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Research Article

Enhancement of eurycomanone biosynthesis in cell culture of longjack (*Eurycoma longifolia*) by elicitor treatment

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Abstract In this study, the effect of elicitors such as yeast extract (YE), methyl jasmonate (MeJA) and salicylic acid (SA) on the accumulation of eurycomanone in *Eurycoma longifolia* cell cultures were investigated. Suspension cells of *E. longifolia* was cultured in Murashige and Skoog (MS) medium supplemented with 30 g/L sucrose, 1.25 mg/L naphthaleneacetic acid (NAA) and 1 mg/L kinetin at a shaking speed of 120 rpm. Elicitors were added in the culture at different concentrations and times to stimulate eurycomanone accumulation in the *Eurycoma longifolia* cells. Eurycomanone content was determined by HPLC with a C18 column, flow rate of 0.8 mL/min, run time of 17.5 min, and a detector wavelength of 254 nm. The stationary phase was silica gel and the mobile phase was acetonitrile: H_2O. Non-elicited cells were used as the control. The study showed the effect of different elicitor concentrations, YE at 200 mg/L, MeJA at 20 μM and SA at 20 μM stimulated high production of eurycomanone. In which, treatment of 20 μM MeJA after 4 days of culture resulted in the highest accumulation of this compound (17.36 mg/g dry weight), approximately 10-fold higher than that of untreated cells (1.70 mg/g dry weight).

Keywords Elicitors, *Eurycoma longifolia*, eurycomanone, suspension cell

Introduction

Elicitors are the chemical compounds from abiotic and biotic sources which have been employed to modify cell metabolism in order to improve the productivity of useful secondary compounds in plant cell or tissue cultures (Jeong and Park 2005).

Methyl jasmonate (MeJA), an abiotic elicitor, plays as intracellular signaling molecules that mediate the activation of gene expression in response to wounding, elicitor treatment, and pathogen infection (Ellard-Ivey and Douglas 1996). According to Durango et al. (2013), another elicitor is salicylic acid (SA) that involved in signal transduction systems to stimulate particular enzymes catalyzing biosynthetic reactions which produce defense compounds for plants against pathogens. Exogenous applications of SA can also result in the induction of these defense compounds in *in vitro* cultures (Vimala and Suriachandraselvan 2009; Mandal 2010). Yeast extract (YE), a biotic elicitor, has been used in plant cell and tissue culture due to their ability to stimulate the defense mechanism, which leads to increase biosynthesis of valuable metabolites and its optimal level may be different for each plant species (Abraham et al. 2011).

There were reports on applications of MeJA, YE and SA to enhance bioactive compounds biosynthesis in cell and tissue cultures of medicinal valuable plants (Naik and Al-Khayri 2016). In *E. longifolia* tree, Abdullah et al. (2016) used MeJA, YE and SA as elicitors to improve the yield of 9-methoxycanthin-6-one (an indole alkaloid) biosynthesis in hairy root culture. Chee et al. (2015) also stimulated 9-methoxycanthin-6-one biosynthesis in root culture by treated with MeJA. Rosli et al. (2009) studied the effect of some factors such as medium compositions, carbon sources, pH, and amino acids on 9-methoxycanthin-6-one production in callus culture. UV elicitation was also used to increase productivity of squalene, quasinoid, canthin-6-one and beta-carboline metabolites in cell suspension culture (Natanael et al. 2014) or terpenoids in callus culture (Parikrama et al. 2014). Study of Keng et al. (2010) showed

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that NaH_2PO_4, an abiotic elicitor, can increase in 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one accumulation in cell suspension culture.

Eurycomanone, a valuable metabolite of *E. longifolia* belongs quassinoid compound, exhibites antimalarial activity (Kardono et al. 1991; Chan et al. 2004), enhances production of testosterone in rat (Low et al. 2013), suppresses lung cancer cells (Wong et al. 2012) and regulates signaling pathways involved in proliferation, cell death and inflammation (Hajjouli et al. 2014). However, we did not find any reports deal on applications of elicitors to improve the yield of eurymaconone biosynthesis in *in vitro* cultures of *E. longifolia*. The present work, therefore, investigated the effects of elicitor concentrations and exposure times on eurycomanone accumulation in cell culture of *E. longifolia*.

Materials and Methods

Cell suspension culture

Cell suspension culture was established by agitation of 3 g callus in a 250 mL Erlenmeyer flasks containing 50 mL of liquid callus culture medium including basal MS medium (Murashige and Skoog 1962) supplemented with 30 g/L sucrose, 1.25 mg/L naphthaleneacetic acid and 1 mg/L kinetin at a shaking speed of 120 rpm under the same conditions for the callus culture as our previous report except the light intensity of 500 lux (Nhan and Loc 2017) (Fig. 1).

Elicitation

Elicitation effects of YE, MeJA and SA were evaluated by adding their different concentrations to the medium at the beginning of culture. The cell biomass was harvested after 14 days of culture for determination of the fresh and dry weights and eurymaconone content.

To measure fresh weight, cell biomass was filtered, washed with distilled water, collected, and weighed. The dry weight (DW) was determined by drying the fresh cells at 50°C until a constant weight was attained. The eurycomanone content of the cell extract was measured by high performance liquid chromatography (HPLC).

Optimal concentration of elicitors will be selected to investigate elicitation time (2 ~ 12 days). The addition of elicitors at the beginning of cell culture was used as the control. The cells were also harvested after 14 days of culture to evaluate the growth and eurycomanone accumulation.

Fig. 1 Callus and suspension cells of *E. longifolia*

Eurycomanone quantification

The dried cell biomass was powdered to fine particles, 0.5 g of this powder was soaked in 10 mL methanol at 60°C and with a shaking speed of 120 rpm for 8 h, repeating this step for 3 times. The extract (30 mL) was then filtered and concentrated completely at 50°C. The precipitate was dissolved in 5 mL methanol (eurycomanone extract) and filtered through Minisart 0.2 μm membrane (Sartorius, Goettingen, Germany) to prepare sample for HPLC.

The HPLC analysis were carried out at ambient temperature with a C18 column (Xbridge: 5 μm, 4.6 × 250 mm), flow rate: 0.8 mL/min, run time: 17.5 min, detector wavelength: 245 nm. The stationary phase was silica gel and the mobile phase was acetonitrile:H_2O (15:85, *v/v*). A 20 μL aliquot of sample was injected into the column using a Hamilton syringe. The HPLC was performed on a LC-20 Prominence system (Shimadzu, Japan) with a SPD-20A UV-VIS detector using LC-Solution software. All solvents were of analytical grade and were purchased from Merck & Co. Inc. (Germany).

A standard curve of eurycomanone (Santa Cruz, USA) was used for measurement of the eurycomanone content in the samples.

Statistical analysis

All experiments were repeated at least three times. The data are presented as mean and the means were compared using a one-way analysis of variance (ANOVA) followed by Duncan's test ($p < 0.05$).

Results

Effect of elicitor concentration

Different concentrations of YE (20-250 mg/L), MeJA

Table 1 Effect of YE on cell growth and eurycomanone accumulation

YE (mg/L)	0	20	50	100	150	200	250
Fresh weight (g/flask)	17.43^{a}	14.7^{bc}	14.98^{b}	15.24^{b}	13.96^{c}	17.08^{a}	16.68^{a}
Dry weight (g/flask)	0.72^{a}	0.42^{e}	0.47^{d}	0.46^{d}	0.41^{e}	0.49^{c}	0.51^{b}
Eurycomanone (mg/g)	1.70^{d}	1.61^{d}	1.71^{d}	1.79^{d}	2.28^{c}	3.71^{a}	3.07^{b}

Different letters in a row represent different means of the repeats (Duncan's test, $p < 0.05$). This note is used for all Tables from 1 to 6.

Table 2 Effect of MeJA on the cell growth and eurycomanone accumulation

MeJA (μM)	0	10	20	50	100	200	500
Fresh weight (g/flask)	17.43^{a}	10.58^{b}	8.14^{c}	5.22^{d}	2.29^{e}	1.63^{f}	0.76^{g}
Dry weight (g/flask)	0.72^{a}	0.43^{c}	0.48^{c}	0.61^{b}	0.33^{d}	0.22^{e}	0.07^{f}
Eurycomanone (mg/g)	1.70^{c}	5.17^{b}	6.60^{a}	1.23^{d}	1.11^{d}	0.51^{f}	0.85^{e}

Table 3 Effect of SA on the cell growth and eurycomanone accumulation

SA (μM)	0	10	20	50	100	200*	500*
Fresh weight (g/flask)	17.43^{b}	16.32^{c}	19.08^{a}	14.67^{d}	2.86^{e}	-	-
Dry weight (g/flask)	0.72^{a}	0.48^{bc}	0.56^{b}	0.52^{b}	0.37^{c}	-	-
Eurycomanone (mg/g)	1.70^{b}	3.30^{a}	3.51^{a}	3.35^{a}	0.9^{c}	-	-

The values with * shows that the cells turned brown and died.

Table 4 Effect of elicitation time with 20 μM MeJA on the cell growth and eurycomanone accumulation

Elicitation time (day)	Control	2	4	6	8	10	12
Fresh weight (g/flask)	8.14^{e}	8.32^{e}	9.21^{d}	10.43^{c}	11.40^{b}	13.83^{a}	14.09^{a}
Dry weight (g/flask)	0.48^{ab}	0.50^{ab}	0.55^{a}	0.56^{a}	0.49^{ab}	0.42^{b}	0.43^{b}
Eurycomanone (mg/g)	6.60^{d}	12.83^{b}	17.36^{a}	9.18^{c}	5.99^{d}	3.81^{e}	2.77^{f}

(10-500 μM) and SA (10-500 μM) were used to investigate their influences on cell growth and eurycomanone accumulation after 14 days of culture. In a previous report, we established the profile of *E. longifolia* cell growth with a maximum biomass obtained on day 14 (Nhan and Loc 2017). Therefore in present work, the effects of elicitors on *in vitro* cultures will be evaluated at this time.

Data in Table 1 show that YE in all applied concentrations inhibited the cell growth, the dry weight ranges between 0.41 and 0.51 g/flask (control: 0.72 g/flask). However, eurycomanone level increased significantly with a maximum value of 3.71 mg/g DW at 200 mg/L of YE, approximately 2-fold higher than that of the control.

Unlike YE, MeJA influenced dramatically on cell cultures of *E. longifolia*. The cell growth decreased strongly when MeJA concentration increased from 10-500 μM. However, eurycomanone content reached a highest value of 6.6 mg/g DW at 20 μM of MeJA, approximately 4-fold higher than that of the control (Table 2).

SA is a signaling elicitor belonging to phenolic compound present in plants and has multiple functions, including hormonal effect on stimulus of plant growth and development and induction of plant defense responses under conditions of biotic and abiotic stresses. Our results in Table 3 show SA exhibites strongly growth inhibition of *E. longifolia* cells in all used concentrations of from 10 to 500 μM. However, eurycomanone content was also enhanced about 2-fold higher than that of the control in the medium containing 20 μM of SA.

Elicitation time

The timing of elicitor addition are critical factors that will affect the response of *in vitro* cultured plant cells. The optimal concentrations of YE (200 mg/L), MeJA (20 μM) and SA (20 μM) from above investigations will be used to find suitable elicitation times for improvement of eurycomanone productivity.

The results showed that eurycomanone content in cells which treated with 20 μM MeJA after 4 days of culture reached a maximum value of 17.36 mg/g DW (Table 4, Figs. 2 ~ 4), 10- and 8-fold higher than that of untreated

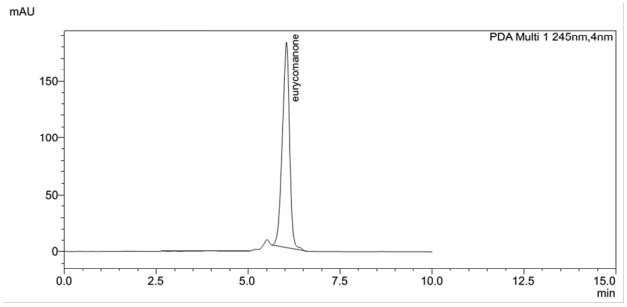

Fig. 2 HPLC chromatogram of standard eurycomanone

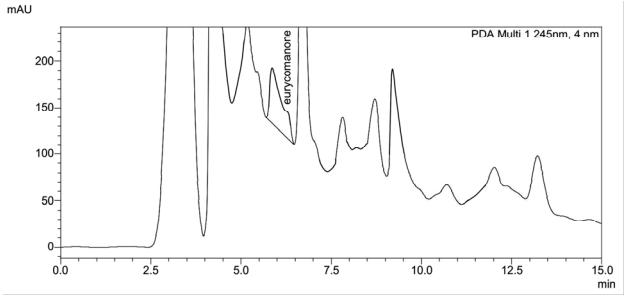

Fig. 3 HPLC chromatogram of eurycomanone extract from untreated cells

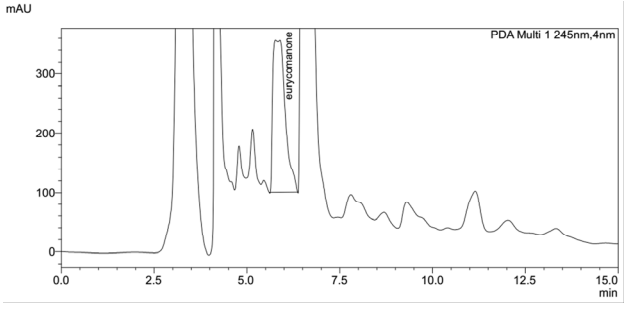

Fig. 4 HPLC chromatogram of eurycomanone extract from cells treated with 20 μM MeJA after 4 days of culture

cells (Tables 1) and roots of 5 years-old tree (Nhan and Loc 2017), respectively. In other treatments, eurycomanone content in cells only reached highest values of 5.2 mg/g

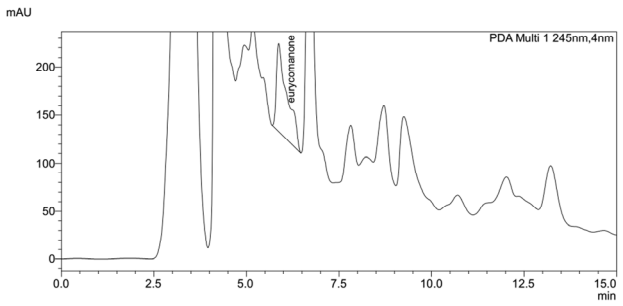

Fig. 5 HPLC chromatogram of eurycomanone extract from cells treated with 20 μM SA after 4 days of culture

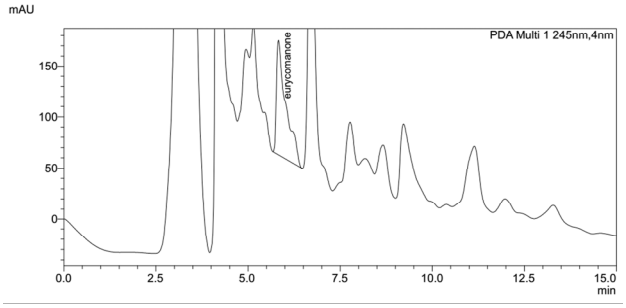

Fig. 6 HPLC chromatogram of eurycomanone extract from cells treated with 200 mg/L YE after 6 days of culture

and 6.25 mg/g DW when 20 μM SA and 200 mg/L YE were added in medium after 4 and 6 days of culture, respectively (Tables 5 ~ 6, Figs. 5 ~ 6). Generally, elicitors stimulated the biosynthesis of eurycomanone in different elicitation times, while the cell growth was not influenced significantly. In most treatments, the cell biomass is from 0.42 ~ 0.56 g DW/flask (control: 0.48 ~ 0.56 g DW/flask) (Tables 4 ~ 6).

Discussion

According to Hussain et al. (2012), elicitor treatments

Table 5 Effect of elicitation time with 20 μM SA on the cell growth and eurycomanone accumulation

Elicitation time (day)	Control	2	4	6	8	10	12
Fresh weight (g/flask)	19.08^{a}	15.58^{c}	16.00^{c}	15.90^{c}	17.09^{b}	18.00^{b}	17.36^{b}
Dry weight (g/flask)	0.56^{a}	0.46^{b}	0.47^{b}	0.47^{b}	0.50^{ab}	0.53^{a}	0.50^{ab}
Eurycomanone (mg/g)	3.51^{c}	4.26^{b}	5.20^{a}	5.01^{a}	2.37^{d}	2.01^{d}	1.97^{d}

Table 6 Effect of elicitation time with 200 mg/L YE on the cell growth and eurycomanone accumulation

Elicitation time (day)	Control	2	4	6	8	10	12
Fresh weight (g/flask)	17.08^{b}	16.86^{b}	17.20^{b}	16.67^{b}	18.21^{a}	17.01^{b}	18.42^{a}
Dry weight (g/flask)	0.49^{a}	0.49^{a}	0.50^{a}	0.50^{a}	0.52^{a}	0.50^{a}	0.50^{a}
Eurycomanone (mg/g)	3.71^{c}	3.87^{c}	4.83^{b}	6.25^{a}	4.49^{b}	2.12^{d}	2.02^{d}

inhibited the growth of the hairy roots of *Panax ginseng*, although simultaneously enhancing saponin production. Study of Yamamoto et al. (1995) indicated that YE stimulated prenylated flavanone accumulation in cell culture of *Sophora flavescens* but the cell growth was decreased. Zhao et al. (2010) investigated the effects of biotic and abiotic elicitors such as metal ions, polysaccharides, plant response-signaling compounds, and hyperosmotic stress on the growth and tanshinone accumulation in *Salvia miltiorrhiza* cell culture. Of these, silver nitrate, cadmium chloride, and YE were most effective to stimulate the tanshinone production, increasing the total tanshinone content of cell by more than 10-fold. Meanwhile, most of the elicitors suppressed the cell growth, decreasing the biomass yield. Cai et al. (2014) investigated the effects of YE on the production of secondary metabolites and on the induction of plant defence responses in cell suspension cultures of *Malus* × *domestica*. The results showed YE enhanced the production of phenolic acids by 2-fold after 3 days of culture. Among the dominant phenolic acids, the production of *p*-coumaric acid was stimulated by YE up to 5.1-fold. Chlorogenic acid concentrations were also increased 2.7-fold within 3 days of culture by YE treatment. However, phenylalanine ammonia-lyase activity was reduced up to 33%. YE maintained control levels of cell growth in culture.

Zabala et al. (2010) obtained results which indicated positive effects of MeJA on peruvoside production in *Thevetia peruviana* cells. Krzyzanowska et al. (2012) also found a highest rosmarinic acid accumulation (12% DW) in *Mentha* × *piperita* cells after addition of 100 μM MeJA, these content was nearly 1.5 times compared to the control. In contrast, the addition of MeJA was found to have no effect on cell biomass of *Melastoma malabathricum* but the presence of higher amount (12.5 ~ 50 mg/L) had caused a reduction in anthocyanin accumulation (See et al. 2011).

Studies related to elicitor action of SA on the synthesis of secondary metabolite in medicinal plants have been conducted in order to increase the economic value of these species. Sesquiterpenes in *Panax ginseng* were highly accumulated after adventitious root culture treated with SA (Rahimi et al. 2014). Study of Gorni and Pacheco (2016) in yarrow (*Achillea millefolium* L.) plant showed 0.5 mM SA stimulated increases in root biomass, total dry matter, ratio root/shoot, chlorophyll (a) and chlorophyll (a+b) content. The use of SA at 0.5 and 1 mM was most effective in eliciting the production of essential oils and total phenolics.

In our study, the data in Tables 1 ~ 3 showed the elicitor treatment at the beginning of culture also caused the inhibition of the *E. longifolia* cell growth. The dry weight of cell biomass was only 0.57 ~ 0.71% (YE treatment), 0.1 ~ 0.85% (MeJA treatment), and 0.51-0.78% (SA treatment) compared to control after 14 days of culture ($p<0.05$). In contrast, the eurycomanone contents have increased from 1.34 to 2.18-fold (YE treatment), 0.1 to 0.85-fold (MeJA treatment), and 3.04 to 3.88-fold (SA treatment) ($p<0.05$).

Suitable elicitation time depends on response of different plant species. Yamamoto et al. (1995) found flavanone accumulation in the callus of *Sophora flavescens* is 5 times higher than that of the control when 2 ~ 8 mg/mL YE was added in medium on the beginning of culture. While Zhao et al. (2002) obtained a tanshinone amount 10-fold higher than that of the control from cell culture of *Salvia miltiorrhiza* when treated 100 mg/L YE at day 18 of culture. Krzyzanowska et al. (2012) added 100 μM MeJA in medium of *Mentha* × *piperita* cell suspension culture after 7 days of inoculation to improve rosmarinic acid production. The results showed that rosmarinic acid content reached a maximum value of 117.95 mg/g DW within 24 h. Zabala et al. (2010) was obtained highest peruvoside content (8.93 mg/L medium) of *Thevetia peruviana* cell culture at a concentration of 100 mg/L MeJA applied at the beginning of the culture. Study of Rahimi et al. (2014) showed that sesquiterpenes in *Panax ginseng* were highly accumulated within 24 h after adventitious roots were cultured in medium supplemented with 200 μM SA. Coste et al. (2011) investigated the effects of SA on accumulation of hypericins (hypericin and pseudohypericin) in shoot cultures of *Hypericum hirsutum* and *H. maculatum*. 50 μM SA treatment showed the biosynthetic enhancements of hypericin (7.98-fold) and pseudohypericin (13.58-fold) in *H. hirsutum*. While in *H. maculatum*, 200 μM SA treatment enhanced the production of hypericin up to 2.2-fold and pseudohypericin up to 3.94-fold.

Our results also indicated that the most suitable elicitor treatment times for *E. longifolia* cells are from 4 to 6 days after inoculation. 20 μM MeJA was added to the medium at 4th day of culture has improved eurycomanone amount up to 17.36 mg/g DW, higher 10-fold than compare to the control that is untreated cells. Similarity, 20 μM SA or 200 mg/L YE was added to the medium at 4th or 6th day also stimulated eurycomanone production up to 5.2 mg/g and 6.25 mg/g DW, higher 1.46 or 1.69-fold than compare to the control, respectively.

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Conflict of interest

We declare that there is no conflict of interest regarding the publication of this article.

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