than the control cells. The 20-hydroxyecdysone content decreased with the increase of cholesterol concentration to 10 mg/L. These results suggest that cholesterol at 5 mg/L favors the production of 20-hydroxyecdysone in *V. glabrata* suspension cultures.

Conclusion

In conclusion, the cell growth and 20-hydroxyecdysone production of *V. glabrata* suspension cultures depended on culture temperature, type of culture medium, sucrose concentration and precursor feeding. It has been demonstrated in this study that *V. glabrata* cell cultures required culture temperature of up to 25°C and half-strength MS medium supplemented with 30 or 40 g/L sucrose. Feeding of cholesterol at 5 mg/L as precursor also improved the production of 20-hydroxyecdysone. Our finding could be recommended as one of the biotechnological-based methodology for large scale production of 20-hydroxyecdysone from *V. glabrata*.

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