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Chitosan Elicitation for Enhancing of Vincristine and Vinblastine Accumulation in Cell Culture of *Catharanthus roseus* (L.) G. Don

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Abstract

Catharanthus roseus (L.) G. Don is an important herbal plant. There are two important alkaloids, vinblastine and vincristine, use in anti-cancer drugs. In this study production of the two alkaloids was enhanced in *C. roseus* cell cultures, in a Murashige and Skoog (MS) liquid medium supplemented with 1.5 mg/L 2,4-D, 0.5 mg/L kinetin and 30 g/L sucrose, by adding 0, 50, 100, 250 or 500 mg/L medium molecular weight chitosan or chitosan derived from shrimp shell. After 14 days of culture, the cell suspension at stationary phase in the 100 mg/L medium molecular weight chitosan could produce the highest amounts of vinblastine and vincristine at 4.15 and 5.48 µg/mg cell dry weight, respectively. At the same time, the controls (0 mg/L chitosan) produced the two alkaloids at only 2.43 and 2.49 µg/mg cell dry weight, respectively. For chitosan from shrimp shell, it was found that 100 mg/L chitosan could lead to the highest quantity of 4.09 µg vinblastine/mg cell dry weight. The highest amount of 5.47 µg vincristine/mg cell dry weight was obtained when 250 mg/L chitosan was added.

Keywords: *Catharanthus roseus*, vinblastine, vincristine, chitosan

1. Introduction

Catharanthus roseus (L.) G. Don is commonly known as the Madagascar periwinkle (Gajalakshmi et al., 2013). It synthesized terpenoid indole alkaloids were used in medicine such as vinblastine and vincristine (Mujib et al., 2014). These alkaloids have commercially important chemotherapy drugs (Zhou et al., 2010). Normally, vinblastine and vincristine could be produced very low approximately 0.0003% of 2.56% total alkaloid content (Shukla et al., 2006), Although, *C. roseus* has turn into one of the best studied herbal plants, in the use of cell suspension cultures for the production of valuable secondary metabolites.

Plant cell suspension culture technologies can be established for production of secondary metabolites. *In vitro* production of secondary metabolites in plant cell suspensions cultures has been reported from various medicinal plants (Zhao et al., 2005). Several strategies, such as manipulating the nutrient, optimizing the culture conditions, feeding of precursor, environmental stress and elicitation, can be applied in order to substantially increase the yields of secondary metabolites in plant cell cultures (Bourgaud et al., 2001; Zhang et al., 2004). Enhancement of secondary metabolites by elicitation is one of the few strategies recently finding commercial application (Namdeo, 2007).

Elicitors are molecules that stimulate defense or stress induced responses in plants. On the basis of their nature, elicitors can be divided into 2 types such as biotic and abiotic (Sreedhar et al., 2009). Both biotic and abiotic elicitors are known to stimulate synthesis of secondary metabolite in plant cells as a result of their defensive reaction against pathogen attack (García-Brugger et al., 2006). The use of both elicitors to activate product formation has become an important develop strategy and has been very useful in reducing the process time required to achieve high product concentrations and increasing product quantity (Cai et al., 2011). Natural elicitors include polysaccharides from insect and fungal pathogen such as chitosan (Brasili et al., 2014) are frequently used in a many plant cell suspension cultures for efficient induction of pharmaceutical secondary metabolites.

Chitosan is the major component of exoskeletons of insects, crustacean and fungal cell wall (Yin et al., 2010). As a natural substance, chitosan mimics the effects of some pathogen to stimulate plant secondary metabolites

production by producing of phytoalexins and the generating of hydrogen peroxide (Abd-Allah & Hashem, 2006). Chitosan has been confirmed as an effective biotic elicitor for improving biosynthesis of secondary metabolites in cell cultures of several medicinal species. The main aim of this study was to examine the effects of chitosan elicitation in the *C. roseus* cell suspension culture, accumulation of vinblastine and vincristine. Moreover, to find the feasible relations between plant chitosan concentrations and vinblastine and vincristine accumulations under chitosan elicitation.

Figure 1. *Catharanthus roseus* (L.) G. Don

2. Method

2.1 Plant Materials and Explants Preparation

A cultivar of *C. roseus* with white flower was used as plant material (Figure 1). The healthy shoot tips, 1 cm in length, were soaked in 70% (v/v) ethanol for 10 sec, followed by sterilization in 1% (v/v) sodium hypochlorite for 15 min, with 2 drops of Tween-20. The samples then washed with sterile distilled water 3 times.

2.2 Callus Induction

The shoot tips were cultured in MS liquid medium supplement with 1.5 mg/L 2, 4-D and 30 g/L sucrose, pH 5.8. They were cultured in the dark at 25±2 °C on orbital shaker at 100 rpm and subcultured every 3 weeks.

2.3 Cell Suspension Culture Preparation

Cell suspension culture was done by transferring 1 g fresh weight friable callus, 12 weeks old after subculture, to a 125 mL Erlenmeyer flask containing 25 mL of MS liquid medium supplement with 1.5 mg/L 2,4-D, 0.5 mg/L kinetin and 30 g/L sucrose, pH 5.8. The suspension cultures were placed on orbital shaker at 100 rpm and cultured at 25±2 °C under 16-h photoperiods with a light fluorescent lamp intensity of 36 µmol/m^2/s. Due to continuous agitation, the friable callus was broken and dispensed releasing cell clump and cells in the medium. Finally the single cells were filtrated by using nylon mesh (120 µm). After that the filtrate contained cell suspension was cultured in MS liquid medium supplemented with 1.5 mg/L 2,4-D, 0.5 mg/L kinetin and 30 g/L sucrose, pH 5.8 and subcultured every 14 days for inoculum cell.

2.4 Cell Suspension Growth Measurement

Measurement of growth of cell culture was performed by culturing 5 mL cell suspension in 125 mL Erlenmeyer flask with 20 mL of MS liquid medium supplement with 1.5 mg/L 2,4-D, 0.5 mg/L kinetin and 30 g/L sucrose, pH 5.8. The cell cultures were cultured at 25±2 °C under 16-h photoperiods with a light fluorescent lamp intensity of 36 µmol/m^2/s and shaked at 100 rpm on orbital shaker. Every day, 100 µl of cell suspension were taken out for cell counting on haemacytometer under light microscope. Growth curve was plotted as shown in Figure 2.

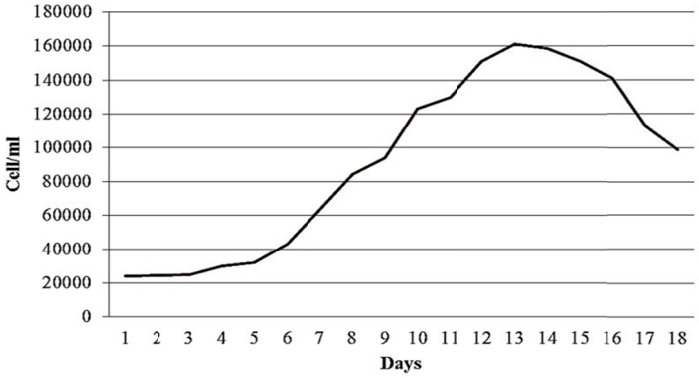

Figure 2. Growth curve of *C. roseus* cell suspension culture

2.5 Elicitor Preparation

In the present study, two chitosans (medium molecular weight and from shrimp shell; Sigma-Alrich Co., Ltd.) were dissolved in 0.1 M acetic acid by gentle heating at 60 °C and continuous stirring until dissolved. The pH of the solution was adjusted to 5.8 with 1M NaOH.

2.6 Elicitation Treatment

The cell suspension, 14 days old, were cultured in 125 mL Erlenmeyer flask with 20 mL of MS liquid medium supplement with 1.5 mg/L 2,4-D, 0.5 mg/L kinetin and 30 g/L sucrose, pH 5.8. The media were added with medium molecular weight or shrimp shell chitosan at the same concentration of 0, 50, 100, 250 and 500 mg/L. Each experiment has three replicates. The suspension cultures were placed on orbital shaker at 100 rpm and 25±2 °C under 16-h photoperiods with light fluorescent lamp intensity of 36 μmol/m^2/s.

2.7 Vinblastine and Vincristine Analysis by HPLC

The cell biomass of the suspension culture was harvested at 14 days after culture initiation by filtrated through 42 μm nylon mesh and freezing dried. Approximately 200 mg of dry cells wasextracted at room temperature in 1 mL of 95% methanol for 60 min in a sonicating bath. The extract was centrifuged at 15,000 *g* for 5 min then the supernatant was filtered through a 0.45 μm Millipore-filter into an amber glass HPLC vial prior to HPLC analysis.

Individual stock solutions of vincristine (vincristine sulfate, CALBIOCHEM) and vinblastine (vinblasine sulfate, CALBIOCHEM) were prepared at a concentration of 5 mg/mL in 95% methanol. These stock solutions were stored at 0 ºC. The analysis was performed by using a Waters (Milford, MA, USA) HPLC system comprising a Waters 2690 programmable photodiode array detector, Waters 717 autosampler and Waters 600 pump. The separations were carried out using a Lichrocart@250-4 column (150 × 4.6 mm, 5 μm particle size). The mobile phase consisted of a gradient mixture of 0.02 mM Na_2HPO_4 [pH adjusted to 6 with H_3PO_4 (solvent A) and 95% methanol (solvent B)]. Flow rate was 2.0 mL/min. The eluent profile (volume of solvent A/volume of solvent B) was 20 min, linear gradient from 80:20 to 20:80; 20 min, isocratic elution with 20:80 (v/v) for column rinsing and 25 min, isocratic elution with 80:20 (v/v) for column equilibration (Tikhomiroff and Jolicoeur, 2002). The retention times of vinblastine and vincristine chromatogram from standard and samples were 3.086 and 3.534 minutes (Figure 3).

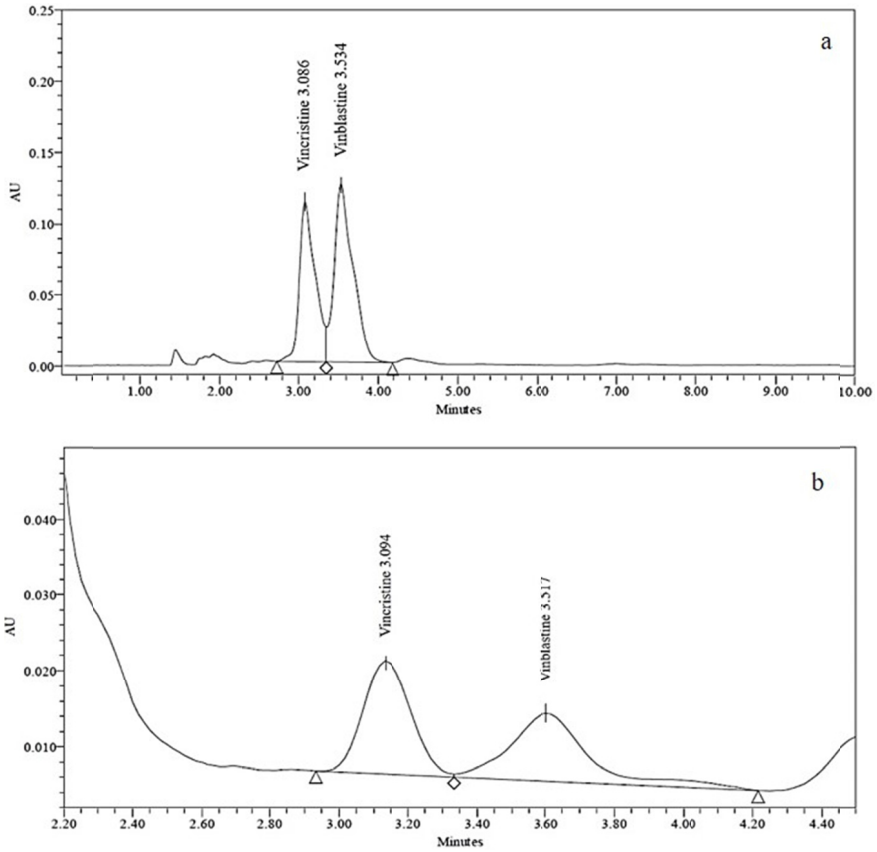

Figure 3. HPLC chromatogram showing standard vincristine and vinblastine (a) with the chromatogram showing the presence of vincristine and vinblastine (b) in cell culture extract of *C. roseus*

2.8 Data Analysis

All data collected from the experiments were analyzed using analysis of variance (ANOVA) with R program. Significant differences between mean assessed by LSD at $P \leq 0.01$.

3. Results

3.1 Accumulation of Vinblastine and Vincristine in Cell Culture

Accumulation of vinblastine and vincristine in growth phase of cell suspension showed that the accumulation of vinblastine and vincristine increased with the growth phase of the cell. The lag phase, 3 days after culture cell accumulated vinblastine and vincristine at 1.74 and 1.83 µg/mg cell dry weight. In the exponential phase 7 day after culture, the cell accumulations of vinblastine and vincristine were 2.11 and 2.27 µg/mg cell dry weight. In the stationary phase 12 day after cultured, the cell accumulations of vinblastine and vincristine were obtained at 2.61 and 2.52 µg/mg cell dry weight respectively (Table 1).

Table 1. Accumulation of vinblastine and vincristine in *C. roseus* cell suspension culture, without stimulant, in lag phase (3 days after culture), exponential phase (7 days after culture) and stationary phase (12 days after culture)

Growth phase	Vinblastine[1] (µg/mg cell dry weight)	Vincristine[1] (µg/mg cell dry weight)
Lag phase	1.74± 0.17^{c}	1.83± 0.16^{c}
Exponential phase	2.11±0.19^{b}	2.27±0.22^{b}
Stationary phase	2.61±0.21^{a}	2.52±0.24^{a}
F-test	**	**
C.V. (%)	6.73	5.89

Note. [1] Data represented mean±S.D. The different letters within the same column showed significant differences at $P \leq 0.01$ analyzed by LSD. ** = significant difference at $P \leq 0.01$.

3.2 Effect of Chitosan Elicitors on Vinblastine and Vincristine Accumulation

The accumulation of vinblastine and vincristine from suspension cells were found to be stimulated by using two types of chitosan as elicitor stimulants. For medium molecular weight chitosan, it was found that the concentration of 100 mg/L was the most effective concentration, enhancing vinblastine and vincristine accumulation at 4.15 and 5.48 µg/mg cell dry weight respectively., The molecular weights of 250, 500 and 50 mg/L were found to produce vinblastine at 3.8,5 2.96 and 2.68 µg/mg cell dry weight, respectively, and produce vincristine at 5.20, 3.91 and 3.66 respectively., The controls produced vinblastine and vincristine only 2.43 and 2.49 µg/mg cell dry weight, respectively (Table 2).

For chitosan from shrimp shell, the most effective concentration for vinblastine induction was 100 mg/L. It led to the yield of vinblastine at 4.09 µg/mg cell dry weight. For vincristine induction, the most effective concentration was 250 mg/L. It could produce the highest amount of 5.47 µg vincristine /mg cell dry weight (Table 3). When comparing of both chitosan efficiencies on enhancing vinblastine and vincristine accumulation in cell suspension, it was found that there was no difference in the accumulation of vinblastine and vincristine.

Table 2. Vinblastine and vincristine accumulation in *C. roseus* cell suspension culture with medium molecular weight chitosan (12 days after culture)

Chitosan (mg/L)	Vinblastine[1] (µg/mg cell dry weight)	Vincristine[1] (µg/mg cell dry weight)
0	2.43±0.23^{e}	2.49±0.22^{e}
50	2.68±0.24^{d}	3.66±0.38^{d}
100	4.15±0.39^{a}	5.48±0.51^{a}
250	3.85±0.36^{b}	5.20±0.49^{b}
500	2.96±0.27^{c}	3.91±0.32^{c}
F-test	**	**
C.V. (%)	6.73	5.89

Note. [1] Data represented mean±S.D. The different letters within the same column showed significant differences at $P \leq 0.01$ analyzed by LSD. ** = significant difference at $P \leq 0.01$.

Table 3. Vinblastine and vincristine accumulation in *C. roseus* cell suspension culture with chitosan from shrimp shell (12 days after culture)

Chitosan (mg/L)	Vinblastine[1] (µg/mg cell dry weight)	Vincristine[1] (µg/mg cell dry weight)
0	2.54±0.23^{e}	2.48±0.22^{d}
50	2.58±0.25^{d}	2.50±0.24^{d}
100	4.09±0.41^{a}	4.28±0.38^{b}
250	3.23±0.31^{b}	5.47±0.52^{a}
500	3.13±0.29^{c}	2.97±0.25^{c}
F-test	**	**
C.V. (%)	5.66	6.53

Note. [1] Data represented mean±S.D. The different letters within the same column showed significant differences at $P \leq 0.01$ analyzed by LSD. ** = significant difference at $P \leq 0.01$

4. Discussion

The present study was carried out to investigate the effect of chitosan on vincristine and vinblastine in cell suspension in *C. roseus*. We could enhance products of vincristine and vinblastine in response to chitosan elicitation. In this study, we found the distinction in the accummulation of vinblastine and vincristine during different development phases. It was found that both alkaloid were highly accumulated in the stationary phase because the substances were accumulated in vacuole and stationary phase had a large of vacuole than the other phase (Roytrakul & Verpoorte, 2007).

For the chitosan as a stimulant, chitosan is known to elicit activities leading to a variety of defensive responses in host plants to microbial infections, including the accumulation of phytoalexins, pathogen related proteins, callus formation and accumulation of secondary metabolite (Yin et al., 2012). This study found that the most effectivechitosan concentrations for inducing vinblastine and vincristine in *C. roseus* cell culture were 100 to 250

mg/L because itinduced programmed cell death and hypersensitive-associated responses in plants cell when concentration increased (Vasil'ev et al., 2009) or chitosan toxicity to the living cells or might be due to the phytotoxic action of vinblastine and vincristine on the cells (Amborabé et al., 2008). These results also agreed with the studies in *Plumbago indica* root cultures of Jaisi and Panichayupakaranant (2016). They found that treatment with chitosan at concentrations of 150 mg/L to 14 days old culture increased the production of plumbagin (13.08 mg/g dry weight) by up to 6.6-fold compared to the level of an untreated root culture (1.97 mg/g dry weight). The cell culture of *Withania somnifera* of Sivanandhan et al. (2012) reported that chitosan at 100 mg/L stimulated higher production of all withanolides about 323.85 mg/g dry weight when compared to control (19.05 mg/g dry weight). Ahmed and Baig (2014) showed that chitosan elicitors at 125 mg/L lead to 8-fold (7,982 µg/g dry weight) higher psoralen accumulation in *Psoralea corylifolia* L. cell culture over control cells (945 µg/g dry weight).

5. Conclusion

This is the first report of the effects of chitosan on vincristine and vinblastine yields in cell suspension cultures of *C. roseus*. The observation suggests that chitosan efficiently enhances vincristine and vinblastine yields especially when stimulated with 100 mg/L chitosan medium molecular weight. The identification of sign of chitosan in biosynthesis could be a very effective reach for large-scale development of both alkaloid yields for pharmaceutical industry.

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