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Effects of Murashige and Skoog medium strength on germination and secondary metabolites production of *Eleutherococcus senticosus*'s somatic embryos in bioreactor

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Abstract

Somatic embryos of *Eleutherococcus senticosus* were germinated into different strength of Murashige and Skoog medium (MS) in balloon type bubble bioreactor (working volume 2Lit) to investigate the effects of medium strength on biomass growth and metabolites production. Fresh weight, dry weight and % dry weight of the germinated somatic embryos increased with the increase of medium strength up to MS2X. Further increase in medium strength decreased all the growth parameters mentioned above significantly. Maximum amount of total eleutheroside (166.37µg/g DW) was obtained in full strength of MS medium but highest chlorogenic acid (2.26 mg/g DW) was obtained in MS2X. Contents of total phenolics and flavonoids were increased with the decrease of MS strength but polysaccharides content increased when medium strength increased. Thus it may explore immense practical applicability in somatic embryos scale up, where the secondary metabolite production from germinated somatic embryos is directly related to MS medium strength.

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Introduction:

Eleutherococcus senticosus (commonly known as Siberian ginseng sp. Araliaceae) is extensively cultivated species in northeast Asia, including much of the far southeastern Russia, northeast China, Korea, and Japan because of its nutraceutical properties. The main active compound in this species is Eleutheroside. Among them, Eleutheroside B (syringin) and E (liriodendrin) were known to have the most pronounced stimulant and anti-stress effects (Brekhman and Dardymov, 1969). These remedial properties help to scavenge free radicals, reduce stress, to improve the immune system, to fight cancer and aging, to reduce other effects of radiation, to improve adrenal function (Schmolz *et al.,* 2001; Gaffney *et al.,* 2001; Shohael *et al.,* 2005). Seed propagation of *E. senticosus* is very difficult because stored seed requires 6 months warm followed by 3 months cold stratification to germinate (Lee, 1979; Isoda and Shoji, 1994). So, the plant tissue culture process has been looked as a potential alternative for the efficient mass propagation method. Plant regeneration through direct somatic embryogenesis (Gui *et al.,* 1991; Choi *et al.,* 1999) and indirect embryogenic callus and cell suspension culture has been reported in *E. sessiliflorus* (Shohael *et al.,* 2005).

Somatic embryogenesis also offer a potential system for large-scale plant propagation in automated bioreactor (Peak and Chakrabarty, 2003; Paek *et al.,* 2005). Conventional micropropagation requires intensive labor and quite often, it limits commercial application. Automation can reduce the labor requirements, which is linked to scale up of cultures. Large-scale somatic embryo culture is an attractive alternative to the traditional method of plantation or plant cell culture. The successful production of somatic embryos through bioreactors has been reported in a number of species (Paek *et al.,* 2005), but still many improvements are required for commercial somatic embryo production.

The use of plant tissue culture for secondary product biosynthesis, particularly in plants of pharmaceutical importance, holds promise for the controlled production of these important plant constituents (Lindsey and Yeoman, 1983). There is no universal medium for *in vitro* culture, since plant species and cultivars are genetically specific with regard to different components of the medium, which include not only organic substances, but also mineral elements (Sari´c *et al.,* 1995). According to Williams, 1995 the *in vitro* chemical microenvironment can be regarded as having three main phases, the medium, the atmosphere or headspace and the plant material. Under *in vitro* conditions, plant growth depends on the mineral elements and organic components of the medium, due to a very low level of photosynthesis and small leaf area of the plants. Hence, the choice of mineral and organic components is very important (Lumsden *et al.,* 1990). The biosynthesis and production yields of secondary metabolites in plant cell cultures are affected by many factors. Secondary metabolites affected by medium composition were reported by Rokem and Goldberg, 1985. It is also reported that germinated somatic embryos contain higher level of secondary metabolites in compare with field-grown leaves and roots (Shohael *et al.,* 2005). Mass production of somatic embryos is now a well establish technology for the production of identical plants and also metabolites. Production of secondary metabolites through germinated embryos is a potential approach that also possible to easy scale up in bioreactor. For large scale production in bioreactor it is very important to determine the appropriate amount of mineral requirements for growth and also secondary metabolites production.

Though production of somatic embryos in bioreactors has been reported in a number of species but still many improvements are needed for practical automated somatic embryos production systems and its germination. As yet there is no reliable method of determining the specific medium composition for untried culture. The present study described the effect of different MS medium strength on growth parameters including fresh weight, dry weight and percent dry weight on germinated somatic embryos

and also production of secondary metabolites in bioreactor.

Materials and Methods

Plant material

In vitro seedlings of *E. senticosus* were maintained in MS medium (Murashige and Skoog, 1962) without plant growth regulators (PGRs). Young leaves (2 cm in length) were collected from sub cultured plants at every 3 weeks. Leaves were cut into 5 x 5 mm pieces and used as an explant source.

In vitro culture conditions

Leaf explants were placed on semisolid MS basal medium supplemented with 1.0 mg l^{-1} 2,4-D with 3 % sucrose and 0.2 % gelrite for callusing and somatic embryogenesis. The medium was adjusted to pH 5.8 prior to addition of gelrite and sterilized at 121 ^{o}C for 15 min and distributed into 15 x 140 mm Petridishes (15 ml of medium). Cultures were maintained in the dark at 25 ^{o}C and evaluated for somatic embryogenesis after 12 weeks. For proliferation of embryogenic cultures on solidified medium, the embryogenic calli were cultured on the same medium as described above and subcultured on every 15 days interval.

Embryogenic cell suspension culture

Embryogenic cells of *E. senticosus* were transferred into MS liquid medium supplemented with 1 mg l^{-1} 2,4-D. The suspension cultures were subcultured into fresh medium on every 14 days of interval. To induce somatic embryos, embryogenic cell clumps were filtered after 2 weeks of subculture through a 90-120 µm stainless steel sieve to remove the larger clumps and allowed the suspension to settle for 5 min. After removal of the remnant 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 agitated at 100 rpm on a gyratory shaker. The culture room was maintained at 25 °C and a 16/8 h (day/night) photoperiod with light supplied by white-fluorescent tubes at an intensity of 35 µmol $m^{-2}s^{-1}$.

Bioreactor culture for somatic embryogenesis

A balloon type bubble bioreactor (BTBB) (Paek *et al.,* 2005; Shohael *et al.,* 2006) was used for the entire regeneration phase using embryogenic suspensions. Ten grams of embryogenic aggregates were placed in the 3L bioreactor along with 2L of MS medium without any PGRs. The pH of the medium was adjusted to 5.8 before autoclaving (at 121 ^{o}C and 1.2 kg cm^{-2} pressure for 32 min). The volume of input air was adjusted to 0.1 vvm (air volume/culture volume, min). All the bioreactors were maintained at 25 ^{o}C in dark. After 21-28 days embryos were well grown and different developmental stages were clearly seen.

Germination of somatic embryos in to the bioreactor

Twenty grams of matured somatic embryos (torpedo and cotyledon type of embryos were separated with bigger sieve in aseptic condition) were transferred to 3L BTBB with 2L of different strengths of MS liquid medium (medium (1/5, 1/3, 1/2, 1 and 2 strength were used) supplemented with 3% sucrose and 4 mg l^{-1} gibberellic acid 3 (GA_3). All the bioreactors were kept at 25^{o}C under 35 □ mol m^{-2} s^{-1} PPFD (photosynthetic photon flux (area) density) under 16 h photoperiod per day.

Measurement of growth

After 45 days of culture, germinated embryos were harvested and washed several times with distilled water then soaked in soft tissue paper to remove water and measured fresh weight. For dry weight, the embryos were dried at 60^{o}C in to an oven drier until completely dry and then measured.

Determination of Eleutherosides and chlorogenic acids

Oven dried embryos were powdered in to the blender. One gm of fine powder was extracted with 60% aqueous methanol (2x50 ml) for 30 min each at 60 ^{o}C separately, and filtered through filter paper (Advantec, Toyo, Japan). The combined extract, evaporated to dryness in vacou, and washed with diethyl ether. The insoluble fraction was dissolve in water and extracted with n-butanol (water

saturated). The organic phase was evaporated to dryness, dissolved in (5 ml) of HPLC grade methanol and filtered through 0.45 µm PVDF (Gelman, USA) filter. Eleutherosides and Chlorogenic acids were quantified by HPLC according to procedures reported by Patrick *et al.,* 1978; Shohael *et al.,* 2006 with use of a liquid chromatograph system (Waters 2690 separation modules, Waters, USA) and a photodiode array detector (Waters 996 photodiode array detector, Waters, USA) on Symmetry^R C 18 (4.6 x 250 mm) column (Waters, USA). Eleutherosides were analyzed by using a flow rate of 0.8 ml/min with water and acetonitrile as the mobile phase with a linear gradient of 10 % acetonitrile for 0-5 min, 20 % acetonitrile for further 20 min, 40 % for another 15 min and reequilibration with 5 % acetonitrile for 5 min. Quantitation was based on ultraviolet absorption at 216 nm. The peak areas corresponding to eleutherosides from the samples, with the same retention time as authentic eleutherosides B, E and E1 and Chlorogenic acid (ChromaDex, Inc.USA) were integrated by comparison with an external standard calibration curve.

Determination of total phenolic contents

The content of total phenolics in plant methanolic extracts were analyzed spectophotometrically by a modification of Folin-Ciocalteu colorometric method (Folin and Ciocalteu, 1927). Hundred microliters of methanolic extracts were mixed with 2.5 ml of deionized water, followed by addition of 0.1 ml (2N) Folin-Ciocalteu reagent. They were mixed well and allowed to stand for 6 min before 0.5 ml of a 20% sodium carbonate solution was added. The color was developed after 30 min at room temperature and the absorbance was measured at 760 nm using a UV visible spectrophotometer (UV-1650PC, Shimadzu, Japan). The measurement was compared to a slandered curve of prepared gallic acid solution and expressed as means (±SE) mg of gallic acid equivalent per gram of plant material for the triplicate extracts.

Determination of total flavonoid contents

Total flavonoid content was determined by colorimetric method (Shohael *et al.,* 2005). Briefly, 0.25 ml of the methanolic plant extract or (+)-catechin standard solution was mixed with 1.25 ml of distilled water, followed by addition of 0.75 ml of a 5 % sodium nitrite solution. After 6 min, 0.15 ml of a 10 % aluminum chloride solution was added and the mixture was allowed to stand for a further 5 min before 0.5 ml of 1M sodium hydroxide was added. The mixture was brought to 2.5 ml with distilled water and mixed well. The absorbance was measured immediately at 510 nm using a spectrophotometer (UV-1650PC, Shimadzu, Japan). The results were expressed as means (±SE) mg of (+)-catechin equivalents per gram of plant material for the triplicate extracts.

Determination of polysaccharides content

During extraction dried powder with 60% methanol, the sediment was collected and desiccated in an oven at 60oC. 0.2g of sediment was dissolved in 5 ml 5% (v/v) sulphuric acid and placed in boiling water for 2 hr. After acidic hydrolysis, the liquid-solid mixture was diluted in 50ml of distilled water. The supernatant was separated by sedimentation, and the polysaccharide in the supernatant assayed according to the carbazole reaction method (Shohael *et al.,* 2006) as followed. 0.2 ml of liquid taken from the above supernatant that was mixed with 6 ml of concentrated sulphuric acid in a boiling water bath for 20 min, and cooled. Then, 0.2 ml of carbazole-absolute ethanol (0-15 %, v/v) was added and mixed. After 2hr of darkness at room temperature, a purplish red color developed and absorbance was measured at 530 nm. D-galacturonic acid (0, 50, 100, 200, 400 and 600 mg ml-1) was used as a standard.

Statistics

Each treatment was consisted by three replications and all the experiments were repeated thrice. Data were subjected to Duncan's multiple range tests using SAS program (Version 6.12, SAS Institute Inc., Cary, USA). Means and standard errors were used

throughout, and statistical significance between mean values was assessed using Duncan's multiple range tests. A probability of P< 0.05 was considered significant.

Results and discussion

Effect of medium strength on growth

The results on the effect of different MS medium strength are presented in Table 1, as fresh weight (FW), dry weight (DW) and % dry weight. Fresh and dry weight was decreased when medium strength was decreased after 45 days of culture. The maximum FW (129.11 g l^{-1}) was recorded with MS2X medium where most of the germinated embryos were found abnormal shaped, and stony. And maximum dry weight was gained (12.76 g l^{-1}) when embryos were germinated in to full strength of MS medium where most of the germinated embryos were normal with green cotyledons or juvenile leaf. On the other hand the lowest FW (37.68g l^{-1}) and DW (5.53 g l^{-1}) were recorded with MS1/5 X of medium, respectively (Table 1). It was observed that higher strength was effective for biomass growth; on the other hand lower strength inhibited the growths. Further increased of medium strength inhibited growth, it was noticed that MS3X inhibited growth in comparison with MS2X (data not shown). The best growth, almost normal embryo shape and multiplication index were achieved on MS1X medium (Table 1). In this study, FW and DW were found to be the best along with the higher concentrations of macroelements, both on MS2X and MS media. However, the utilization of mineral elements usually has an adverse effect on a higher level of the element supply, and it can depend on the level of elements in a plant tissue. The inhibitory effect of a high level of mineral elements in the medium on their uptake and plant growth can be avoided if the current supply is high enough, i.e. if the plants were cultured in a high pretreatment, and then transplanted again to the medium with a high content of mineral elements (the high-high treatment) (Williams, 1993). This response was similar on Gisela 5 cultures (Ruzic *et al.,* 2000). According to Ingestad and Ågren, 1992, one of the models for study of *in vitro* mineral nutrition and plant growth is based on the observation that the relative plant growth is linearly related to the external concentration of the elements in the medium. However, the effect of the concentration of mineral elements in the medium on plant growth is closely related to the uptake of mineral elements from the medium and to the culture growth. Williams, 1995 also reported that a limited addition of an essential ion is one of the possible factors leading to a limited plant growth. Sakano *et al.,* 1995 also reported the correlation between the uptake of Phosphorus from MS medium and *Catharanthus roseus* (L.) on cell growth. Phosphorous is not only the element, which plants take up in the highest amount and most rapidly, but its concentration in the medium is of great importance as well. Pryce *et al.,* 1993 found out that the growth rate of *Iris* plants increases with the increase in the levels of mineral elements or only in the phosphate concentration in the medium. The effect of nitrogen sources on secondary metabolism is conditioned by several factors including the type of metabolic pathway, the producing organism, the type and concentration of the nitrogen sources and whether cultures are stationary or submerged.

Table 1. Effect of MS medium strength on growth of germinated somatic embryos in bioreactor after 45 days of initial culture.

MS strength	Biomass (g l^{-1})		
	FW	DW	% DW
MS 1/5X	37.68 e[y]	5.53 d	14.68a
MS1/3X	47.84 d	6.97 c	14.57a
MS1/2X	71.32 c	9.75 b	13.67b
MS 1X	119.83 b	12.76 a	10.65c
MS 2X	129.11 a	10.31 b	7.98d

[y]Mean separation within column by Duncan's multiple range test at p ≤ 0.05.

Effect of medium strength on accumulation of secondary metabolites

The contents of total phenolic, total flavonoid, polysaccharides, eleutherosides and chlorogenic

acids were measured in germinated embryos were grown in to different MS medium strengths. Total phenolic, flavonoid and polysaccharides contents are shown in Fig. 1. It was observed that contents of total phenolic and flavonoids were increased with the decrease of MS strength, on the other hand polysaccharides contents were increased when MS strength were increased (Fig. 1). The maximum contents of total phenolic (24.8 mg g^{-1} DW) and total flavonoid (28.11 mg g^{-1} DW) were observed when embryos were germinated in MS 1/5X medium (Fig.1). On the other hand the maximum polysaccharides (99.65 mg g^{-1} DW) were obtained with MS2X medium (Fig. 1).

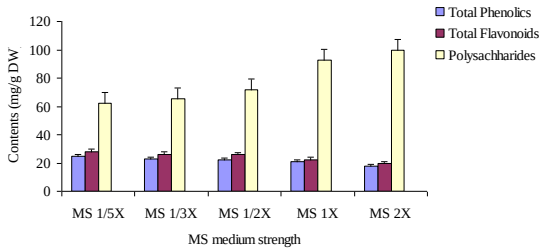

Fig. 1. Contents of total phenolics, total flavonoids and polysaccharides in germinated somatic embryos of *Eleutherococcus senticosus* affected by medium strength.

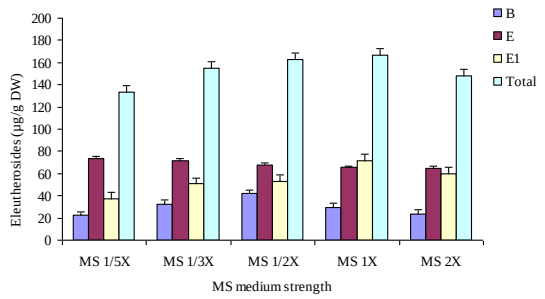

Fig. 2. Contents of eleutheroside B, E, E1 and total eleutherosides in germinated somatic embryos of *Eleutherococcus senticosus* affected by medium strength.

Amounts of different Eleutherosides (B, E, E_1) and Chlorogenic acids are shown in (Fig. 2 & 3). The results revealed that maximum amount of Elutherosides B (41.72 µg g^{-1} DW) were accumulated when embryos were germinated in half strength of MS medium. Maximum amount of eleutherosides E (73.40 and 71.75µg/ g^{-1} DW) were found in cultures with MS1/5X and MS1/3X of medium respectively. Eleutherosides E_1 accumulated in large amount (71.54 µg g^{-1} DW) in full MS medium (Fig. 2). Maximum amount of total eleutheroside was obtained in full strength of MS medium (166.37 µg g^{-1} DW). However no significant differences were observed between the total amounts of eleutherosides (166.37 & 162.71µg g^{-1} DW) when embryos were cultured in either full strength of MS medium of half strength (Fig. 2). Higher and lower than MS full strength inhibited the accumulation of eleutherosides. Opposite results were recorded for chlorogenic acid accumulation, in this case maximum amount was obtained in MS2X (2.26mg g^{-1} DW) and significantly decreased with the decrease of medium strength (Fig. 3).

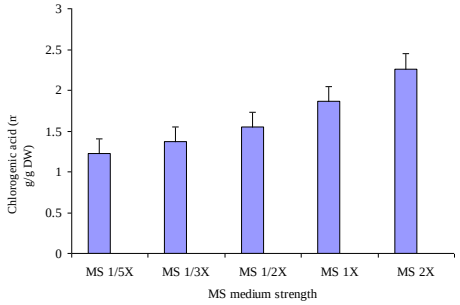

Fig. 3. Contents of chlorogenic acid in germinated somatic embryos of *Eleutherococcus senticosus* affected by medium strength.

Variation of carbon and nitrogen sources, phosphate, trace elements, growth factors and addition of precursors can greatly affect the production of secondary metabolites (Rokem and Goldberg, 1985). A medium composition, which favors cell growth, however, often inhibits the production of secondary metabolites and vice versa. For example, during the production of shikonin in *Lithospermum erythrorhizon* cell culture (Fujita *et al.*, 1981), nitrate and ammonium salt was found indispensable for cell growth. However, ammonium salt must be removed from the medium to enhance shikonin production (Fujita *et al.*, 1981)

Very often, secondary metabolic pathways are negatively affected by nitrogen sources favorable for growth (Betina, 1994). Negative effects of ammonium salts have been reported in the production of cephalosporin and other

metabolites/antibiotics (Demain, 1992). Adjustment of the medium composition to enhance the cell growth and secondary metabolite production is necessary to obtain high concentrations of secondary metabolites.

Conclusions

At constant culture condition, the germination of somatic embryos is mostly affected by MS medium strength. Double strength of MS medium increased the biomass though the stress condition affected the cell size, shape and structure. Abnormal embryos were found in higher strength of medium stress in compare to the normal. Best accumulation of eleutheroside contents that's the main secondary metabolites in eleuthorococcus species was obtained in normal MS strength. Growth and some secondary metabolites increased with the increase of MS strength, even in the phenolics and flavonoid contents reduced. Lower MS medium strength were found better for total phenolics and flavonoid accumulation in this species, but the double strength of MS medium was more favorable for growth, polysaccharides and chlorogenic acid production.

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