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Short communication

Increased eleutheroside production in *Eleutherococcus sessiliflorus* embryogenic suspension cultures with methyl jasmonate treatment

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Abstract

The effects of methyl jasmonate elicitation were investigated in embryogenic suspension cultures of *Eleutherococcus sessiliflorus* in bioreactors for the production of eleutherosides and chlorogenic acid. Eleutherosides and chlorogenic acid increased with methyl jasmonate elicitation (in the range 50–400 μM); however, fresh weight, dry weight and growth ratio of the cells were inhibited by increasing methyl jasmonate concentration. There were 3.5-, 2.6-, 2.5- and 3.2-fold increments in eleutherosides B, E, E1 and chlorogenic acids were evident, respectively, with 200 μM methyl jasmonate treatment. These results suggest that methyl jasmonate elicitation is beneficial for eleutheroside accumulation in the embryogenic suspension cultures.

Keywords: *Eleutherococcus sessiliflorous*; Eleutherosides; Elicitation; Methyl jasmonate

1. Introduction

Eleutherococcus sessiliflorus (Rupr. & Maxim.) S.Y. Hu is an important medicinal plant belonging to the family Araliaceae, and is distributed mainly in northeastern Asia. Extracts from roots are used primarily for the analgesic, anti-inflammatory, antipyretic and diuretic action [1]. *Eleutherococcus* species contain eleutherosides including A, B, C, D, E, F, G and complex polysaccharides as active ingredients from roots and leaves [5]. *E. sessiliflorus* is listed as threatened species because of excessive commercial harvest from natural habitat. Propagation of the plants by seed is difficult because over 18 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, we reported induction of somatic embryogenesis in *E. sessiliflorus* and *Eleutherococcus chiisanensis* and subsequent mass production of somatic embryo as medicinal raw materials by suspension (bioreactor) cultures, but the accumulation of physiologically active eleutherosides in somatic embryos were low [6,11].

Development of an efficient cell culture system for commercial production of eleutherosides requires enhancement strategies. Elicitation has been effective in increasing product yields of a wide variety of secondary metabolites. Jasmonates, as key signal compounds, are widely used elicitors for the production of secondary metabolites in plant cells [14]. In the present study, methyl jasmonate (MJ) was added to embryogenic suspension cultures of *E. sessiliflorus* and result showed that it enhanced accumulation of eleutherosides. This work is considered beneficial to the commercial production of eleutherosides.

2. Materials and methods

2.1. Somatic embryogenesis

Embryogenic callus was induced from young leaves of *E. sessiliflorus* (Rupr. & Maxim.) S.Y. Hu by following the procedures of Shohael et al. [11]. Embryogenic callus was maintained on Murashige and Skoog (MS, pH 5.8) [9] medium supplemented with 1 mg l^{-1} 2,4-D, 3% (w/v) sucrose and 0.2% (w/v) gel rite

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in dark at 25 °C by subculturing to fresh medium every 2-week interval.

2.2. Embryogenic cell suspension culture

Five hundred milligrams of embryogenic cells of *E. sessiliflorus* were transferred to 300 ml flasks containing 100 ml MS liquid medium supplemented with 1 mg l^{-1} 2,4-D and were maintained on rotary shaker at 100 rpm, in dark at 25 °C. Suspension cultures were sub-cultured at every 2-week interval. To induce somatic embryos, 2-week-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 growth regulators in 300 ml Erlenmeyer flasks. The cultures were incubated at 100 rpm on a gyratory shaker in dark at 25 °C. At the end of 4 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.

2.3. Establishment of large-scale suspension cultures in bioreactors and methyl jasmonate elicitation

Ten grams of cotyledonary somatic embryos were transferred to 3 l balloon type bubble bioreactor (Fig. 1; Samsung Scientific Company, Seoul, Korea) 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. The volume of input air was adjusted to 0.1 vvm (air volume/culture volume, min). Cultures were kept under a 16 h photoperiod at 35 μmol m^{-2} s^{-1} photosynthetic photon flux. In an elicitation experiment different concentrations of methyl jasmonate (MJ, 0, 50, 100, 200 and 400 μM) was added to the cultures on the day of inoculation. The fresh and dry weights of germinated embryos and levels of eleutherosides and chlorogenic acid in the embryos were recorded at weekly intervals up to 6 weeks. Biomass and secondary metabolite (eleutherosides B, E, E1 and chlorogenic acid) levels were recorded after 6 weeks of culture in another set of cultures. Dry weight was determined after drying the biomass for 24 h at 60 °C. The growth ratio was calculated as: increased dry weight [harvested dry weight (g) − inoculated dry weight (g)]/inoculated dry weight (g). Data were subjected to Duncan's multiple range tests using SAS program.

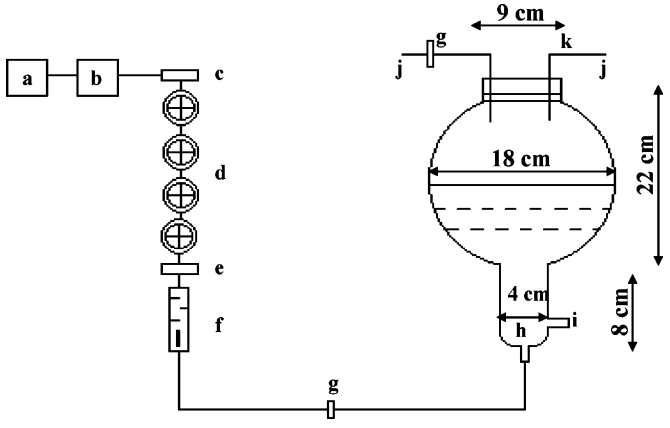

Fig. 1. Schematic diagram of a 3 l capacity air lift bioreactor. (a) Air compressor; (b) air reservoir; (c) air cooling device; (d) air filter system; (e) air dryer, (f) air flow meter; (g) membrane filter; (h) glass sparger; (i) medium sampling port; (j) vent and (k) prefilter.

2.4. Determination of eleutherosides

Germinated somatic embryos were dried and powdered (2 g), extracted with 60% aqueous methanol (2× 50 ml) for 30 min each at 60 °C, and filtered through filter paper (Advantec, Toyo, Japan). The combined extract, was evaporated to dryness in vacuum and washed with ether. The insoluble fraction was dissolved in water and extracted with *n*-butanol (water saturated). The organic phase was evaporated to dryness, dissolved in methanol (10 ml) and filtered through 0.45 μm (Gelman, USA) filter. Eleutherosides were quantified by HPLC (Waters 2690 separation modules, Waters, USA) equipped with a Symmetry C18 column (4.6 mm × 250 mm, Waters, USA) according to Yat et al. [13] and using a photodiode array detector (Waters 996 photodiode array detector, Waters, USA). Eleutherosides and

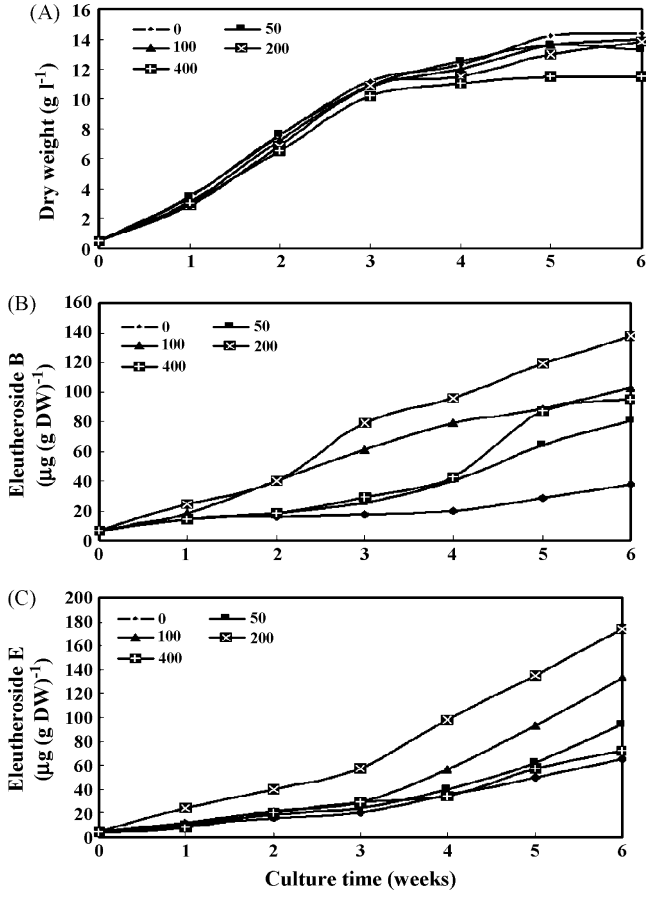

Fig. 2. Dynamic profiles of biomass of embryos (by dry weight) (A), eleutheroside B content (B) and eleutheroside E content (C) in embryonic suspension cultures of *Eleutheococcus sessiliflorus* treated by MJ elicitation.

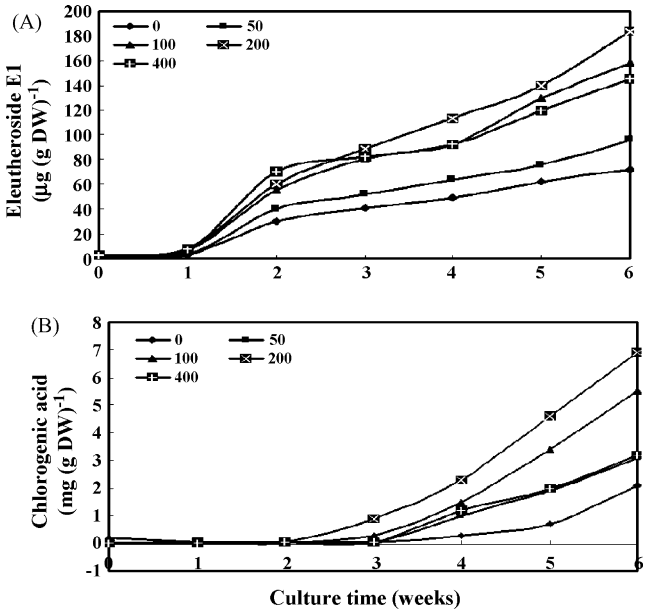

Fig. 3. Dynamic profiles of eleutheroside E1 content (A) and chlorogenic acid content (B) in embryonic suspension cultures of *E. sessiliflorus* treated by MJ elicitation.

chlorogenic acid were separated using a flow rate 0.8 ml min^{-1} with water and acetonitrile as the mobile phase with a step-wise gradient of 10% acetonitrile for 0–5 min, 20% acetonitrile for further 20 min, 40% for another 15 min and equilibration with 5% acetonitrile for 5 min. 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 B, E, E1 (ChromaDex, USA) and chlorogenic acid (Sigma, USA) were integrated by comparison with an external standard calibration curve.

3. Results and discussion

The results of dynamic profiles of growth of embryos (dry biomass) and secondary metabolites (eleutheroside B, E, E1 and chorogenic acid) accumulation during elicitation experiment are presented in Figs. 2 and 3. Biomass of embryos was not much affected by the application of lower concentration of MJ up to 200 μM. However, biomass was reduced substantially with the cultures treated with 400 μM MJ (Fig. 1). There was 21% and 7% growth reduction in fresh and dry biomass of embryos at the end of 6 weeks with 400 μM MJ elicitation compared to control (Table 1). The growth ratio was reduced from 19.7 (control) to 15.5 with 400 μM MJ treatment. On the other hand, the level of eleutherosides B, E, E1 and chlorogenic acid were increased by MJ elicitation (Figs. 2B and C and 3A and B). The amount of eleutherosides B, E, and E1 was 138, 174 and 184 μg g^{-1} DW, respectively, with the cultures 200 μM MJ elicitation; while these values were 38, 65 and 71 μg g^{-1} DW, respectively, for the control (Table 2). Thus, there were 3.5-, 2.6- and 2.5-fold increments in eleutherosides B, E, and E1 was evident with 200 μM MJ treatment, respectively, compared to the control. Chlorogenic acid content was increased to 6.9 mg g^{-1} DW with 200 μM MJ treatment, whereas it was 2.1 mg g^{-1} DW for the control. Thus 3.2-fold increment of chlorogenic acid is evident with MJ elicitation.

Table 1
The effect of methyl jasmonate (MJ) on biomass of *Eleutherococcus sessiliflorus* embryogenic suspension after six of bioreactor culture[a]

MJ concentration (μM)	Biomass		Growth ratio[b]
	Fresh weight (FW) (g l^{-1})	Dry weight (DW) (g l^{-1})	
0	235 b	14.4 a	19.7
50	236 a	14.3 a	19.8
100	230 ab	14.0 ab	19.1
200	225 b	13.9 b	19.0
400	153 c	11.5 c	15.5

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

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 compound of plant defense responses. Therefore, the treatment of plant cells with biotic and abiotic elicitors has been a useful strategy to enhance secondary metabolite production in cell/organ cultures [2,4]. The elicitors most frequently used in previous studies include fungal carbohydrates, yeast extract, MJ and chitosan. The present results illustrate higher accumulation of eleutherosides and chlorogenic acid by MJ elicitation. Similarly, MJ and jasmonate derivatives have been used as elicitors for higher accumulation secondary metabolites in cell cultures of *Taxus chinensis* [3,7,10] and *Panax ginseng* [8,12]. Elicitor dosage is one of important factor which affect the biomass and secondary metabolite accumulation; for example 100 μM methyl jasmonte or dihydro-methyl jasmonate addition found suitable accumulation taxnae production in suspension cultures of *T. chinensis* and these compounds elicits taxane accumulation without any inhibitory effects in the cell growth; whereas higher levels (above 100 μM) inhibited cell growth [3]. Ketchum et al. [7] reported that 200 μM MJ was best for paclitaxel production, and elicitation effect was greatly decreased under a higher level of MJ (600 or 800 μM). In our culture system, 200 μM MJ found suitable for optimum accumulation of eleutherosides B, E and E1 as well as chlorogenic acid (Figs. 2 and 3; Tables 1 and 2), however

Table 2
The effect of methyl jasmonate (MJ) on accumulation of eleutherosides and chlorogenic acid in somatic embryos of *E. sessiliflorus* cultured in bioreactors[a,b]

MJ concentration	Eleutherosides (μg g^{-1} DW)				Chlorogenic acid
(μM)	B	E	E1	Total	(mg g^{-1} DW)
0	38 d	65 d	72 d	175 e	2.1 d
50	81 c	94 c	96 c	271 d	3.1 c
100	102 b	133 b	158 b	393 c	5.5 b
200	138 a	174 a	184 a	496 a	6.9 a
400	95 bc	72 d	145 b	312 c	3.2 c

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

this concentration did not affect the biomass accumulation. Whereas higher concentration (400 μM) MJ inhibited embryogenic biomass as well as accumulation of eleutherosides and chlorogenic acid. Increment in eleutheroside B content was highest (3.5-fold increment) compared to eleutheroside E (2.65-fold increment) and eleutheroside E1 (2.5-fold increment) produced by the suspended somatic embryos due to 200 μM MJ elicitation. Similar to the present observation differential accumulation of secondary compounds have been reported during cell/organ cultures of *P. ginseng* [8,12]. Eleutheroside E (syringaresinol di-*O*-β-D-glucosedie D) and eleutheroside B (syringin) are thought to be the pharmacologically active among eleutherosides [13] and their concentration was enhanced in the present experiments due to MJ elicitation and thus MJ elicitation is beneficial for production of pharmacologically important eleutherosides.

4. Conclusion

In conclusion, the present work demonstrates the beneficial effect of MJ elicitation on embryogenic suspension cultures of *E. sessiliflorus* which is accountable for enhanced accumulation of eleutherosides and chlorogenic acid. Among the various levels of MJ (50–400 μM) tested, 200 μM MJ treatment was found suitable and this concentration was responsible for 3.5-, 2.6-, 2.5- and 3.2-fold increments in eleutherosides B, E, E1 and chlorogenic acid, respectively, compared to control. These results illustrate the possibilities of production of desirable eleutherosides on large scale by using bioreactor cultures. The further investigation on the time of addition of MJ and combination of MJ and sucrose feeding may stimulate higher accumulation of eleutherosides in embryogenic suspension cultures of *E. sissiliflorus*.

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