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SHORT COMMUNICATION

Methyl jasmonate induced overproduction of eleutherosides in somatic embryos of *Eleutherococcus senticosus* cultured in bioreactors

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Abbreviations: 2,4-D: 2,4-dichlorophenoxy acetic acid
HPLC: high performance liquid chromatography
MJ: methyl jasmonate
MS: Murashige and Skoog

This study was concentrated on the production of eleutherosides and chlorogenic acid in embryogenic suspension cultures of *Eleutherococcus senticosus* by exposing them to different concentrations (50-400 µM) of methyl jasmonate (MJ) during the culture period. In the bioreactor cultures, eleutheroside content increased significantly by elicitation of MJ, however, the fresh weight, dry weight and growth ratio of embryos was strongly inhibited by increasing MJ concentrations. The highest total eleutheroside (7.3 fold increment) and chlorogenic acid (3.9 fold increment) yield was obtained with 200 µM MJ treatment. There was 1.4, 3.4 and 14.9 fold increase in the eleutheroside B, E, and E1 production respectively with such elicitation treatment. These results suggest that MJ elicitation is beneficial for eleutheroside accumulation in the embryogenic cell suspension cultures.

Eleutherococcus senticosus Rupr. & Maxim is popularly known as Siberian ginseng or Eleuthero is an important medicinal plant belonging to the same Araliaceae family as ginseng (*Panax* spp.) and was naturally distributed in northeastern Asia and now extensively used in USA (Yat et al. 1998). Roots and rhizome of the plant have long been used as a stimulant and enhance overall resistance to diseases and stress (Farnsworth et al. 1985). Extracts from the plant showed analgesic, anti-inflammatory, antipyretic

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and diuretic action. Active components of *Eleutherococcus* species include (1) phenylpropane compounds: synergine = eleuthroside B, sinaptic alcohol, chorogenic acid, caffeic acid derivatives; (2) lignans: syringaresinol-4,4"-O-B-D-diglycoside E (D); syringaresinol-4-O-B-D-glycoside, syringaresinol; (3) coumarins: isofraxidine 7-O glucoside and its aglycone-isoraxidine; (4) polysaccharides; and (5) other compounds, such as oleanic acid, aromatic oils, sugar. These active components showed positive effects on cellular defense and physical fitness in man (Szolomicki et al. 2000). *E. senticosus* is listed as threatened species because of excessive commercial harvest from natural habitat. Propagation of the plants by seed is difficult because over eighteen months stratification is required for germination of zygotic embryos. So, the plant tissue culture process has been looked at as a potential alternative for the more efficient mass propagation method. Recently, induction of somatic embryogenesis has been reported (Choi et al. 1999). Somatic embryos were successfully cultivated in bioreactors and germinated somatic embryos are used as raw material for medicinal purposes, but the accumulation of physiologically active eleutherosides in germinating embryos was low.

Elicitation has been proved to be effective way to increase secondary metabolite production. A number of elicitors and precursors such as methyl jasmonate (MJ) have been used successfully for enhancing production of secondary metabolites such as saikosaponins, taxoids, plaxitaxel and baccatins, ginsenosides during cell cultures of many plant species (Yukimune et al. 1996; Ketchum et al. 1999; Yu et al. 2000; Aoyagi et al. 2001; Yu et al. 2002; Kim et al. 2004; Thanh et al. 2005). In recent years, we have been searching for a strategy that could significantly affect the accumulation of eleutherosides by focusing on commercially valuable eleutherosides as a research target. In the present study, MJ was employed in embryogenic suspension of *E. senticosus* in order to examine the impacts on eleutherosides production.

MATERIALS AND METHODS

Induction of somatic embryogenesis and maintenance of stock cultures of embryos

Young leaves (2 cm in length) of *E. senticosus* were collected from *in vitro* grown plants and cut into 5 x 5 mm pieces, cultured on Murashige and Skoog medium (MS, Murashige and Skoog, 1962; pH 5.8; Duchefa, Haarlem, Netherlands) with 1 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D), 3% (w/v) sucrose and 0.2% (w/v) gel rite and cultures were maintained in dark at 25ºC. Embryogenic callus was developed from the leaves within twelve weeks after culture. Embryogenic callus was maintained on MS liquid medium supplemented with 1 mg L^{-1} 2,4-D, 3% (w/v) sucrose and 0.2% (w/v) gel rite by sub-culturing once in four weeks.

Embryogenic cell suspension culture

Embryogenic cells of *E. senticosus* were transferred to MS liquid medium supplemented with 1 mg L^{-1} 2,4-D and suspension cultures were sub-cultured at every two weeks interval. To induce somatic embryos, 2 weeks old embryogenic cell clumps were filtered through a sterile 212 µm stainless steel sieve to remove the larger clumps. The suspension was allowed to settle for 5 min for easier removal of the used medium. About 500 mg of cell clumps was transferred to 100 mL MS liquid medium without 2,4-D in 300 mL Erlenmeyer flasks. The cultures were incubated at 100 rpm on a gyratory shaker (SI-600R, Jeio Tech, Seoul, South Korea) in dark at 25ºC. At the end of four weeks of culture, the content of flask was passed through different stainless steel sieves to separate different stages of embryos (>800 µm = cotyledonary; 600 µm = torpedo; 420 µm = heart; <420 µm globular). Cotyledonary embryos were used as explants for establishing subsequent cultures.

Table 1. The effect of MJ on *Eleutherococcus senticosus* embryogenic suspension after six weeks of bioreactor culture.

MJ concentration (µM)	Biomass		Growth ratio[z]
	Fresh weight (FW) (g L^{-1})	Dry weight (DW) (g L^{-1})	
0	102.65a[y]	11.32a	20.36
50	103.16a	10.60a	19.01
100	104.66a	10.10b	18.05
150	102.52a	9.52c	16.96
200	99.25b	9.29c	16.52
300	88.81c	7.58d	13.30
400	18.30d	2.91e	4.49

[y]Mean separation within column by Duncan's multiple range test at $p < 0.05$.
[z]Growth ratio is the quotients of the dry weight after cultivation and the dry weight of the inoculum.

Establishment of large scale suspension cultures in bioreactors

Ten grams of cotyledonary somatic embryos were transferred to 3 L balloon type bubble bioreactor with 2 L MS liquid medium with 3% (w/v) sucrose and 4 mg L^{-1} GA_3. The pH of the medium was adjusted to 5.8 before autoclaving by using 0.1 N hydrochloric acid or 0.1 N sodium hydroxide. The volume of input air was adjusted to 0.1 v/v (air volume/culture volume) per min. Cultures were kept under a 16 hrs photoperiod at 35 µmol m^{-2} s^{-1} photosynthetic photon flux. In an elicitation experiment different concentrations of MJ (0, 50, 100, 150, 200, 300 or 400 µM) was added to the cultures on the day of inoculation. The fresh and dry weights were recorded after 6 weeks of culture. Dry weight was determined after drying the biomass for 24 hrs at 60ºC. Data were subjected to Duncan's multiple range tests using SAS program (Version 6.12, SAS Institute Inc., Cary, USA).

Determination of eleutherosides

Germinated somatic embryos were dried and powdered (2 g) with a blender and extracted with 60% aqueous methanol (2 x 50 mL) for 30 min. each at 60ºC, and filtered through filter paper (No. 2, 90 mm, Advantec, Toyo, Japan). The combined extract, was evaporated to dryness in vacuum and washed with 50 ml of ether. The insoluble fraction was dissolved in water and extracted with *n*-butanol (water saturated). The organic phase was evaporated to dryness, dissolved in (10 mL) high performance liquid chromatography (HPLC) grade methanol and filtered through 0.45 µm membrane filter (Polyvinylidene difluoride, Whatman, USA) filter. Eleutherosides were quantified by HPLC (Waters 2690 separation modules, Waters, USA) equipped with a Symmetry® C 18 column (4.6 mm x 250 mm, Waters, USA) by following the procedure described previously (Apers et al. 2005). Eleutherosides and chlorogenic acid were separated using a flow rate 0.8 mL min^{-1} with water (solvent A) and acetonitrile (solvent B) as the mobile phase. The elution programme was: initially 90:10 (A:B) with isocratic elution for 5 min followed by linear gradient to 80:20 in 22 min, linear gradient to 60:40 in 15 min, isocratic for 5 min, linear gradient to the starting conditions (90:10) in 5 min and isocratic for 5 min (equilibration time). Quantification was based on ultraviolet absorption at 216 nm. The peak areas corresponding to eleutherosides from the samples, with same retention time as authentic eleutherosides and chlorogenic acid. Retention times were 9.86, 13.10, 21.90 and 31.20 min for eleutheroside B, chlorogenic acid, eleutheroside E and E1 respectively.

RESULTS AND DISCUSSION

The growth and secondary metabolite accumulation by the embryos of *E. senticosus*, cultivated in bioreactor cultures are presented in Table 1 and Table 2. The embryos in the untreated cultures reached 102.65 g L^{-1} fresh weight and 11.32 g L^{-1} dry weight. Growth of embryos was significantly affected by the application of MJ. There was slight increment in fresh weight of embryos (104.66 g L^{-1}) when compared to the control (Table 1). However, the fresh weight, dry weight and growth ratio were decreased with increasing MJ concentration. On the other hand, eleutheroside content was significantly enhanced by the addition of MJ. Amount of total eleutherosides and chlorogenic acid increased with increasing MJ concentration, and reached a maximum at 200 µM MJ representing 7.3 fold (649.95 µg g^{-1} DW) and 3.9 fold (4.48 mg g^{-1} DW) increases over controls respectively. There were 1.4, 3.4 and 14.9 fold increments in eleutheroside B, E and E1 respectively compared to the control.

The accumulation of secondary metabolites in plants is part of the defense response against pathogenic attack, which is triggered and activated by elicitors, the signal compounds of plant defense responses (Zhao et al. 2005). Therefore, the treatment of plant cells with biotic and/or abiotic elicitors has been a useful strategy to enhance secondary metabolite production in cell cultures. The most frequently used elicitors in previous studies were fungal

Table 2. The effect of MJ on accumulation of eleutherosides and chlorogenic acid in somatic embryos of *Eleutherococcus senticusus* cultured in bioreactors[z].

MJ concentration (µM)	Eleutherosides (µg g^{-1} DW)				Chlorogenic acid
	B	E	E1	Total	(mg g^{-1} DW)
0	25.55c[y]	28.60e	34.55f	88.70f	1.14e
50	26.73c	89.05c	119.96e	235.74e	2.01d
100	27.95c	93.95b	150.00d	271.90d	2.38c
150	32.70b	89.00c	315.50b	437.20b	4.04b
200	37.40a	99.40a	517.50a	649.95a	4.48a
300	33.05b	90.90c	238.15c	366.45c	2.43c
400	31.51b	85.26d	159.56d	276.33d	1.33e

[y]Mean separation within column by Duncan's multiple range test at $p < 0.05$.
[z]Data was taken after 6 weeks of culture.

carbohydrates, yeast extract, MJ and chitosan. MJ, a proven signal compound, is the most effective elicitor of taxol production in *Taxus chinensis* Roxb. (Wu and Lin, 2003) and ginsenoside production in *Panax ginseng* C.A. Meyer(Yu et al. 2000; Yu et al. 2002; Kim et al. 2004; Thanh et al. 2005) cell/organ culture. In the present study, the effect of different concentrations of MJ on embryogenic cell growth and eleutheroside accumulation was tested and results reveled that addition of 200 µM MJ was suitable for optimum accumulation of eleutheroside B, E, E1 and chlorogenic acid. However, addition of MJ at higher concentration (above 100 µM) was detrimental for biomass accumulation. Similar to the present results, MJ inhibited the cell growth and promoted the secondary metabolite production with cell/adventitious root cultures of *Bupleurum falcatum* L. (Aoyagi et al. 2001), *Taxus* spp. (Yukimune et al. 1996; Ketchum et al. 1999) and *Panax ginseng* C.A. Meyer (Kim et al. 2004; Thanh et al. 2005). Differential accumulation of eleutherosides was observed during elicitation experiments (Table 2). Eleutheroside E1 content was highest among the different eleutherosides produced by the suspended somatic embryos. Similar to the present observation differential accumulation of secondary compounds have been reported during cell/organ cultures of *Panax ginseng* (Kim et al. 2004; Thanh et al. 2005).

The results from this study demonstrate that MJ elicitation strategy was quite useful to improve the yield of eleutherosides and chlorogenic acid in embryogenic cell cultures of *E. senticosus*. The biomass produced in the bioreactor cultures may be used as source of medicinal raw material for the extraction of eleutherosides and chlorogenic acid.

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