PHOTOSYNTHETICA 44 (2): 221-226, 2006

Changes in contents of photosynthetic pigments and ribulose-1,5-bisphosphate carboxylase activity during the development of globular somatic embryo into the plantlet of Siberian ginseng

D.M. PANDEY[*], J.-W. KIM[**], W.-S. LEE[**], and U.-D. YEO[*,***]

Faculty of Biological Science, Chonbuk National University, Jeonju 561-756, Republic of Korea[*]
Department of Bioscience and Biotechnology, Microplants Co. Ltd., Jeonju 561-203, Republic of Korea[**]

Abstract

During the development of the globular embryos *via* heart-shaped, torpedo-shaped, and cotyledonary embryos into plantlets, contents of chlorophyll (Chl) *a* and *b* and carotenoids, and activity of ribulose-1,5-bisphosphate carboxylase (RuBPC, EC 4.1.1.39) were investigated. In the solid cultivation (SC) the contents of Chl *a*, Chl *b*, Chl *a/b,* and total pigments increased up to plantlet stage. In the liquid cultivation (LC), contents of Chl *a*, Chl *b*, and total pigments increased till the torpedo-shaped stage, but decreased with the further development up to plantlets stage. During SC, RuBPC activity increased up to the torpedo-shaped embryo stage, but in the LC RuPBC activity increased continuously with the progress in the developmental stages. The correlations between Chl *a* and RuBPC activity on the SC and LC were negative, $r = -0.26$ and -0.56, respectively.

Additional key words: carotenoids; chlorophyll; *Eleutherococcus senticosus*.

Introduction

Greening is the biosynthesis of chlorophyll (Chl) and construction of the photosynthetic apparatus, and is an essential event in green plants (Sato-Nara *et al*. 2004). During the expression of somatic embryogenesis, embryogenic cells and aggregates continue their growth by passing through a series of developmental stages, namely (in dicots) globular, oblong, heart-shaped, and finally torpedo-shaped (Schiavone and Cooke 1985). Plastids in green embryos differentiate chloroplasts with rudimentary grana (Mansfield and Briarty 1991). Rao *et al*. (1985) reported that callus cells induced from the embryonal end of the bamboo seeds differentiated into chlorophyllous embryoids. Nsangou and Greenwood (1998) described that in red spruce (*Picea rubens* Sarg.) seedlings that were produced from zygotic embryo indicated lower total Chl content compared with the somatic embryos.

Suspension-cultured cells of carrot are easily and synchronously transformed to somatic embryos by removal of auxin from the medium and whole plant regenerates through a succession of globular to heart- to torpedo-shaped embryos (Halperin 1966). Although the appearance, or not, of greening during embryogenesis differs among taxa, the expression of several photosynthesis-related genes during embryogenesis has been reported in both green and non-green embryos (Borroto and Dure 1986, Medford and Sussex 1989, Degenhardt *et al*. 1991). Similarly, expression of photosynthesis-related genes and their regulation by irradiation during somatic embryogenesis of carrot has already been described (Sato-Nara *et al*. 2004). The chloroplast has photosynthetic pigments and a Chl antenna system consisting of pigment-protein complexes. Ribulose-1,5-bisphosphate carboxylase (RuBPC, EC 4.1.1.39) is one of the major proteins in plants, and acts as a key enzyme, fixing CO_2 in the Calvin cycle (Wildman 1979). Using the carrot somatic embryogenesis system, Aleith and Richter (1991) reported that in mature somatic embryos blue radiation induced Chl

Received 20 June 2005, *accepted* 8 December 2005.
[***]Corresponding author; fax: (+82) 63 270 3362, e-mail: y520419@chonbuk.ac.kr
Abbreviations: Cars – carotenoids; Chl – chlorophyll; 2,4-D – 2,4-dichlorophenoxyacetic acid; LC – liquid cultivation; RuBP – ribulose-1,5-bisphosphate; RuBPC – ribulose-1,5-bisphosphate carboxylase; SC – solid cultivation.
Acknowledgements: This work was supported by the grant of Post-Doc Program, Chonbuk National University (2004) and the research grant (PF033062-01) from Plant Diversity Research Center of 21st Century Frontier Research Program funded by Ministry of Science and Technology of Korean government. We thank Dr. C.L. Goswami, CCS HAU Hisar, India for critically reading the manuscript.

biosynthesis and gene expression of RuBPC.

Siberian ginseng (*Eleutherococcus senticosus*) is an endangered medicinal woody plant species. Conventional propagation is very difficult because long-term stratification is required to induce both maturation and germination of the zygotic embryos (Isoda and Shoji 1994). Initiation and development of somatic embryos of Siberian ginseng has already been described (Chakrabarty *et al*. 2003). Similarly, the embryogenic cells that were established in a liquid medium and used to produce plantlets through somatic embryogenesis on a larger scale have been described (Kim and Kim 2001). To the best of our knowledge, studies on the evolution of photosynthetic pigments and RuPBC activity during the development of the somatic embryos of Siberian ginseng have not been carried out. In the present study we investigated the changes in the photosynthetic pigments and activity of RuBPC and their correlations during development of the globular somatic embryos into the plantlets in the solid and liquid cultivation systems.

Materials and methods

Culture conditions of somatic embryogenesis and preparation for analysis: The hypocotyl segments (1 mm in length) of Siberian ginseng (*Eleutherococcus senticosus* Maxim) seedlings were placed on the surface of a MS (Murashige and Skoog 1962) medium supplemented with 4.52 μM 2,4-D, 3 % sucrose, and 0.8 % agar. The somatic embryos were induced directly from the hypocotyl explants (Choi *et al*. 1999). The embryogenic cells were formed from somatic embryos by culture on the solid (agar 0.8 %) medium containing 4.52 μM 2,4-D (Choi *et al*. 1999). The embryogenic cells were inoculated on the solid medium supplemented with 2.26 μM 2,4-D in 87×15 mm Petri-dishes (*GCM Co.*, Korea) and maintained by sub-culturing on the same medium at 2 week-intervals in the dark room (Fig. 1*A*). When they were transferred into the solid medium without growth regulators, globular embryos developed after 2 weeks of cultivation (Fig. 1*B*). The globular embryos were transferred on the same solid medium for 2 weeks under 8.05 W m^{-2} of white fluorescent radiation with a 16-h photoperiod at 24 °C. The globular embryos developed into plantlets *via* heart-shaped, torpedo-shaped, and cotyledonary embryos (Fig. 1*C-F*). After 2 weeks of cultivation, stages of somatic embryo and plantlet were selected under a stereoscopic microscope (*KL1500*, *Zeiss*) and stored in liquid nitrogen until they were used.

For liquid culture, 1.0 g fresh mass of embryogenic cells, which were maintained on the solid MS medium (Fig. 1*A*), were transferred into 5 m^3 of air-lift type bioreactor containing 2 m^3 of MS liquid medium without growth regulators (Fig. 2*A*) under 8.05 W m^{-2} of white fluorescent radiation with a 16-h photoperiod at 24 °C. The embryogenic cells also developed to the plantlets *via* the globular, heart-shaped, torpedo-shaped, and cotyledonary embryos (Fig. 2). For sample homogeneity, after 2 weeks of cultivation, stages of somatic embryo and plantlet were selected under the above stereoscopic microscope and stored in liquid nitrogen until they were used.

Photosynthetic pigments such as Chl *a*, *b*, and carotenoids (Cars) were determined by the Pandey *et al*. (2003) method and were calculated using the Wellburn method (1994). Photosynthetic pigments were extracted by homogenization [0.5 g(f.m.)] of each stage of somatic embryo and plantlet in a crucible mortar with quartz sand. Sodium carbonate (0.05 g) was added to minimize acid-catalyzed isomerisation of Cars. Following homogenization, 2 cm^3 of 80 % acetone was added, macerate was again homogenized with 2 cm^3 of acetone, and centrifuged with a *Beckman J2-MC* centrifuge (USA) at 12 000×*g* for 20 min. The supernatant was kept in the dark at 4 °C. Absorbances at 470, 646, and 663 nm were recorded with an *Ultrospec 3000* UV/Visible Spectrophotometer [*Pharmacia Biotech* (*Biochrom*), Cambridge, England]. For the estimation of dry mass, samples were dried in an oven at 72 °C for 3 d.

Extraction and activity assay of RuBPC was made according to the Borland *et al*. (1998) and Pandey *et al*. (2000), with some modifications. Fresh mass (0.5 g) of each stage of somatic embryo and plantlet was homogenized in 2 cm^3 of extraction buffer [100 cm^3 of 50 mM Tris-HCl (pH 7.5) containing 1 % polyvinylpolypyrrolidine (PVPP), 1 mM dithiothreitol (DTT), 0.2 mM EDTA, 5 mM 2-mercaptoethanol (MCE), 0.1 % *Triton X-100*, 20 mM $MgCl_2 \times 6\,H_2O$, 5 mM $NaHCO_3$] at 4 °C. The homogenate was centrifuged with a *Beckman J2-MC* centrifuge at 14 000×*g*, 4 °C for 20 min. The content of total soluble proteins of the homogenate was determined by the Bradford (1976) method using bovine serum albumin as standard.

Assay of RuBPC activity was conducted in a 1.0 cm^3-cuvette with 950 mm^3 of reaction mixture [100 mM Bicine-KOH (pH 8.0), 20 mM $NaHCO_3$, 5 mM $MgCl_2$, 3.5 mM ATP, 3.5 mM P-creatine, 0.4 mM NADH, 4 units creatine P-kinase, 4 units glyceraldehyde-3-P-dehydrogenase, 4 units 3-phosphoglyceric phosphokinase, and 0.05 mM RuBP]. The reaction was started with the addition of 50 mm^3 of the enzyme extract. A decrease in OD at 340 nm and 25 °C for 3 min was recorded by a spectrophotometer [*Ultrospec 3000* UV/visible spectrophotometer, *Pharmacia Biotech* (*Biochrom*), Cambridge, England].

Statistics: All data represent mean ± SE from three independent experiments. Asterisks indicate the level of significant difference from the globular embryo according to the LSD tests: $p<0.05^{*}$, $p<0.01^{**}$.

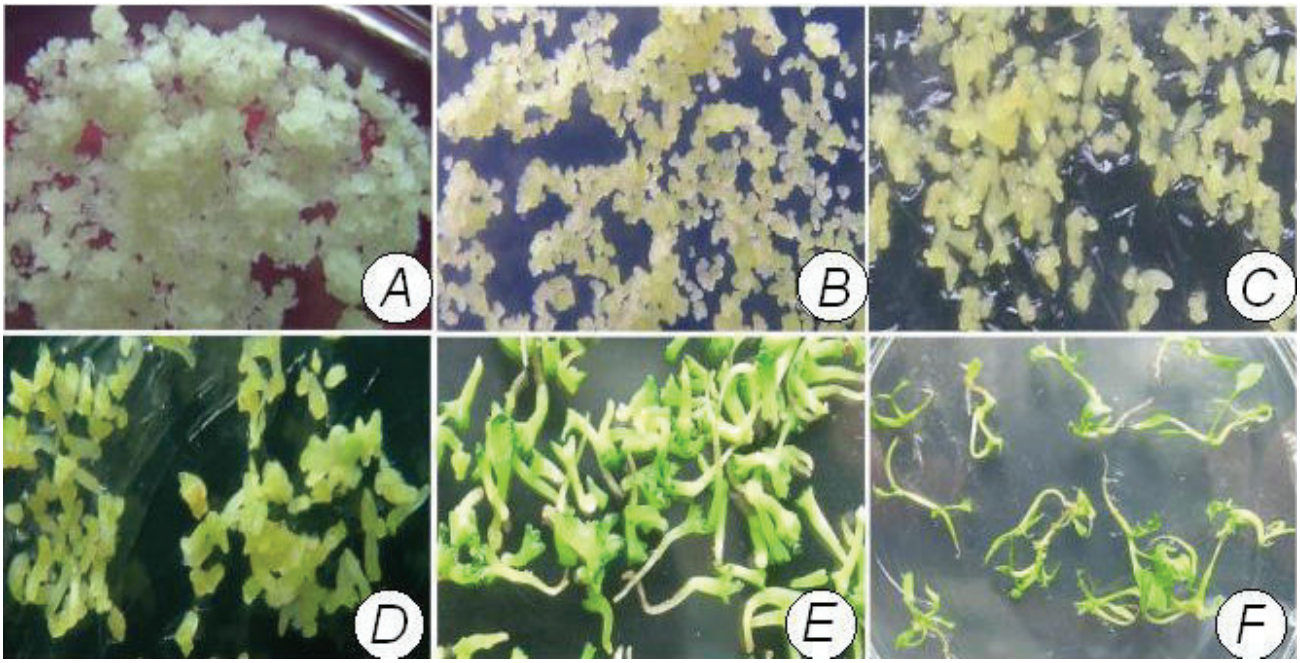

Fig. 1. Somatic embryogenesis and plantlet development from the embryogenic cells of Siberian ginseng on growth regulator-free MS solid (agar, 0.8 %) medium in Petri-dishes. *A*, embryogenic cells; *B*, globular embryos; *C*, heart-shaped embryos; *D*, torpedo-shaped embryos; *E*, cotyledonary embryos; *F*, plantlets.

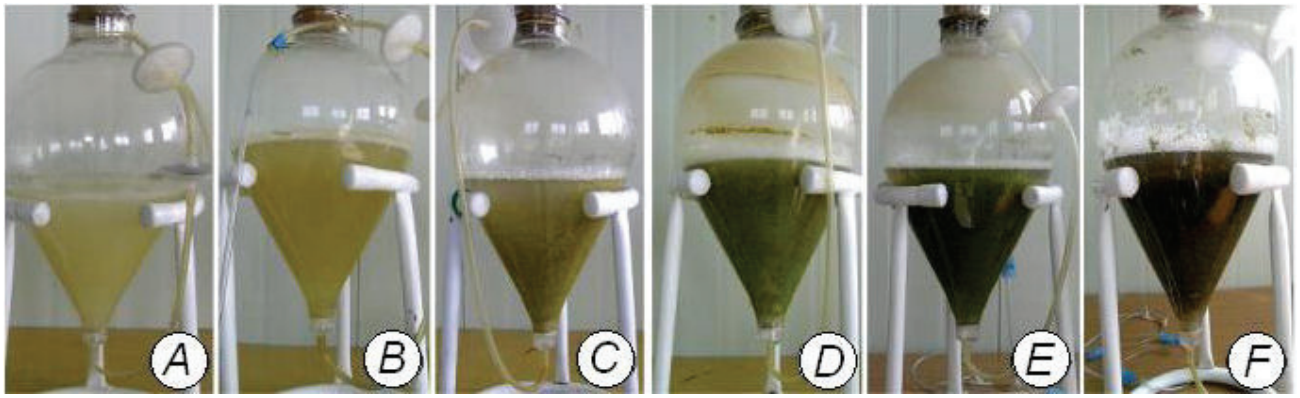

Fig. 2. Somatic embryogenesis and plantlet development from the embryogenic cells of Siberian ginseng in growth regulators-free MS liquid medium (5 m^3 air-lift type bioreactor). *A*, embryogenic cells; *B*, globular-shaped embryos; *C*, heart-shaped embryos; *D*, torpedo-shaped embryos; *E*, cotyledonary embryos; *F*, plantlets.

Results

Changes in photosynthetic pigments: In the SC the contents of Chl *a*, Chl *b*, Cars, and total pigments, and Chl *a/b* increased during the development of globular somatic embryos to plantlets (Table 1) by 10.0, 3.6, 2.8, 5.0, 6.3, and 6.0-fold, respectively. Similarly, the ratios of Cars/total Chl and Cars/total pigments increased from globular embryos to heart-shaped embryos by 1.5- and 1.7-fold, respectively. However, with the further development of torpedo-shaped embryos into plantlets these ratios decreased (Table 1).

In the LC the photosynthetic pigments increased with the development of globular embryos up to torpedo-shaped embryos (Table 1) by 1.2, 1.7, and 1.3-fold, respectively. However, with the further development of cotyledonary embryos into plantlets their values decreased. The ratio of Chl *a/b* continuously increased up to the plantlet stage. From the globular embryos to the heart-shaped embryos, the ratios of Cars/total Chl and Cars/total pigments increased by 1.7- and 1.4-fold, respectively. With the further development of the torpedo-shaped and cotyledonary embryos into the plantlets these ratios decreased (Table 1).

Table 1. Changes in the contents of photosynthetic pigments during the development of globular somatic embryo into the plantlet of Siberian ginseng (*Eleutherococcus senticosus* Maxim) in MS solid (SC) and liquid (LC) cultivations. All data represent mean ± SE from three independent experiments. *Asterisks* indicate the level of significant difference in LSD tests: $^{*}p<0.05$, $^{**}p<0.01$. CE: cotyledonary embryo, GE: globular embryo, H-SE: heart-shaped embryo, Pl: plantlet, T-SE: torpedo-shaped embryo.

Parameter	Cultivation method	Pigment content [g kg^{-1}(d.m.)]				
		Developmental stage				
		GE	H-SE	T-SE	CE	Pl
Chl *a*	SC	0.17±0.01	0.16±0.00	0.33±0.01**	1.14±0.01**	1.70±0.02**
	LC	0.36±0.01	0.32±0.01	0.43±0.01*	0.34±0.01	0.28±0.01*
Chl *b*	SC	0.23±0.02	0.15±0.00*	0.30±0.01*	0.64±0.02**	0.83±0.03**
	LC	0.70±0.01	0.63±0.02	0.77±0.01	0.51±0.01**	0.31±0.01**
Chl *a*/*b*	SC	0.72	1.04	1.12	1.80	2.04
	LC	0.52	0.51	0.56	0.67	0.89
Cars	SC	0.15±0.03	0.17±0.00	0.27±0.00**	0.57±0.00**	0.75±0.01**
	LC	0.47±0.01	0.69±0.01**	0.80±0.00**	0.49±0.00	0.23±0.00**
Total Chl	SC	0.40±0.03	0.31±0.00	0.63±0.02**	1.78±0.03**	2.53±0.06**
	LC	1.06±0.01	0.95±0.03	1.20±0.01*	0.85±0.02**	0.58±0.02**
Cars/total Chl	SC	0.37	0.57	0.42	0.32	0.30
	LC	0.44	0.73	0.67	0.57	0.40
Total pigments	SC	0.55±0.01	0.48±0.01	0.90±0.02**	2.34±0.03**	3.28±0.06**
	LC	1.53±0.02	1.64±0.03	2.01±0.01**	1.34±0.02*	0.82±0.02**
Cars/total pigments	SC	0.27	0.36	0.30	0.24	0.23
	LC	0.31	0.42	0.40	0.36	0.28

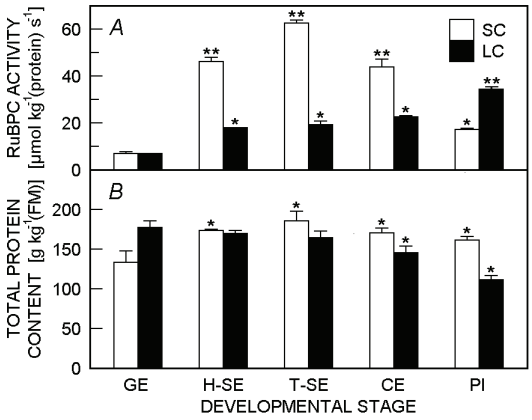

Fig. 3. Changes in the activity of RuBPC (*A*) and total soluble protein content (*B*) during development of globular somatic embryo into the plantlet of Siberian ginseng in the solid (SC, 0.8 % agar) and liquid (LC) cultivations. Means ± SE from three independent experiments. *Asterisks* indicate the level of significant difference in LSD tests: $^{*}p<0.05$, $^{**}p<0.01$. CE: cotyledonary embryo, GE: globular embryo, H-SE: heart-shaped embryo, Pl: plantlet, T-SE: torpedo-shaped embryo.

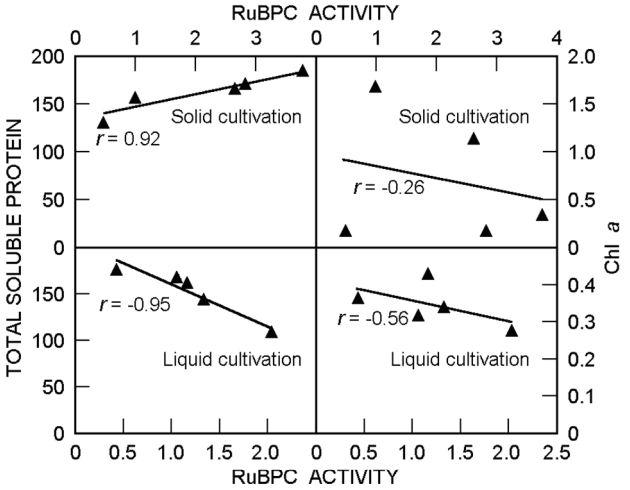

Fig. 4. Correlations of total soluble protein and Chl *a* with the activity of RuBPC during the development of globular somatic embryo into the plantlet of Siberian ginseng in the solid (agar, 0.8 %) and liquid cultivations.

The RuBPC activity (Fig. 3) in SC increased with the progress in the developmental stages. Compared to the globular embryo, RuBPC activity in the torpedo-shaped embryo increased by 5.0-fold. However, with the further development of cotyledonary embryos and plantlets the activity decreased. In contrast, in the LC the activity continuously increased with the progress in the developmental stages. Compared to the globular embryos, RuBPC activity at the plantlet stage was higher by 4.7-fold (Fig. 3*A*).

In the SC, the content of total soluble protein increased with the progress in the developmental stage. Compared to the globular embryos, total soluble protein of the torpedo-shaped embryos was 1.4-fold higher. However, with the further development of cotyledonary embryos and plantlets, the content of total soluble protein decreased. In the LC, total soluble protein content decreased continuously with the progress in the developmental stage. Compared to the globular embryos, total soluble protein content at the plantlet stage was 40 % lower (Fig. 3*B*).

Discussion

Developmental patterns (Fig. 1*A*) of globular somatic embryos into plantlets were similar to those described previously (Choi *et al*. 1999, Kim and Kim 2001). Either on SC or LC, the ratio of Chl *a/b* of the globular somatic embryo was lower as compared to plantlet stage, which is the result of a lower content of Chl *a* as compared with Chl *b* (Table 1). However, with the development of globular, torpedo-shaped, and cotyledonary embryos into the plantlets, Chl *a*, Chl *b*, as well as their ratio increased. This might be explained by these reasons: (*1*) the synthesis of Chl *a* was higher as compared with Chl *b*, (*2*) Chl *b* was converted to Chl *a*. Similar to our findings, Ito *et al*. (1993) found that the inter-conversion of Chl *a* and *b* plays a significant role in the establishment of required Chl *a/b* ratio during the adaptation of leaves to irradiance. Moreover, the ratios of Chl *a/b*, Cars/total Chl, and Cars/total pigment in soybean callus on a solid MS medium were found as 3.22, 0.37, and 0.27, respectively by Pandey *et al*. (2003), and in spinach leaves they were 3.40, 1.90, and 0.67, respectively (Pandey *et al*. 2005), showing significant changes in the photosynthetic pigments. In present study, in LC the higher ratio of Cars/total Chl than on the SC might be explained by the fact that Chl biosynthesis has higher requirement for oxygen than Cars biosynthesis, and LC lacks oxygen. Similarly, in the LC the increase in the contents of Chl *a*, Chl *b*, and Cars from the development of globular embryos to torpedo-shaped embryos was followed by a slight reduction in their contents. This might be explained by the reason that during the development of globular embryos to torpedo-shaped embryos irradiance was sufficient for the synthesis of both Chls and Cars. However, with the further development of cotyledonary and plantlet stages, irradiance may become a limiting factor that results in the decreased synthesis of both Chls and Cars.

In the SC, the increases in the activity of RuBPC and content of total soluble protein during the development of globular somatic embryos to torpedo-shaped embryos followed by a slight reduction indicated that either the rate of protein synthesis decreased and/or proteins were utilized in other metabolic processes. Under *in-vitro* culture system with the progress of rooting stage, RuBPC activity increased, which is an indication of a gradual transition from heterotrophic to autotrophic mode of carbon fixation (Hidder and Desjardins 1994). Our unpublished data indicate that activity of phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) decreased with the development of globular somatic embryos up to torpedo-shaped embryos, and activity of RuBPC increased sharply, showing the transition of heterotrophic to autotrophic mode of carbon fixation. Up to the torpedo stage, the content of total soluble protein as well as the activity of RuBPC increased in the SC (Fig. 3*A*). From the cotyledonary stage to the plantlets stage, synthesis of cell-wall polysaccharides, lignin, and other compounds took place, resulting in the reduction of total soluble protein on the basis of fresh mass. Kemp and Sutton (1972) reported that both protein synthesis and accumulation increased over the first 50 h in culture followed by decrease in the rate of protein accumulation to an equal rate as the fresh mass accumulation. Also, at this stage reduction in RuBPC transcript, lower availability of RuBP and/or higher availability of 3-phosphoglyceric acid (3-PGA), due to its decreased metabolism, might be responsible for the decrease in RuBPC activity. Similarly, in LC, the decrease in total soluble protein content during the developmental stages indicated that either the protein synthesis decreased and/or protein was further utilized in the metabolic processes. Hence, when the changes of total soluble protein content during the development of globular somatic embryo were plotted against the RuBPC activity (Fig. 4), a clear positive correlation ($r = 0.92$) was seen in the SC while a strong negative correlation ($r = –0.95$) was found in the LC medium. On the other hand, changes of Chl *a* and RuBPC activity during the development of globular somatic embryo in the SC and LC were negatively correlated ($r = –0.26$ and $–0.56$, respectively).

References

Aleith, F., Richter, G.: Chloroplast differentiation in somatic embryos of carrot: Efficiency of blue and red light irradiation on gene expression. – J. Plant Physiol. **138**: 304-308, 1991.

Borland, A.M., Técsi, L.I., Leegood, R.C., Walker, R.P.: Inducibility of crassulacean acid metabolism (CAM) in *Clusia* species; physiological/biochemical characterisation and intracellular localization of carboxylation and decarboxylation processes in three species which exhibit different degrees of CAM. – Planta **205**: 342-351, 1998.

Borroto, K.E., Dure, L., III: The expression of chloroplast genes during cotton embryogenesis. – Plant mol. Biol. **7**: 105-113, 1986.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. – Anal. Biochem. **73**: 248-254, 1976.

Chakrabarty, D., Yu, K.W., Park, K.Y.: Detection of DNA methylation changes during somatic embryogenesis of Siberian ginseng (*Eleuterococcus senticosus*). – Plant Sci. **165**: 61-68, 2003.

Choi, Y.E., Kim, J.W., Yoon, E.S.: High frequency of plant production via somatic embryogenesis from callus or cell suspension cultures in *Eleutherococcus senticosus*. – Ann. Bot. **83**: 309-314, 1999.

Degenhardt, J., Fiebig, C., Link, G.: Chloroplast and nuclear transcripts for plastid proteins in *Arabidopsis thaliana*: Tissue distribution in mature plants and during seedling development and embryogenesis. – Bot. Acta **104**: 455-463, 1991.

Halperin, W.: Alternative morphogenetic events in cell suspensions. – Amer. J. Bot. **53**: 443-453, 1966.

Hidder, C., Desjardins, Y.: Changes in ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoenolpyruvate carboxylase activities and $^{14}CO_2$ fixation during the rooting of strawberry shoots *in vitro*. – Can. J. Plant Sci. **74**: 827-831, 1994.

Isoda, S., Shoji, J.: Studies on the cultivation of *Eleuterococcus senticosus* Maxim. II. On the germination and raising of seedlings. – Nat. Med. **48**: 75-81, 1994.

Ito, H., Tanaka, Y., Tsuji, H., Tanaka, A.: Conversion of chlorophyll *b* to chlorophyll *a* by isolated cucumber etioplasts. – Arch. Biochem. Biophys. **306**: 148-151, 1993.

Kemp, D.J., Sutton, D.W.: Protein metabolism in cultured plant tissues. – Plant Physiol. **49**: 596-601, 1972.

Kim, J.W., Kim, H.S.: Mass production of Siberian ginseng (*Eleutherococcus senticosus*) somatic embryos by cell culturing. – Orient. Pharm. exp. Med. **1**: 34-38, 2001.

Mansfield, S.G., Briarty, L.G.: Early embryogenesis in *Arabidopsis thaliana*. II. The developing embryo. – Can. J. Bot. **69**: 461-476, 1991.

Medford, J.I., Sussex, I.M.: Regulation of chlorophyll and Rubisco levels in embryonic cotyledons of *Phaseolus vulgaris*. – Planta **179**: 309-315, 1989.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue culture. – Plant Physiol. **15**: 473-493, 1962.

Nsangou, M., Greenwood, M.: Physiological and morphological differences between somatic, in vitro germinated, and normal seedlings of red spruce (*Picea rubens* Sarg.). – Can. J. Forest Res. **28**: 1088-1092, 1998.

Pandey, D.M., Goswami, C.L., Kumar, B., Jain, S.: Hormonal regulation of photosynthetic enzymes in cotton under water stress. – Photosynthetica **38**: 403-407, 2000.

Pandey, D.M., Kang, K.-H., Yeo, U.-D.: Effects of excessive photon on the photosynthetic pigments and violaxanthin de-epoxidase activity in the xanthophyll cycle of spinach leaf. – Plant Sci. **168**: 161-166, 2005.

Pandey, D.M., Kim, K.-H., Yeo, U.-D.: Dynamic changes of photosynthetic pigments in soybean callus under high irradiance. – Photosynthetica **41**: 311-314, 2003.

Rao, I.U., Rao, I.V.R., Narang, V.: Somatic embryogenesis and regeneration of plants in the bamboo *Dendrocalamus strictus*. – Plant Cell Rep. **4**: 191-194, 1985.

Sato-Nara, K., Demura, T., Fukuda, H.: Expression of photosynthesis-related genes and their regulation by light during somatic embryogenesis in *Daucus carota*. – Planta **219**: 23-31, 2004.

Schiavone, F.M., Cooke, T.J.: A geometric analysis of somatic embryo formation in carrot cell cultures. – Can. J. Bot. **63**: 1573-1578, 1985.

Wellburn, A.R.: The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. – J. Plant Physiol. **144**: 307-313, 1994.

Wildman, S.G..: Aspect of fraction 1 protein evolution. – Arch. Biochem. Biophys. **196**: 598-610, 1979.