ELSEVIER

Process Biochemistry 41 (2006) 512–518

PROCESS BIOCHEMISTRY

www.elsevier.com/locate/procbio

Enhancement of eleutherosides production in embryogenic cultures of *Eleutherococcus sessiliflorus* in response to sucrose-induced osmotic stress

A.M. Shohael [a], D. Chakrabarty [a,b], M.B. Ali [a], K.W. Yu [a], E.J. Hahn [a], H.L. Lee [c], K.Y. Paek [a,*]

[a] *Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju, Republic of Korea*
[b] *Floriculture Section, National Botanical Research Institute, Lucknow, India*
[c] *Department of Urology, College of Medicine, Chungbuk National University, Cheongju, Republic of Korea*

Received 2 June 2005; received in revised form 10 September 2005; accepted 13 September 2005

Abstract

The effect of initial sucrose concentration was investigated in embryogenic cell cultures of *Eleutherococcus sessiliflorus* in bioreactors for production of eleutherosides, phenol, flavonoids and polysaccharide. The final dry cell weight was increased from 6.16 to 23.33 g l^{-1} with an increase of initial sucrose concentration from 10 to 70 g l^{-1}, but an even higher sucrose concentration of 90 g l^{-1} seemed to repress the cell growth. Furthermore, it was found that a high sugar level was favorable to the synthesis of eleutherosides, phenol and flavonoids, which may be due to higher osmotic pressure. A marked increase in malondialdehyde (MDA) content in germinating embryos was observed in high sucrose treatment. MDA, a decomposition product of polyunsaturated fatty acids hydroperoxides, has been utilized very often as a suitable biomarker for lipid peroxidation, which is an effect of osmotic stress. This hypothesis is also confirmed by the higher lipoxygenase (LOX) activity under this treatment. The much higher antioxidant enzyme activities in the culture fed with higher initial sucrose suggest that osmotic stress arising from a sudden increase in medium osmotic pressure. A hypothesis of osmotic stress and eleutheroside accumulation was confirmed from these results.

Keywords: Antioxidant enzymes; Eleutherosides; Flavonoids; Osmotic stress; Phenols; Somatic embryo

1. Introduction

Eleutherococcus sessiliflorus is important medicinal woody plant belonging to the family Araliceae, and distributed mainly in northeastern Asia. The cortical tissues of its roots are used primarily for their analgesic, anti-inflammatory, antipyretic and diuretic action [1]. *Eleutherococcus* species contain eleutherosides including eleutheroside A, B, C, D, E, F, G etc., and complex polysaccharides as an active ingredients from roots and leaves [2]. *E. sessiliflorus* is listed as threatened species because of excessive commercial harvest from the natural habitat. Propagation of the plants by seed is difficult because over 18 months of stratification are required for germination of zygotic embryos [3]. So, the plant tissue culture process has been looked at as a potential alternative for the more efficient mass propagation method. Recently, we reported somatic embryogenesis induction from leaf expants of *E. sessiliflorus* and subsequent mass production of germinated somatic embryos as medicinally raw materials by bioreactor culture, but the accumulation of physiologically active eleutherosides and phenolic compounds in germinating embryos are low [4]. In plant cell cultures, metabolite productivity is a key factor in determining its process efficiency and commercial potential. A relatively high sucrose concentration can be used to increase cell density and volumetric productivity of secondary metabolites [5–7]. An increase of sucrose concentration in the medium above the normally used 2–3% level was reported to stimulate the production of ginseng saponin and polysaccharide in suspension cultures of *Panax notoginseng* [5]. For the cell cultures of *Taxus chinensis*, both cell density and taxane accumulation were improved by sucrose feeding [6] and a combination of the aqueous–organic two-phase culture and sucrose feeding increased the paclitaxel yield by six-fold in shake flasks [7].

* Corresponding author.
E-mail address: paekky@chungbuk.ac.kr (K.Y. Paek).

1359-5113/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2005.09.005

In addition for being major carbon source for in vitro growth, sucrose acts as osmotic agents that may introduce osmotic stress above certain concentration, in which reactive oxygen species, such as superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and alkoxyl radical ($RO^{\bullet}$) are produced [8]. It is already known that free radical-induced peroxidation of lipid membranes is a reflection of stress-induced damage at the cellular level. Therefore, the level of malondialdehyde (MDA), produced during peroxidation of membrane lipids, is often used as an indicator of oxidative damage [9]. Fortunately, plants have developed two antioxidant systems, enzymatic and non-enzymatic scavenging systems to protect plant cells by controlling the intra-cellar ROS content [10,11]. Enzymatic scavenging system includes superoxide dismutases (SOD), which convert the superoxide radical to H_2O_2, and the catalase (CAT) and peroxidases (primarily ascorbate peroxidase), which trigger the conversion of H_2O_2 to water and oxygen [12]. In non-enzymatic scavenging system, antioxidant compounds, such as ascorbate (AA), glutathione (GSH), β-carotene and α-tocopherol also play important roles in the removal of toxic oxygen compounds. AA and GSH are two key metabolites of the ascorbate–glutathione cycle and the xanthophylls cycle generally considered as the important ROS detoxification systems in plant cells [13,14].

In this paper, the kinetics of growth, eleutherosides, phenol, flavonoids and polysaccharide accumulation by germinating somatic embryos were investigated in detail under various initial sucrose concentrations. Furthermore, based on our newly obtained information, a hypothesis of osmotic stress and elutheroside accumulation was confirmed from these results.

2. Materials and methods

2.1. Plant material

In vitro seedlings of *E. sessiliflorus* were maintained in MS medium [15] without plant growth regulators (PGRs). Young leaves (2 cm in length) were collected from subcultured plants every 3 weeks. Leaves were cut into 5 mm × 5 mm pieces and were placed on semisolid MS basal medium supplemented with 1 mg l^{-1} 2,4-D, with 3% sucrose and 0.2% gelrite for somatic embryogenesis. Cultures were maintained in the dark at 25 °C and after 12 weeks embryogenic cells of *E. sessiliflorus* were transferred to MS liquid medium supplemented with 1 mg l^{-1} 2,4-D. The suspension cultures were subcultured every 15 days. To induce formation of somatic embryos, 2 weeks old embryogenic cell clumps were transferred to 3 l balloon type bubble bioreactor with 2 l MS liquid medium without PGRs (Fig. 1A). At the end of 4 weeks of culture, the content of each bioreactor 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 this study (Fig. 1B).

2.2. Germination in bioreactors: effect of sucrose feeding

Ten grams of cotyledonary somatic embryos were transferred to 3 l balloon type bubble bioreactor with 2 l MS liquid medium supplemented with different concentrations of sucrose (0, 1, 3, 5, 7 and 9%) and 4 mg l^{-1} GA_3. The pH of the medium was adjusted to 5.8 before autoclaving (at 121 °C and 1.2 kg cm^{-2} pressure for 30 min). The volume of input air was adjusted to 1.0 vvm (air volume/culture volume, min). Cultures were kept under a 16 h photoperiod at 35 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF). The germination rate, fresh

Fig. 1. (A) Somatic embryo production in an air-lift balloon type bubble bioreactor. (B) Embryo fractions obtained after 4 weeks of culture, after filtration through different sieves to enrich in specific developmental stages (cotyledonary). (C and D) Germinated somatic embryos in a bioreactor after 6 weeks of culture.

weigh, dry weight, were recorded after 6 weeks of culture (Fig. 1C and D). Dry weight was determined after drying for 24 h at 60 °C.

2.3. 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 (10 ml) HPLC grade methanol and filtered through 0.45 μm PTEF (Gelman, USA) filter. Eleutherosides were quantified by HPLC (Waters 2690 separation modules, Waters, USA) equipped with a Symmetry^{R} C 18 (4.6 mm × 250 mm) column (Waters, USA) according to Patric et al. [16] and using a photodiode array detector (Waters 996 photodiode array detector, Waters, USA). Eleutherosides and chlorogenic acid were separated using a flow rate of 0.8 ml min^{-1} with water and acetonitrile as the mobile phase with a stepwise 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. 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, E1 (ChromaDex, USA) and chlorogenic acid (Sigma, USA) were integrated by comparison with an external standard calibration curve.

2.4. Determination of total phenolic contents

The content of total phenolics in plant methanolic extracts was analyzed spectrophotometrically by a modification of Folin–Ciocalteu colorometric method [17]. One hundred microlitres of methanolic extracts were mixed with 2.5 ml 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 standard curve of prepared gallic acid solution and expressed as means (±S.E.) mg of gallic acid equivalent per gram of plant material for the triplicate extracts.

2.5. Determination of total flavonoid contents

Total flavonoid content was determined by colorimetric method [18–20]. 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 0.075 ml of a 5% sodium nitrite solution. After 6 min, 0.150 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 1 M 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 (±S.E.) mg of (+)-catechin equivalents per gram of plant material for the triplicate extracts.

2.6. Determination of polysaccharides content

After eleutherosides extraction (2 g) with 60% methanol, the sediment was collected and desiccated in an oven at 60 °C. The sediment of 0.2 g was resuspended in 5 ml 5% (v/v) sulphuric acid and placed in boiling water for 2 h. After acidic hydrolysis, the liquid–solid mixture was diluted to 50 ml with distilled water. The supernatant was separated by sedimentation, and the polysaccharide in the supernatant assayed according to the carbazole reaction method [5] as follows. A sample of 0.2 ml taken from the above supernatant was mixed with 6 ml concentrated sulphuric acid, held in a boiling water bath for 20 min and cooled. Then, 0.2 ml carbazole-absolute ethanol (0–15%, v/v) was added and the contents mixed vigorously. After a reaction time of 2 h in 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.

2.7. Antioxidant enzyme assay

For determination of antioxidant enzyme activities, 0.5 g of germinated embryos was homogenized in 1 ml of respective extraction buffer in a pre-chilled mortar and pestle by liquid nitrogen. The homogenate was filtered through four layers of cheesecloth and centrifuged at 22,000 × *g* for 20 min at 4 °C. The supernatant re-centrifuged again at 22,000 × *g* for 20 min at 4 °C for determination of antioxidant enzyme activities. The preparation was applied to a column of sephadex G-25, equilibrated with the same buffers and kept in an ice bath until the assays were completed. Protein concentration of the enzyme extract was determined according to Bradford [21].

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Beyer and Fridovich [22]. Germinated embryos were homogenized in 1 ml cold 100 mM K-phosphate buffer (pH 7.8) containing 0.1 mM ethylenediamine tetraacetic acid (EDTA), 1% (w/v) polyvinyl-pyrrolidone (PVP) and 0.5% (v/v) Triton X-100. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm. For the determination of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) activities, germinated embryos were homogenized in 100 mM sodium phosphate buffer (pH 7.0) containing 5 mM ascorbate, 10% glycerol and 1 mM EDTA. APX (EC 1.11.1.11) activity was determined in 1 ml reaction mixture containing 50 mM K-phosphate (pH 7.0), 0.1 mM ascorbate (extinction coefficient, 2.8 mM^{-1} cm^{-1}), 0.3 mM H_2O_2. The decrease in absorbance was recorded at 290 nm for 3 min [23]. MDHAR (EC 1.6.5.4) activity was assayed following the decrease in absorbance at 340 nm due to NADH oxidation using an extinction coefficient of 6.22 mM^{-1} cm^{-1} [24]. The 1.0 ml reaction mixture consisted of 90 mM K-phosphate buffer (pH 7.0), 0.0125% Triton X-100, 0.2 mM NADH, 2.5 mM L-ascorbic acid, and required amount of enzyme extract. One unit of asccorbate oxidase (AO) is defined by the manufacturer (units as defined by Sigma Chem. Co.) as the amount that causes the oxidation of 1 μmol of ascorbate to monodehyadroascorbate per minute. DHAR (EC 1.8.5.1) activity was measured by measuring the reduction of dehydroascorbate at 265 nm for 4 min [25]. The 1.0 ml reaction mixture contained 90 mM K-phosphate buffer (pH 7.0), 1 mM EDTA, 5.0 mM glutathione (GSH) and required amount of enzyme extract. The reaction was initiated by the addition of 0.2 mM dehyadro ascorbate (DHA) (extinction coefficient, 14 mM^{-1} cm^{-1}). For determination of CAT, GR and GST, germinated embryos were homogenized in 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA under liquid nitrogen. Catalase (CAT; EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 (extinction coefficient, 39.4 mM^{-1} cm^{-1}) at 240 nm for 3 min [26]. Glutathione reductase (GR; EC 1.6.4.2) activity was assayed by following the reduction of DTNB at 412 nm (extinction coefficient, 13.6 mM^{-1} cm^{-1}) with some modifications as described by Smith et al. [27]. The assay mixture (1 ml) contained 100 mM K-phosphate buffer (pH 7.5), 1 mM oxidized glutathione and 0.1mM NADPH and 100 μl of enzyme extract. Glutathione-*S*-transferase (GST; EC 2.5.1.18)

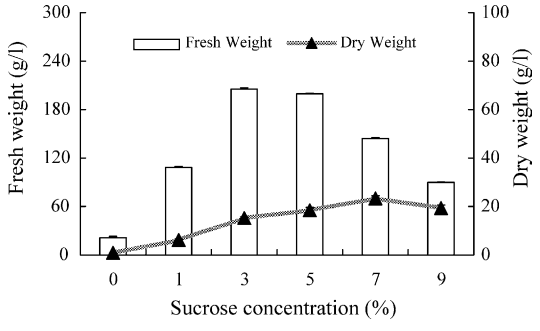

Fig. 2. Growth and biomass production in embryogenic cultures of *Eleutherococcus sessiliflorus* at 3 l balloon type bubble bioreactor with 2 l MS liquid medium supplemented with different concentrations of sucrose (0, 1, 3, 5, 7 and 9%) and 4 mg l^{-1} GA_3.

Table 1
The content of eleutherosides and chlorogenic acid in embryogenic cultures of *Eleutherococcus sessiliflorus* at different initial sucrose concentration

Sucrose (%)	Eleutherosides (μg g^{-1} dry weight)				Chlorogenic acid (μg g^{-1} dry weight)
	B	E	E_1	Total	
0	6.10e	4.25f	2.70f	13.05f	125.00f
1	14.85d	11.62e	3.97e	30.44e	615.50e
3	16.08c	59.95c	65.97c	142.00c	856.00d
5	16.78c	74.77a	72.53b	164.08b	1749.50c
7	33.78b	70.20a	75.30a	179.28a	1997.00b
9	70.10a	27.27d	24.20d	121.57d	3626.00a

Mean separation with columns by different letters (a–f) are significantly different according to Duncan's multiple range test at $p \leq 0.005$.

activity was determined by measuring the increase in absorbance at 340 nm (extinction coefficient, 9.6 mM^{-1} cm^{-1}), incubating reduced glutathione (GSH) and 1-chloro-2,4-dinithrobenzene (CDNB) as substrate, according to Drotar et al. [28]. The 1 ml reaction mixture contained 100 mM K-phosphate buffer, pH 6.25 and 0.8 mM 1-chloro-2,4-dinitrobenzene (CDNB).

2.8. Measurement of lipid peroxidation and lipoxygenase (LOX) activity

Malondialdehyde (MDA) content was determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer [29]. LOX activity was determined according to Axerold et al. [30].

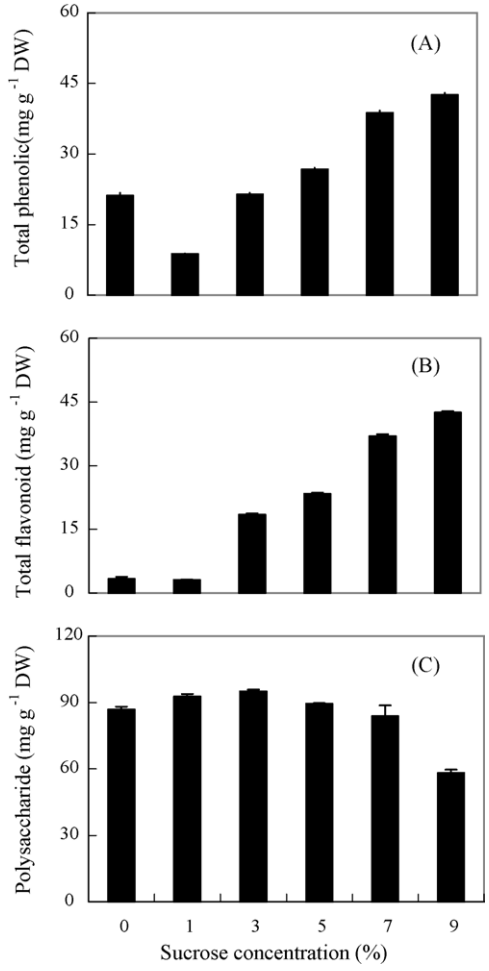

Fig. 3. The content of total phenolic (A), total flavonoid (B) and polysaccharide (C) in embryogenic cultures of *Eleutherococcus sessiliflorus* at different initial sucrose concentration.

2.9. Statistics

Data were subjected to Duncan's multiple range tests using SAS program (Version 6.12, SAS Institute Inc., Cary, USA).

3. Results and discussion

The final dry weight accumulated in the cultures was significantly affected by the initial sucrose level in the medium. The maximum dry biomass obtained was 23.33 g l^{-1} at an initial sucrose concentration of 70 g l^{-1} (Fig. 2). The result also suggests that the biomass growth was repressed by relatively higher initial sucrose concentration (90 g l^{-1}), which led to a relatively higher osmotic pressure. For cell cultures of *Coleus blumei*, a high initial sucrose concentration of 60 g l^{-1} led to a

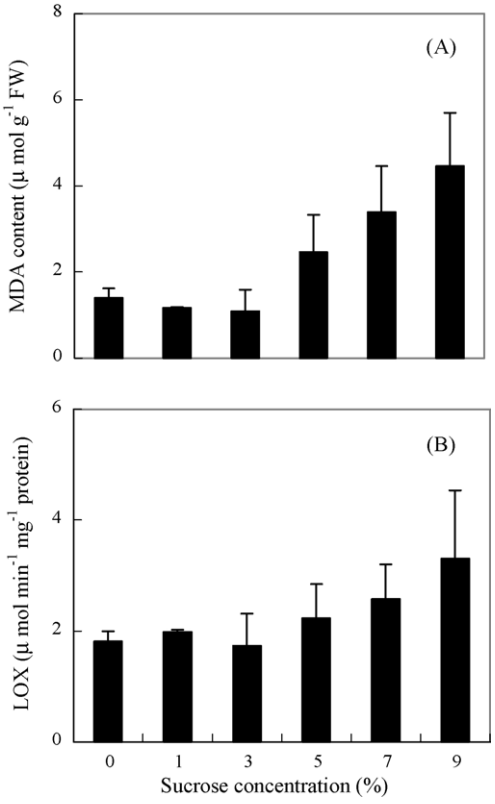

Fig. 4. Changes in the level of MDA content (A) and LOX activity (B) in embryogenic cultures of *Eleutherococcus sessiliflorus* at different initial sucrose concentration.

high biomass accumulation without an obvious lag phase [31]. Such a phenomenon was also claimed in suspension cultures of *Perilla frutescens*, the growth rate increased with an increase of initial sucrose level up to 60 g l^{-1} in the medium [32]. It is clear that initial sucrose concentration is important to the growth of plant cells and its effect is dependent on a specific cell line.

The profile of eleutherosides content of the germinating embryos was dependent on a different initial sucrose concentration in medium (Table 1). At a relatively high initial sucrose concentration of 70 g l^{-1}, a significant increase of total eleutherosides accumulation was observed; after that, the eleutherosides content showed a decrease. However, a significant increase of eleutheroside B was observed in relatively higher sucrose concentration (90 g l^{-1}). Similarly total phenols and flavonoids were also higher in germinating embryos that cultured in high sucrose medium (Fig. 3A and B). However, polysaccharide content has not been influenced by high sucrose treatment (Fig. 3C). The positive effects of a relatively high initial sucrose concentration on metabolite production were also reported in a number of other plant cell cultures [5–7]. At high initial sucrose concentrations, the increase of eleutherosides content may be due to the effects of high osmotic pressure, as claimed by Zhang et al. [5] in suspension cultures of *Panax notoginseng* for production of ginseng saponin.

In order to investigate that the osmotic stress at elevated osmotic pressure was involved to stimulate secondary metabolites production in *E. sessiliflorus* somatic embryo cultures, the potential role of antioxidant enzymes were detected as further evidence for the activation of plant cell stress responses by the high osmotic pressure. A marked increase in malondialdehyde (MDA) content in germinating embryos was observed in high sucrose treatment (Fig. 4A). MDA, a decomposition product of polyunsaturated fatty acids hydroperoxides, has been utilized very often as a suitable biomarker for lipid peroxidation, which is an effect of osmotic stress. This hypothesis is also confirmed by the higher lipoxygenase (LOX)

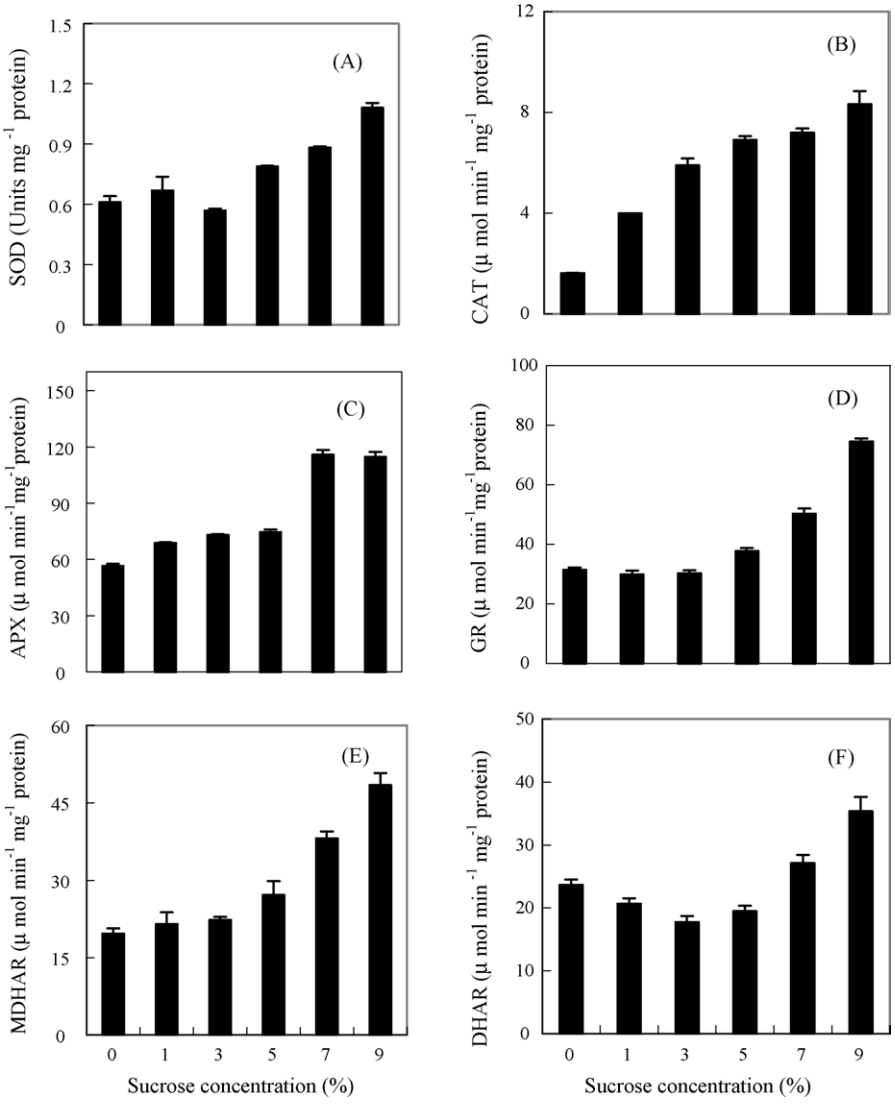

Fig. 5. Changes in the level of SOD (A), CAT (B), APX (C), GR (D), MDHAR (E) and DHAR (F) activities in embryogenic cultures of *Eleutherococcus sessiliflorus* at different initial sucrose concentration.

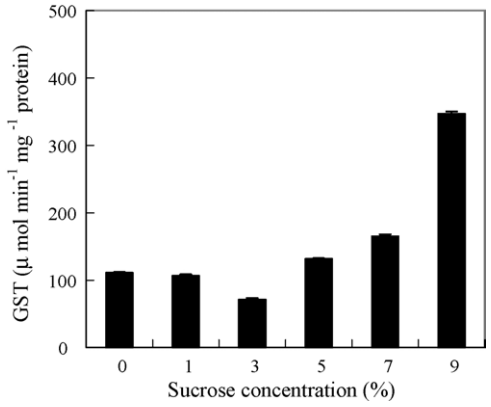

Fig. 6. Changes in the level of GST activity in embryogenic cultures of *Eleutherococcus sessiliflorus* at different initial sucrose concentration.

activity under this treatment (Fig. 4B). Osmotic stress in plant cells produces subcellular damage that impairs electron transport systems and leads to the production of active oxygen species (AOS) [33]. AOS unbalance the cellular redox systems in favor of oxidized forms, inactivate enzymes, cause lipid peroxidation and potentially damage DNA [34]. To scavenge these toxic radicals, plant cells have developed a complex system of antioxidant enzymes that includes superoxide dismutases (SODs), catalases (CATs), ascorbate peroxidases (APXs) and glutathione reductases (GRs), which have specific subcellular locations. The connection between some of the responses observed under high sucrose concentration, and some antioxidant enzymes, namely with SOD, CAT, APX, MDHAR, DHAR, GR and GST enzymes was studied (Figs. 5 and 6).

Total SOD activity increased after exposure to high sucrose treatment (Fig. 5A). Similarly, CAT and APX activity were also increased significantly in high sucrose treatment (Fig. 5B and C). The observed changes in SOD, CAT and APX activities demonstrate that the embryos have an enhanced capacity to scavenge AOS during the experimental conditions tested. Under these conditions, the product of the SOD reaction, H_2O_2, is eliminated by CAT or APX.

Total GR activity also increased in high sucrose treatment (Fig. 5D). The role of GR and glutathione in the H_2O_2 scavenging in plant cells has been well established in Halliwell–Asada pathway [35]. GR is involved in the recycling of reduced glutathione, providing a constant intracellular level of GSH [36], the main cell antioxidant [37,38].

In the present study, we also investigated enzymes like MDHAR and DHAR related to ascorbate metabolism (Fig. 5E and F). The function of MDHAR is to limit the formation of MDHA content through the enzymatic disproportionation, thus generating DHA [39]. However, DHA accumulation is harmful to the plant cell [39,40]. Increased DHAR activity could have generated more ascorbate (AA) from the DHA pool before hydrolysis. Thus, the increased activities of DHAR and MDHAR in high sucrose treatment indicated that these two enzymes catalyzed the regeneration of ascorbate for scavenging of H_2O_2.

In our study, a significant increase in GST activity was also observed in high sucrose treatment (Fig. 6). The GSTs play an important role in the detoxification mechanism by catalyzing the GSH conjugation of toxic substances under stress.

The results obtained in the present study show that the eleutherosides, total phenols and flavonoids were increased considerably in response to high sucrose treatments. Increase in activities of antioxidant enzymes in germinating embryos after high sucrose treatment indicates evidence of osmotic stress and that the embryos have an enhanced capacity to scavenge AOS.

Acknowledgements

This work was supported by grants from Korea Science and Engineering Foundation (KOSEF) and Rural Development Administration, Korea (Biogreen 21) to Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Korea. One of the authors (DC) also wishes to acknowledge the KOSEF for providing financial assistance in the form of "Long-term Foreign Scientist Fellowship".

References

[1] Brekhman II, Dardymov IV. New substances of plant origin which increase nonspecific resistance. Annu Rev Pharmacol 1969;9:415.
[2] Farnsworth NR, Kinghorn AD, Soejarto DD, Waller DP. Siberian ginseng (*Eleutherococcus senticosus*): current status as an adaptogen. In: Wagner H, Hikino H, Farnsworth NR, editors. Economic and medicinal plant research. London: Academic Press; 1985. p. 155–215.
[3] Isoda S, Shoji J. Studies on the cultivation of *Eleutherococus senticosus* Maxim. II. On the germination and rising of seedling. Nat Med 1994;48:75–81.
[4] Shohael AM, Chakrabarty D, Yu KW, Hahn EJ, Paek KY. Application of bioreactor system for large scale production of *Eleutherococcus sessiliflorus* somatic embryos in a air-lift bioreactor and their production of eleutherosides. J Biotechnol 2005;120:228–36.
[5] Zhang YH, Zhong JJ, Yu JT. Enhancement of ginseng saponin production in suspension cultures of *Panax notoginseng* manipulation of medium sucrose. J Biotechnol 1996;51:49–56.
[6] Wang HQ, Yu JT, Zhong JJ. Significant improvement of taxane production in suspension cultures of *Taxus chinensis* by sucrose feeding strategy. Process Biochem 2000;35:479–83.
[7] Wang C, Wu J, Mei X. Enhanced taxol production and release in *Taxus chinensis* cell suspension cultures with selected organic solvents and sucrose feeding. Biotechnol Prog 2001;17:89–94.
[8] Kilayri JM, Bahrany AM. Callus growth and proline accumulation in response to sorbitol and sucrose induced osmotic stress in rice. Biol Plant 2002;45:609–11.
[9] Chakrabarty D, Park SY, Ali MB, Shin KS, Paek KY. Hyperhydricity in apple: ultrastuctural and physiological aspects. Tree Physiol, in press.
[10] Ahmed S, Nawata E, Hosokawa M, Domae Y, Sakuratani T. Alterations in photosynthesis and some antioxidant enzymatic activities of mungbean subjected to waterlogging. Plant Sci 2002;163:117–23.
[11] Sairam RK, Deshmukh PS, Saxena DC. Role of antioxidant systems in wheat genotypes tolerance to water stress. Biol Plant 1998;41:387–94.
[12] Jeffrey AA. Catalase activity, hydrogen peroxide content and thermotolerance of pepper leaves. Sci Hort 2002;95:277–84.
[13] Hodges DM, Andrews CJ, Johnson DA, Hamilton RI. Antioxidant compound responses to chilling stress in differentially sensitive inbred maize lines. Physiol Plant 1996;98:685–92.
[14] Francisco JP, Daniel V, Nilo M. Ascorbic acid and flavonoid-peroxidase reaction as a detoxifying system of H_2O_2 in grapevine leaves. Phytochemistry 2002;60:573–80.
[15] Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962;15:473–97.

[16] Patrick NY, Arnason JT, Anwang DVC. An Improved extraction procedure for the rapid, quantitative high-performance liquid chromatographic estimation of the main eleutherosides (B and E) in *Eleutherococcus senticosus* (Eleuthero). Phyto Anal 1998;9:291–5.
[17] Folin O, Ciocalteu V. On tyrosine and tryptophane determination in proteins. J Biol Chem 1927;27:627–50.
[18] Dewanto V, Wu X, Adom KK, Liu RH. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J Agric Food Chem 2002;50:3010–4.
[19] Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem 1999;64:555–9.
[20] Sakanaka S, Tachibana Y, Okada Y. Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha). Food Chem 2005;89:569–75.
[21] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[22] Beyer WF, Fridovich I. Assaying for superoxide dismutase activity: some large consequences of minor changes in condition. Anal Biochem 1987;161:559–66.
[23] Chen GX, Asada K. Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiol 1989;30:987–98.
[24] Hossain MA, Nakano Y, Asada K. Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. Plant Cell Physiol 1984;25:385–95.
[25] Doulis AG, Debian N, Kingston-Smith AH, Foyer CH. Differential localization of antioxidants in maize. Plant Physiol 1997;114:1031–7.
[26] Aebi H. Catalases. In: Bergmeyer HU, editor. Methods of enzymatic analysis, vol. 2. NY: Academic Press; 1974. p. 673–84.
[27] Smith IK, Vierheller TL, Thorne CA. Assay of glutathione reductase in crude tissue homogenates using 5, 5′-dithiobis(2-nitrobenzoic acid). Anal Biochem 1988;175:408–13.
[28] Droter A, Phclps P, Fall R. Evidence for glutathione peroxidase activities in cultured plant cells. Plant Sci 1985;42:35–40.
[29] Heath RL, Packer L. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 1968;125:189–98.
[30] Axerold B, Chesbrough TM, Laakso S. Lipoxygenase from soybean. In: Lowenstein JM, editor. Methods enzymology. NY: Academic press; 1981. p. 441–51.
[31] Zenk MH, El-Shagi HE, Ulbrich B. Production of rosmarinic acid by cell suspension cultures *Coleus hlumei*. Naturwissenschaften 1977;64:585–6.
[32] Zhong JJ, Xu GR, Yoshida T. Effect of initial sucrose concentration on excretion of anthocyanin pigments in suspended cultures of *Perilla fruktescens* cells. World J Microb Biotechnol 1994;10:590–2.
[33] Price AH, Atherton NH, Hendry GAF. Plants under drought-stress generate activated oxygen. Free Radic Res Commun 1989;8:61–6.
[34] Smirnoff N. The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol 1993;125:27–58.
[35] Bray EA, Bailey-Serres J, Weretilnyk E. Responses to abiotic stress. In: Buchanan BB, Gruissem W, Jones RL, editors. Biochemistry and molecular biology of plants. Waldorf: American Society of Plant Biologists; 2000. p. 1158–203.
[36] Calbert I, Mannervik B. Glutathione reductase. Methods Enzymol 1985;113:484–90.
[37] Meister A. Metabolism and functions of glutathione. Trends Biochem Sci 1981;231–4.
[38] Alscher RG. Biosynthesis and antioxidant function of glutathione in plants. Plant Physiol 1989;77:457–64.
[39] Arrigoni O. Ascorbate system in plant development. J Bioenerg Biomembr 1994;26:407–19.
[40] Gara LD, Paciolla C, Tullio MC, Motto M, Arrigoni O. Ascorbate-dependent hydrogen peroxide detoxification and ascorbate regeneration during germination of a highly productive maize hybrid: evidence of an improved detoxification mechanism against reactive oxygen species. Physiol Plant 2000;109:7–13.