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Keng, Chan Lai; Sze Wei, Ang; Bhatt, Arvind
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Elicitation effect on cell biomass and production of alkaloids in cell suspension culture of the tropical tree *Eurycoma longifolia*

Chan Lai Keng¹, Ang Sze Wei² and Arvind Bhatt³

^{1,2,3}School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia

³arvin_bhatt@rediffmail.com

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ABSTRACT

The present study tests the effect of different elicitor on cell biomass and alkaloid production in *Eurycoma longifolia* cells. The cell suspension cultures were prepared by inoculating 0,5g FW (fresh weight) of cells obtained from first batch of suspension cultures prepared from callus in liquid MSBs medium. Different concentration of chitosan, NaH₂PO₄, Na₂CO₃ and polyvinylpyrrolidone (PVP) were added as elicitor to optimize cell biomass and alkaloid production. MSBs medium supplemented with 100mg/L chitosan induced significant increment in the cell biomass while higher amount of chitosan (150mg/L) induced the highest production of 9-hydroxycanthin-6-one (0,44%) but not 9-methoxycanthin-6-one. The addition of 2mg/L and 20mg/L NaH₂PO₄ induced the highest increment in cell biomass and alkaloid production respectively. However, addition of different concentration Na₂CO₃ and polyvinylpyrrolidone showed inhibitory effect on cell growth with no significantly increased in alkaloid production.

KEY WORDS

Cell suspension culture, *Eurycoma longifolia*, medicinal plant, secondary metabolites.

RESUMEN

Analizamos el efecto de varios "reveladores" en la biomasa celular y la producción de alcaloides en células del árbol asiático *Eurycoma longifolia*. Las suspensiones celulares fueron preparadas por inoculación 0,5g FW (peso fresco) de células extraídas de suspensiones celulares de primer procesamiento preparadas de callos celulares en medio líquido MSBs. Agregamos quitosan, NaH₂PO₄, Na₂CO₃ y polivinilpirrolidona (PVP) como reveladores para aumentar la biomasa celular y la producción de alcaloides. El medio MSBs con 100mg/L quitosan induce un incremento significativo en la biomasa celular mientras que un aumento de quitosan (150mg/L) induce una alta producción de 9-hidroxicantina-6-uno (0,44%), pero no de 9-metoxicantina-6-uno. La adición de 2mg/L y 20mg/L NaH₂PO₄ aumenta la biomasa celular y la producción de alcaloides respectivamente. Sin embargo, la adición de Na₂CO₃ y polivinilpirrolidona muestran un efecto inhibitorio en el crecimiento celular y en la producción de alcaloides no hay un aumento significativo.

PALABRAS CLAVE

Suspensión celular, *Eurycoma longifolia*, planta medicinal, metabolitos secundarios.

Eurycoma longifolia Jack is an evergreen tree growing up to 10-15m in height. It is widely distributed in South East Asian countries such as Malaysia, India, China, Indonesia, Philippines and Thailand. It is commonly found in understory of the lowland forests up to 500m above sea level (Goh et al. 1995). It is commonly known as Tongkat Ali in Malaysia, Singapore and Brunei.

E. longifolia is an important medicinal plant and every parts of the plants are utilized for medicinal purposes (Jiwajinda et al. 2002, Osman et al. 2003). Leaves are used to treat diarrhea, fever, glandular swelling, bleeding, dropsy, persistent cough, hypertension, relief of pain in the bones and malaria. The

pulverized bark is used by aboriginal folks to treat wounds, ulcers, syphilitic sores and headache. The pulverized root or the root extract is used as tonic for women to recover after child birth (Bedir et al. 2003). In Vietnam, the bark is used to reduce difficulties of swallowing food and to treat lumbago. Vietnamese also uses the plant extract to treat cancer (Ueda et al. 2002). In Cambodia, the root is used as an antidotal remedy and to treat icterus, the yellowish discoloration of the whites of the eyes, skin, and mucous membranes caused by deposition of bile salts in these tissues (Perry & Metzger 1980).

The roots of *E. longifolia* were found to contain quassinoids and some alkaloids that had been proven to possess

anti-malarial, anti-ulcer, antipyretic, cytotoxic, antiproliferative, aphrodisiac properties and plant growth inhibition activities (Morita et al. 1990, Zhari et al. 1999, Kuo et al. 2003, Nurhanan et al. 2005, Chan et al. 2005, Tee & Azimzhtol 2005, Hout et al. 2006).

E. longifolia is dioecious in nature and produces fruits only once a year (August-September). Approximately 200-300 fruits are produced by a tree in one season. Moreover, the plant takes a long time to reach maturity and usually the whole plant is uprooted for medicinal preparation. This has caused an adverse effect on the natural population of *E. longifolia*. Production of bioactive compounds via cell suspension culture technology can be used as an alternative to solve this problem. The present study was hence carried out to evaluate the elicitation effect on *E. longifolia* cell biomass and the production of alkaloids, 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one. At the same time to identify a suitable elicitor that could increase the cell biomass and the content of both of the alkaloids.

MATERIALS AND METHODS

Callus culture of Eu9 line of *E. longifolia* that produced stable and moderate amount of alkaloids and maintained at Plant Tissue and Cell Culture Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia for three years was used in the present study. The cell suspension cultures was prepared by inoculating 0,5g homogenous Eu9 callus in 25 ml of MSBs, a modified MS medium formulated by Lufthi (2004). The pH of the medium was adjusted to 5,75 prior to autoclave at 121°C and 1,05kg/cm³ for 13 minutes with Elite Sterilizer (EAC-400°C). The cultures were maintained at 22°C ± 2°C and exposed to 24 hours photoperiod with light intensity of 32,5µE m⁻²s⁻¹. The culture were placed on an orbital shaker and agitated at 120rpm for 13 days. The subcultures were performed at 13-day intervals with 0,5g fresh cell as inoculum.

Different concentration of chitosan, NaH₂PO₄, Na₂CO₃ and polyvinylpyrrolidone were added as elicitors into MSBs, the cell proliferation medium, a day before harvesting the cell. The experiment was carried out using randomized complete block design. The cell FW was determined on the 13th day of culture. Thereafter the cells were air dried to constant weight for determination of cell dried weight (DW).

About 0,25g of dried cell powder was weighed and soaked with 20mL hexane for 24 hours in a sample bottle. After 24 hours, hexane was discarded and the cells were again soaked with 20mL methanol for additional 24 hours. This process were repeated three times. Finally the cells

were filtered through filter paper (Whatman no.1). The filtrate was evaporated with a rotary evaporator at 45°C. The dry residue was redissolved in 1,25mL methanol and filtered with milipore membrane (0,45µm, Whatman). The alkaloids content was determined by HPLC system (LC-10 ADVp Shimadzu). Cell extract of 20µL was injected into injector (Rheodyne, USA) connected to a reverse phase column with 5µm particle size and 250 x 4,6mm (Hypersil BDS column). The Ultra-violet detector (SPD-10 AVp Shimadzu UV-VIS) was used for the detection of the presence of 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one in the extract. Mobile phase of the HPLC system comprised of acetonitrile (Fisher Scientific) and 0,2% acetic acid (Merck, Germany) in the ratio of 42 : 58. The flow rate was set to 2mL/m. The elution of alkaloids was detected at 280nm. The standards of 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one were dissolved in methanol in a dilution series of 0,75, 3, 15, 30, 60, 120, and 250mg/L to obtain the calibration curve for these two alkaloids. The area under the peak of the chromatogram for the standards was plotted against their respective concentration to obtain a regresion linear equation as below:

$$Y = mX + c$$

Y = area under the peak of the chromatogram

X = concentration of the alkaloid (mg/L)

m and c = constants

This regresion linear equation was used to obtained the concentration of alkaloids in the tested samples. The 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one content in the sample could be obtained as:

$$\%(w/w) = \frac{\text{alkaloid content in sample (mg)}}{\text{extract weight (mg)}} \times 100$$

All experiments were carried out with 6 replicates. The data obtained for cell mass and alkaloids content were analysed separately using Two-Way analysis of Variance (ANOVA) followed by HSD Tukey Test at p = 0,05.

RESULTS

Results revealed that certain elicitors affect the cell biomass and production of alkaloids from *E. longifolia* cells. MSBs medium supplemented with 100mg/L chitosan induced significant increment in the cell biomass (FW 2,83 g; DW 0,26 g) as compared to the control (FW 1,47g; DW 0,14g) after 13 days of cell culture. The medium supplemented with 50mg/L chitosan also showed increment in cell biomass (FW 1,70g; DW 0,17g). Lower amount of chitosan (10mg/L, 25mg/L) and high chitosan

level (150mg/L) present in the culture medium reduced the cell biomass. However, MSBc medium supplemented with 150mg/L chitosan induced the highest production of 9-hydroxycanthin-6-one (0,44%). The presence of chitosan in the culture medium did not induced the production of 9-methoxycanthin-6-one. In fact the absent of chitosan in the cell cultures had induced low production of 9-methoxycanthin-6-one (0,08%) (Table 1).

The MSBs medium supplemented with different concentration of NaH_2PO_4 also resulted in increment of cell biomass. The MSBs medium supplemented with 2mg/L NaH_2PO_4 showed the highest increment in the cell biomass (FW 3,10g; DW 0,22g) as compared to the control (FW 0,66g; DW 0,07). Most of the NaH_2PO_4 treated cells showed increasing effect on the production of 9-hydroxycanthin-6-one except 5 mg/L NaH_2PO_4 . The addition of

TABLE 1
Effect of different elicitors supplemented into MSBs liquid medium on cell biomass and production of 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one from *E. longifolia* cells after 13 days of culture

Concentration of Elicitor (mg/L)	Fresh cell biomass (g) (n=6)	Dry cell biomass (g) (n=6)	% w/w of 9-hydroxycanthin-6-one	% w/w of 9-methoxycanthin-6-one
Chitosan				
0	1,47 ± 0,18	0,14 ± 0,03	0,08 ± 0,05	0,08 ± 0,04
10	0,56 ± 0,04	0,06 ± 0,01	0,39 ± 0,02	—
25	0,88 ± 0,30	0,07 ± 0,02	0,34 ± 0,01	—
50	1,70 ± 0,28	0,17 ± 0,03	0,28 ± 0,04	—
100	2,83 ± 0,25	0,26 ± 0,03	0,25 ± 0,01	—
150	1,07 ± 0,32	0,07 ± 0,01	0,44 ± 0,07	—
NaH_2PO_4				
0	0,66 ± 0,03	0,07 ± 0,01	0,08 ± 0,02	0,08 ± 0,01
2	3,10 ± 0,44	0,22 ± 0,05	0,34 ± 0,01	0,94 ± 0,00
5	1,20 ± 0,35	0,18 ± 0,02	0,08 ± 0,01	0,06 ± 0,01
10	1,29 ± 0,55	0,11 ± 0,02	0,40 ± 0,11	0,16 ± 0,04
15	1,56 ± 0,48	0,13 ± 0,03	0,39 ± 0,01	0,10 ± 0,02
20	1,53 ± 0,17	0,13 ± 0,02	0,75 ± 0,06	0,41 ± 0,02
Na_2CO_3				
0	0,50 ± 0,01	0,05 ± 0,00	0,11 ± 0,02	0,12 ± 0,02
2	0,50 ± 0,01	0,05 ± 0,01	0,17 ± 0,02	0,22 ± 0,02
4	0,46 ± 0,04	0,04 ± 0,01	0,31 ± 0,02	0,24 ± 0,11
6	0,57 ± 0,02	0,05 ± 0,00	0,32 ± 0,01	0,27 ± 0,06
8	0,60 ± 0,01	0,06 ± 0,00	0,23 ± 0,00	0,12 ± 0,01
10	0,43 ± 0,07	0,04 ± 0,00	0,31 ± 0,02	0,24 ± 0,07
Polyvinylpirrolidone				
0	0,50 ± 0,01	0,05 ± 0,00	0,10 ± 0,01	0,11 ± 0,03
10	0,44 ± 0,01	0,04 ± 0,00	0,28 ± 0,01	0,31 ± 0,01
100	0,37 ± 0,01	0,03 ± 0,00	0,38 ± 0,01	0,22 ± 0,01
500	0,43 ± 0,02	0,04 ± 0,00	0,55 ± 0,04	0,28 ± 0,02
1000	0,48 ± 0,01	0,04 ± 0,00	0,26 ± 0,04	0,03 ± 0,01
1500	0,43 ± 0,01	0,04 ± 0,00	0,50 ± 0,00	0,14 ± 0,02

20mg/L NaH_2PO_4 into the culture medium induced the highest production of 9-hydroxycanthin-6-one. While MSBs medium supplemented with 10 mg/L NaH_2PO_4 induced the highest production of 9-methoxycanthin-6-one in the cells (Table 1).

The addition of Na_2CO_3 into the cell cultures of *E. longifolia* showed marginal effect on cell biomass. The MSBs medium supplemented with 8mg/L of Na_2CO_3 induced only slight increment in the cell biomass (FW 0,60g; DW 0,06g) as compared to the control (FW 0,50g; DW 0,05g). MSBs medium supplemented with 6mg/L Na_2CO_3 increased production of 9-hydroxycanthin-6-one (0,32%) and 9-methoxycanthin-6-one (0,27%) in the cells as compared to the control (9-hydroxycanthin-6-one 0,11%) and 9-methoxycanthin-6-one 0,12%).

On the other hand, the cell culture medium of *E. longifolia* supplemented with polyvinylpyrrolidone caused reduction in cell biomass. However, the addition of 500mg/L

polyvinylpyrrolidone into the cell cultures induced the highest production of 9-hydroxycanthin-6-one (0,55%) as compared to the other concentration. The presence of 10mg/L polyvinylpyrrolidone in the cell cultures induced approximately equal amount of 9-hydroxycanthin-6-one ($0,28 \pm 0,01\%$) and 9-methoxycanthin-6-one ($0,31 \pm 0,01\%$) and high cell biomass (Table 1).

The presence of 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one were determined by comparing the retention time between the alkaloid standard and the samples. The retention time for 9-hydroxycanthin-6-one was 2,092 minute and for 9-methoxycanthin-6-one was 4,317 minute (Fig. 1 and 2).

Observation under light microscope showed that the cell walls of *E. longifolia* were broken after soaking in methanol for 72 hours. Even the cell lysis, there was only about 20% of the extract obtained after the evaporation process (data not shown). This indicated that there was

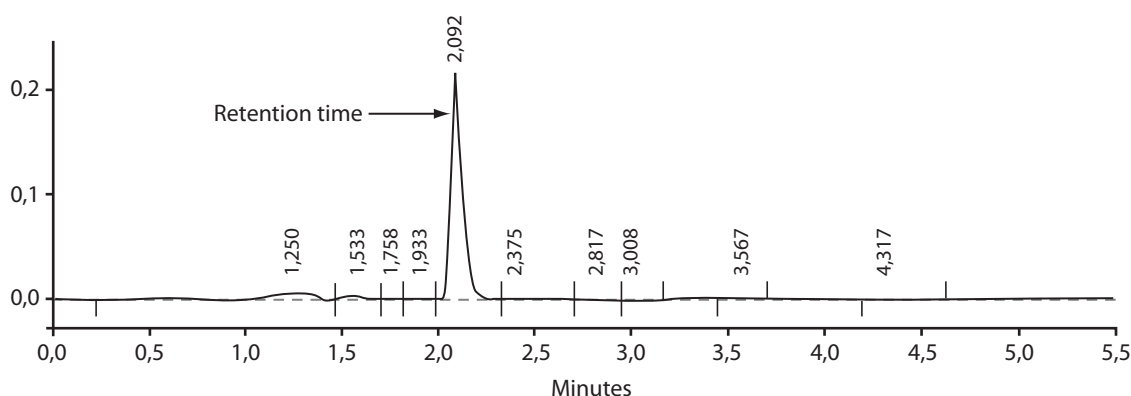


FIG. 1. Chromatogram of 9-hydroxycanthin-6-one standard with retention time of 2,092 min.

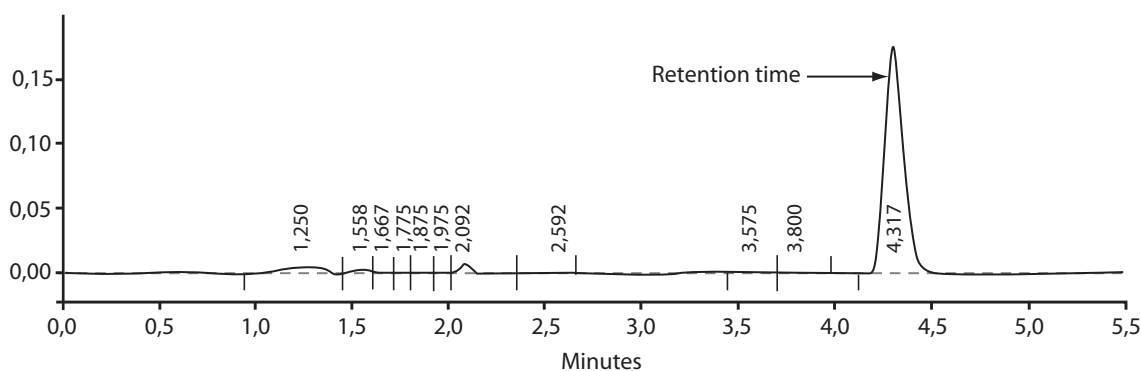


FIG. 2. Chromatogram of 9-methoxycanthin-6-one standard with retention time of 4,317 min

still a lot of alkaloids retained in the cells. Therefore, the cells can be soaked for a longer period in methanol solvent to release more alkaloids.

DISCUSSION

Generally, most of the pharmaceutically important secondary metabolites are isolated from wild or cultivated plants because their chemical synthesis is not economically feasible. The production of useful and valuable secondary metabolites via cell cultures system could be a better alternative. Plant cell culture system is a potential source of valuable secondary metabolites which can be used as food additives, nutraceuticals and pharmaceutical purposes (Zhong 2001). Elicitation is one of the most effective ways to promote secondary metabolites production in plant cell cultures (Zhang et al. 2000). Elicitors have been successfully used for the production of secondary metabolites in cell cultures of different species such as *Thalictrum rugosum* (Brodelius et al. 1989), *Tagetes patula* (Buitelaar et al. 1992), *Catharanthus roseus* (Vázquez-Flota et al. 1994), *Taxus* sp. (Ciddi et al. 1995) and many others. Production of secondary metabolites in plants is mostly occurred due to stress created by abiotic and biotic factors. Elicitors may be biotic or abiotic, which possess the ability to induce physiological changes in the living organism such as plant cells (Zhao et al. 2005).

MSBs medium supplemented with 50 and 100mg/L chitosan, significantly increased the cell biomass of *Eurycoma longifolia*. The MSBs medium supplemented with lower amount (10mg/L, 25mg/L) and higher amount of chitosan (150mg/L) reduced the cell biomass. However, the higher concentration of chitosan (150mg/L) showed the highest production of 9-hydroxycanthin-6-one but it did not affect the production of 9-methoxycanthin-6-one. From the biosynthesis pathway, 9-methoxycanthin-6-one was derived from 9-hydroxycanthin-6-one. The biosynthesis pathway might be blocked by chitosan which probably induced some enzymatic reaction or production of some inhibitors that inhibited the production of 9-methoxycanthin-6-one. These findings were in tune with Palazón et al. (2003) who reported the similar effect of chitosan in *Panax ginseng*.

MSBs medium supplemented with different concentration of NaH_2PO_4 showed the increment of cell biomass and also increased the production of 9-hydroxycanthin-6-one except 5mg/L NaH_2PO_4 . The similar results were reported by Toivonen et al. (1989) in *Catharanthus roseus*, where the addition of NaH_2PO_4 was effective in increasing the cell biomass and alkaloid production. The active mechanisms of elicitors are considered to be special and complex. Since little information is available on the biosynthetic

pathways of most secondary metabolites in plants, the effect of elicitation on the plant cell culture cannot be predicted easily. The effect of elicitors also depends on many factors such as elicitor concentration, the growth stage of the culture at the time of elicitation and the contact time of elicitation. Addition of polyvinylpyrrolidone in MSBs medium caused the reduction in cell biomass. However, the addition of 500mg/L polyvinylpyrrolidone induced the highest production of 9-hydroxycanthin-6-one (0,55%). These results are in agreement with Zhao et al. (2000), who reported that polyvinylpyrrolidone inhibited the cell growth but it increase the ajmalicine production in *Catharanthus roseus*.

This study indicated that different elicitors had different effect on cell biomass and alkaloids production from *E. longifolia* cells. The addition of 100mg/L chitosan or 2mg/L NaH_2PO_4 into the cell cultures caused 5,7 and 6,2 folds increased in cell biomass while the addition of Na_2CO_3 and polyvinylpyrrolidone did not accelerate cell growth. Culture media supplemented with higher concentration of Chitosan (150mg/L) or NaH_2PO_4 (20mg/L) induced significantly higher alkaloid production.

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