

Full Length Research Paper

Enhance production of 20-hydroxyecdysone in cell suspension cultures of *Vitex glabrata* R.Br. by elicitor feeding

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The effects of chitosan and methyl jasmonate on growth and 20-hydroxyecdysone production in cell suspension cultures of *Vitex glabrata*, a medicinal plant in Thailand, were investigated. Elicitation with chitosan at 50 mg/L resulted in 17.16 g/L biomass and 377.09 mg/100 g dry weight (DW) 20-hydroxyecdysone, which were 1.62 and 8.33 times higher than the control cultures, respectively. Likewise, addition of methyl jasmonate at 100 µM also enhanced growth and production of 20-hydroxyecdysone. The highest growth and 20-hydroxyecdysone production reached 14.44 g/L and 621.76 mg/100 g DW, which were 1.35 and 14.54 times higher in comparison to the control cultures, respectively. This is the first report to indicate that elicitation with chitosan and methyl jasmonate enhanced the production of 20-hydroxyecdysone in cell suspension cultures of *V. glabrata*.

Key words: *Vitex glabrata*, 20-hydroxyecdysone, cell suspension culture, chitosan, methyl jasmonate.

INTRODUCTION

Vitex glabrata R.Br. (commonly known as “Kai Nao”), an important medicinal plant of the family Verbenaceae, has been reported to accumulate high content of ecdysteroid, 20-hydroxyecdysone or beta-ecdysone in stem bark (Werawattanametin, 1986). This ecdysteroid plays a crucial role in molting, metamorphosis, reproduction and diapause of arthropods (Butenandt and Karson, 1954; Makka et al., 2003). A wide range of applications of this ecdysteroid and its analogues have been reported, e.g. as insecticides (Dhadialla and Tzertzinis, 1998), a growth stimulating agent for shrimp culture (Chaiwatcharakool, 1986), an anabolic steroid in sport and bodybuilding and tonic supplement for male and female reproductive system (Ahmad et al., 2000c; Bathori, 2002; Dinan and Lafont, 2006). Beside, some other compounds, e.g. vitexcarpan, 6a, 11a-dihydro-6H-[1] benzofuro [3,2-c][1,3] dioxolo [4,5-g] chromen-9-ol, of the related plant from the

same family (Verbenaceae), such as *Vitex agnus castus* have been reported for their effectiveness in anti-inflammatory, antibacterial activity, phytotoxic activity and urease- and chymotrypsin-inhibitory activity (Ahmad et al., 2010a; Ahmad et al., 2010b).

Like many other species of Thai medicinal plants, the *V. glabrata* is now almost extinct due to over-exploiting. In addition, this plant grows quite slowly and the stem bark suitable for extraction takes years to grow. Therefore, cell cultures of this medicinal plant offer great potential for 20-hydroxyecdysone production (Rao and Ravishankar, 2002). Production of 20-hydroxyecdysone from *V. glabrata* using plant cell cultures has been reported. Many strategies have been used to increase the production of 20-hydroxyecdysone from *V. glabrata* cell cultures, such as medium optimization (Thavornnithi, 1990; Prasertsom, 1990), precursor feeding and cell line selection (Duanghakang, 2001; Sinlaparaya et al., 2007). However, little is known about the effect of elicitor feeding on production of 20-hydroxyecdysone in this plant. Although, feeding of elicitors has been reported to enhance the production of secondary metabolites in

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several plants, e.g. *Artemisia annua* (Liu et al., 1999; Putalun et al., 2007; Baldi and Dixit, 2008), *Panax notoginseng* (Wang and Zhong, 2002), *Taxus chinensis* (Lan et al., 2002), *Lavandula vera* (Georgiev et al., 2007), *Catharanthus roseus* (Lee-Parsons et al., 2004), *Linum nodiflorum* (Berim et al., 2005), *Saussurea medusa* (Xu et al., 2007a), *Hydrilla verticillata* (Xu et al., 2007b), *Cocos nucifera* (Chakraborty et al., 2009) and *Thevetia peruviana* (Zabala et al., 2010), and to our knowledge, this is the first study to investigate the effects of chitosan and methyl jasmonate on 20-hydroxyecdysone accumulation in *V. glabrata* cell cultures. Our results demonstrated that elicitation with chitosan and methyl jasmonate significantly increased the production of 20-hydroxyecdysone in suspension cultures of *V. glabrata*.

MATERIALS AND METHODS

Plant

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 Skoog'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 $25 \pm 2^\circ\text{C}$ 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 on a rotary shaker at 120 rpm under 2000 lux intensity of light and 16/8 h light/dark photoperiod at $25 \pm 2^\circ\text{C}$. Cells from 7-day-old suspension culture were used for further experiments.

Growth of suspension cultures and 20-hydroxyecdysone content

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, and was incubated on a rotary shaker at 120 rpm under 2000 lux intensity of light and 16/8 h light/dark photoperiod at $25 \pm 2^\circ\text{C}$ for 12 days. Cells were harvested every 2 days to determine the dry weight and 20-hydroxyecdysone content by using high performance liquid chromatography (HPLC). Each experiment was performed in triplicate.

Effect of elicitors on 20-hydroxyecdysone production

The method of elicitation with chitosan (Fluka Biochemika) and methyl jasmonate (Sigma-Aldrich) was adopted according to Putalun et al. (2007). A stock solution of chitosan was prepared by dissolving in dilute HCl by gentle heating. The pH of this solution was adjusted to 5.0 with 1 N NaOH and then sterilized at 121°C for 15 min. Methyl jasmonate was prepared by dissolving in ethanol and then filter-sterilized through a microfilter (0.2 μm). Each elicitor

in the following final concentration was added: (1) chitosan (Ch) 50, 100 and 200 mg/L; (2) methyl jasmonate (MJ) 50, 100 and 200 μM . Elicitors were added to 6-day-old suspension cultures under aseptic condition. Control cultures were treated with sterile distilled water and ethanol for chitosan and methyl jasmonate treatment, respectively. The cultures were further cultivated on a rotary shaker at 120 rpm under 2000 lux intensity of light and 16/8 h light/dark photoperiod at $25 \pm 2^\circ\text{C}$ for 10 days. Samples were taken every 2 days and analyzed for biomass and 20-hydroxyecdysone content. The data including means of three replicates and \pm standard deviation (SD) values are presented as error bars.

Determination of dry cell weight

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.

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 extracts were 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 elution 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 20-hydroxyecdysone purchased from Sigma-Aldrich was used as the standard control in the measurement. For each sample, the injection volume was 20 μl , and the results were analyzed with Empower data system.

RESULTS AND DISCUSSION

Time course of cell growth and 20-hydroxyecdysone production in *V. glabrata* suspension cultures

The time course of cell growth and 20-hydroxyecdysone production in suspension cultures are as shown in Figure 1. Cells grew faster over 4 days and gave the highest dry weight of 16.42 g/L at day 8. The 20-hydroxyecdysone contents increased from day 2 and reached its maximum level at 31.60 mg/100 g DW on day 8. These results are similar to the observation of Duanghaklang (2001) who showed that the maximum peak of 20-hydroxyecdysone accumulation was detected at day 8. After day 8, cell dry weight was decreased, probably due to the depletion of nutrient which was correlated to the reduction of sucrose in the culture medium (data not shown). 20-

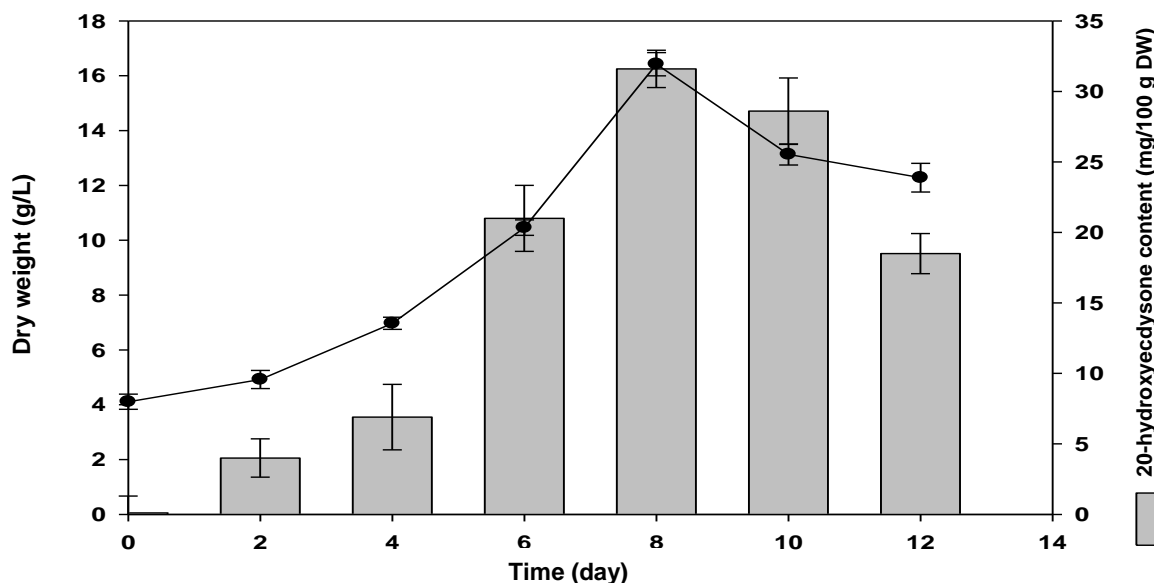


Figure 1. Time course of *V. glabrata* cell growth and 20-hydroxyecdysone production in cell suspension culture at $25 \pm 2^\circ\text{C}$. Vertical bars represent standard deviation of three replications.

hydroxyecdysone was produced intracellularly and most production occurred during the active growth phase of *V. glabrata* cells (Sinlaparaya et al., 2007). Therefore, the 6-day-old suspension cultures were chosen for elicitation experiments.

Effect of chitosan on cell growth and 20-hydroxyecdysone production in *V. glabrata* suspension cultures

Figure 2 shows the effect of chitosan on cell growth and 20-hydroxyecdysone production in suspension cultures of *V. glabrata*. 2 h after addition of chitosan, the amounts of accumulated biomasses were significantly increased at all concentrations tested. The highest value of biomass (17.16 g/L), which was 1.62-fold higher than the control cultures, was obtained when cells were treated with 50 mg/L chitosan (Figure 2A).

The influences of chitosan on 20-hydroxyecdysone production are illustrated in Figure 2B. Chitosan at 50 mg/L gave the highest concentration of 20-hydroxyecdysone (377.09 mg/100 g DW) after 8 days, which was 8.33-fold higher than the control cultures. Chitosan has been proved to enhance various secondary metabolites, such as indirubin (Kim et al., 1997), plumbagin (Komaraiah et al., 2002), paclitaxel (Luo and He, 2004), syringin (Xu et al., 2007a), artemisinin (Putalun et al., 2007) and *p*-hydroxybenzoic acid (Chakraborty et al., 2009). In the suspension cultures of *Plumbago rosea*, more than 6-fold increase of plumbagin was detected when subjected to chitosan at 150 mg/L (Komaraiah et al., 2002). In the hairy root cultures of *A.*

annua, 6-fold higher artemisinin was accumulated when treated with chitosan at 150 mg/L (Putalun et al., 2007). In our study, chitosan at 50 mg/L was found to be the most effective and highest amount of 20-hydroxyecdysone accumulation was obtained as compared to other concentrations. It should be noted from these findings that the optimal concentrations of chitosan for enhancing the production of secondary metabolites are normally dependent on plant species (Vasconsuelo and Boland, 2007). It was also observed that the accumulation of 20-hydroxyecdysone in cells of *V. glabrata* declined sharply when the chitosan concentration was higher than 100 mg/L. This might be due to change in cell membrane permeability, as evident from increase in medium conductivity on addition of chitosan (data not shown). Our findings are similar to those reported by Zhang et al. (2007) and Baldi and Dixit (2008). Chitosan can be used not only as elicitor, but also as a permeabilizing agent (Young et al., 1982; Knorr and Teutonicw, 1986). Previous study by Komaraiah et al. (2002) showed that 70% of plumbagin was produced extracellularly when suspension cultures of *P. rosea* were treated with chitosan at concentration of more than 150 mg/L.

Effect of methyl jasmonate on cell growth and 20-hydroxyecdysone production in *V. glabrata* suspension cultures

A typical growth curve and 20-hydroxyecdysone production in *V. glabrata* suspension cultures with and without methyl jasmonate is as shown in Figure 3.

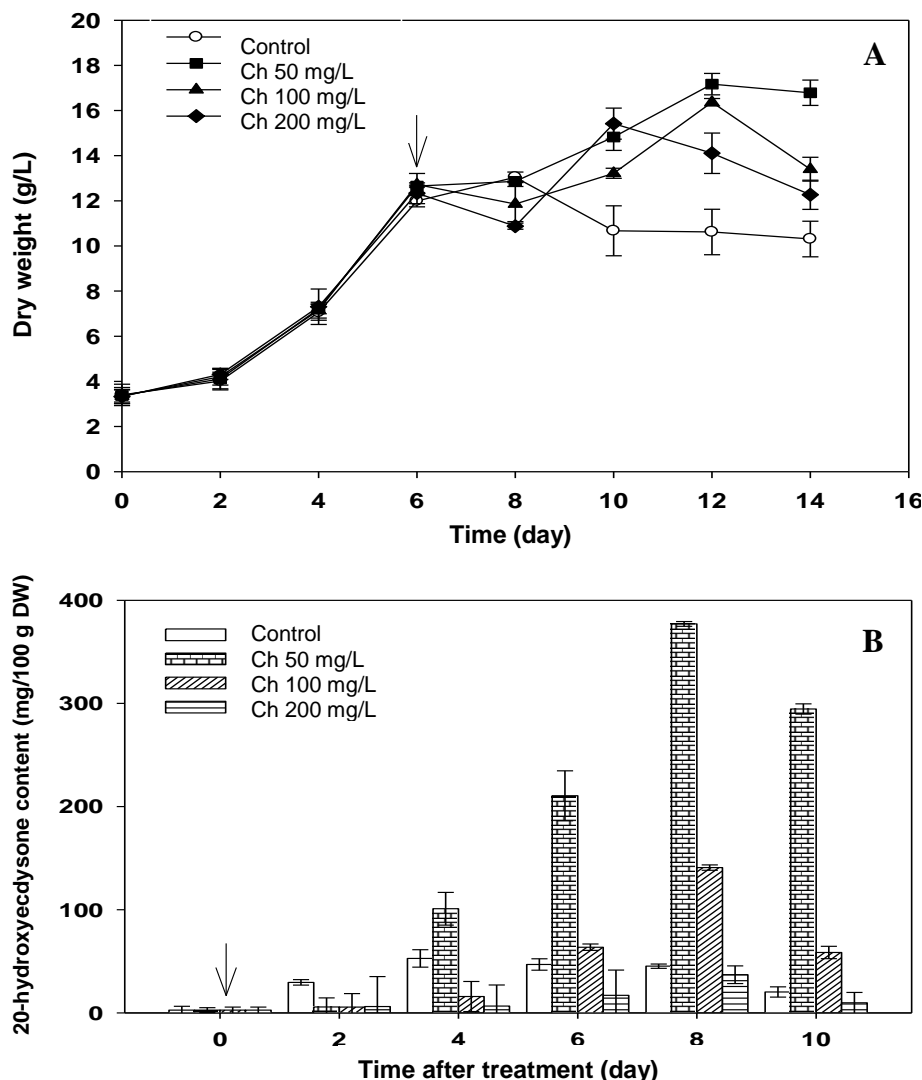


Figure 2. Effect of chitosan on growth (A) and 20-hydroxyecdysone production (B) in cell suspension cultures of *V. glabrata*. Chitosan (50, 100 and 200 mg/L) was added to 6-day-old cultures as indicated by the arrowhead and cultivated at $25 \pm 2^\circ\text{C}$ for a further 10 days. Vertical bars represent standard deviation of three replications.

Addition of methyl jasmonate influenced the *V. glabrata* growth, like that observed with chitosan treatment. Four days after its addition, the amounts of accumulated biomasses were enhanced as compared to the control cultures. When the cells were treated with 100 or 200 μM methyl jasmonate, the yield of accumulated biomass reached about 14.44 g/L, almost 1.35-fold increased over control cells (Figure 3A). After 6 days of elicitation until the end of the culture period, the growth patterns of treated cells followed that of non-treated and the decrease in accumulated biomasses might be due to the prolonged elicitation with methyl jasmonate, like that observed in suspension cultures of *C. nucifera* (Chakraborty et al., 2009).

Beside the growth of *V. glabrata* cells, methyl jasmonate also strongly influenced the production of 20-

hydroxyecdysone (Figure 3B). At all concentrations of methyl jasmonate investigated, accumulation of 20-hydroxyecdysone in *V. glabrata* cells was increased, and the maximum accumulation appeared at 4 days after elicitation. Methyl jasmonate at 100 μM gave the highest concentration of 20-hydroxyecdysone (621.76 mg/100 g DW), which was 14.54-fold higher as compared to the control cells. The calculated 20-hydroxyecdysone productivity was 155.44 mg/100 g DW, which is one of the highest reported up to now. At lower (50 μM) and higher (200 μM) concentration of methyl jasmonate, the amounts of 20-hydroxyecdysone were 363.10 and 489.01 mg/100 g DW, which were 8.48-fold and 11.43-fold higher than the control cells, respectively. The optimal concentration of methyl jasmonate (100 μM) for maximizing the production of 20-hydroxyecdysone found

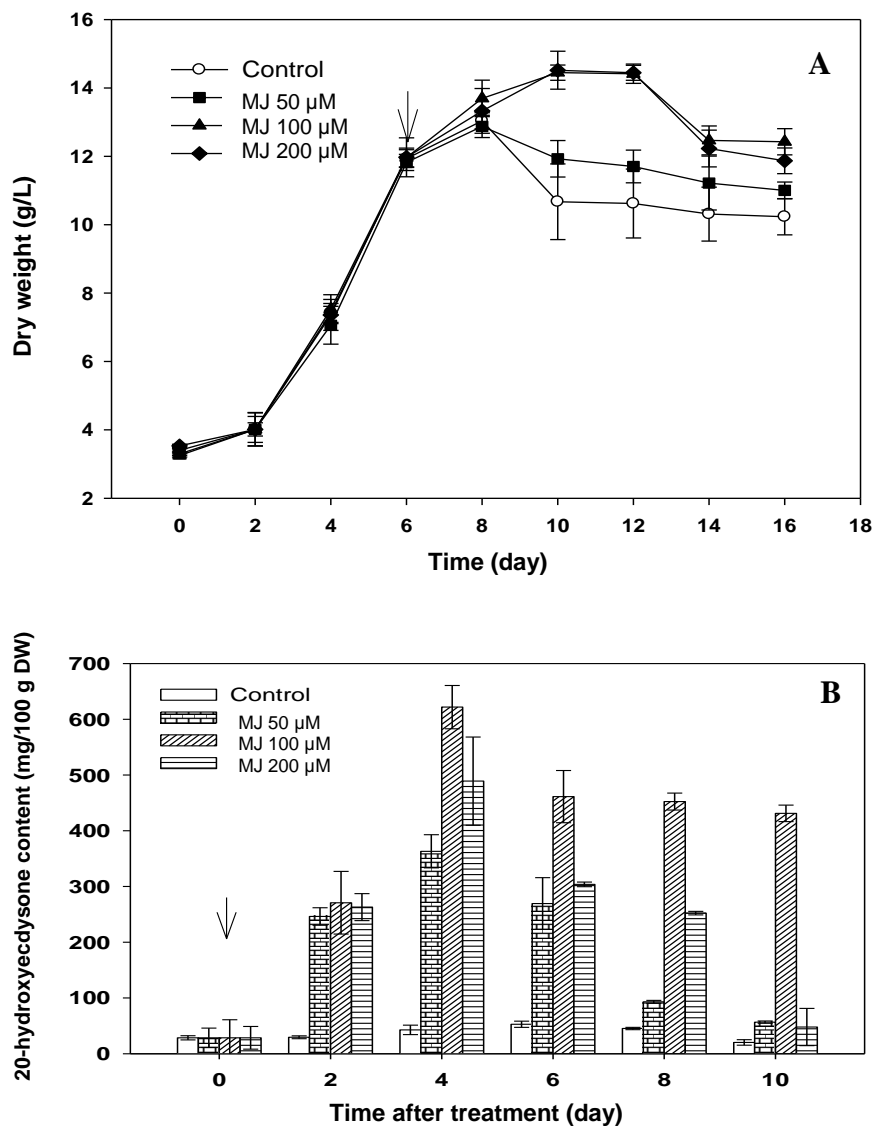


Figure 3. Effect of methyl jasmonate on growth (A) and 20-hydroxyecdysone production (B) in cell suspension cultures of *V. glabrata*. Methyl jasmonate (50, 100 and 200 μ M) was added to 6-day-old cultures as indicated by the arrowhead and cultivated at $25 \pm 2^\circ\text{C}$ for a further 10 days. Vertical bars represent standard deviation of three replications.

in the present study is similar to that reported for the production of cephalomanine in *T. chinensis* suspension cultures (Lan et al., 2002). In contrast to our finding, other researchers also reported the lower and higher concentrations of methyl jasmonate for maximizing the production of several secondary metabolites. For example, Georgiev et al. (2007) reported that the optimal concentration of methyl jasmonate for production of rosmarinic acid in suspension cultures of *L. vera* was 50 μ M, while Putalun et al. (2007) reported that the optimal concentration of methyl jasmonate for the production of artemisinin in hairy root cultures of *A. annua* was 200 μ M. These findings, together with the results from the present

study, suggested that methyl jasmonate is an effective elicitor for production of plant secondary metabolites like 20-hydroxyecdysone and the optimal concentrations of methyl jasmonate are normally dependent on the plant species.

The application of methyl jasmonate and other jasmonic acid derivatives as elicitor has been successful in other plant species, in which a production improvement of a particular metabolite was observed, as previously described. Many culture characteristics brought about by the addition of methyl jasmonate could be explained by its participation in the secondary metabolism of several plant species, serving as a signal molecule in various

Table 1. Comparison of 20-hydroxyecdysone production by different yield enhancement strategies.

Enhancement strategy	20-hydroxyecdysone concentration (mg/100 g DW)	Reference
Medium optimization	40.32	Duanghaklang (2001)
Precursor feeding	45.10	Sinlaparaya et al. (2007)
Elicitor feeding		
-Chitosan	377.09	This study
-Methyl jasmonate	621.76	

pathways (Zhao et al., 2005; Zabala et al., 2010).

Comparison of 20-hydroxyecdysone production by different yield enhancement strategies

The improvement of 20-hydroxyecdysone production in *V. glabrata* suspension cultures by different enhancement strategies is summarized in Table 1. Addition of chitosan as elicitor at 50 mg/L resulted in 20-hydroxyecdysone production of 377.09 mg/100 g DW, which was about 9.35 and 8.36-fold higher than those of 20-hydroxyecdysone production by the medium optimization and precursor feeding strategies, respectively. Likewise, addition of methyl jasmonate at 100 μ M resulted in the maximum enhancement of 20-hydroxyecdysone accumulation by 15.42 and 13.79 folds as compared to those obtained by the medium optimization and precursor feeding strategies, respectively. In contrast, the yield of 20-hydroxyecdysone derived from elicitation with methyl jasmonate was about 1.65-fold higher than that derived from elicitation with chitosan. From a biotechnological point of view, the productivity increment after elicitation with chitosan or methyl jasmonate is of practical value. To reach industrial application, further studies on downstream processing and scale-up production must be investigated.

Conclusion

The present study reports the first successful approach for enhancing the production of 20-hydroxyecdysone by suspension cultures of *V. glabrata*. The problem of low content in the cell cultures of *V. glabrata* was overcome by elicitation with chitosan or methyl jasmonate, which resulted in significant improvement in 20-hydroxyecdysone production. Our present work could be recommended as one of the biotechnology-based methodology for large-scale production of 20-hydroxyecdysone.

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