

Effect of sucrose concentration on somatic embryogenesis in carnation (*Dianthus caryophyllus* L.)

A. Deljou*, O. Karami and P. Ostad Ahmadi

Department of Biotechnology, Faculty of Agriculture, Bu-Ali Sina University, Hamadan, Iran.

*E-mail: alideljou@yahoo.com

Abstract

The effect of sucrose concentration was investigated on callus induction and differentiation of embryogenic callus derived from petal explants of carnation cultivar 'Nelson'. Embryogenic calli were produced on MS culture medium containing 9 μM 2,4-dichlorophenoxy acetic acid (2,4-D), 0.8 μM 6-benzyladenine (BA) and different concentrations of sucrose alone or in combination with sorbitol. In constant osmotic potential medium, number of explants containing embryogenic calli was significantly enhanced by increasing the sucrose concentration. Somatic embryos were induced on a hormone-free media containing various concentrations of sucrose alone or in combination with sorbitol. Different sucrose concentrations from 50 to 150 mM significantly increased somatic embryos. No callus and embryo formed when sorbitol was the sole carbon source. In the presence of a constant sucrose concentration, increasing the osmotic potential with sorbitol led to increase in the frequency of somatic embryos. In medium containing low concentration of sucrose (50 and 100 mM), reduced development of embryos was recorded. 90% of somatic embryos were regenerated to form the entire plantlets when they were transferred onto the half-strength MS culture medium containing 3% sucrose. Plantlets also continued to grow under greenhouse condition.

Key words: Carnation, embryogenic callus, somatic embryos, sucrose.

Introduction

Carnation (*Dianthus caryophyllus* L.) is an important floricultural crop with high commercial interest worldwide (Burich *et al.*, 1996). Somatic embryogenesis is a desirable mode of plant regeneration (Williams and Maheswaran, 1986). Besides being the fastest method of plant micropropagation, somatic embryos may also be encapsulated in various gels to form synthetic seed that can be easily stored and transported to long distances (Ghosh and Sen, 1994). Due to the presence of well-developed root and shoot primordia, somatic embryos germinate easily to produce plantlets without additional step of rooting (Laux and Jugens, 1997).

Carbohydrates play various essential roles in plants. They are substrate for respiration, play a role in the synthetic pathways of many compounds and are building blocks of macromolecules. In addition, carbohydrates may control developmental processes (Gibson, 2000; Smeekens, 2000).

Plant cell, tissue or organ culture normally requires the incorporation of a carbon source to the culture medium (Karhu, 1997). The nutritional importance of an adequate carbon source in a culture medium is widely known. However, the addition of medium components, especially macronutrients and carbon sources, represent considerable decrease in the medium osmotic potential (George, 1993). The substitution of the culture medium carbon source by osmotically active solutes has shown that sugars act as a carbon source and as osmotic regulators. In this case, the most frequently used solutes are the two alcohol sugars sorbitol and mannitol (George, 1993).

It has been observed that sugar concentration affects the formation of somatic embryos in culture medium (Kamada *et al.*, 1989; Gray *et al.*, 1993; Lou and Kako, 1995; Lou *et al.*, 1996; Nakagawa *et al.*, 2001). Among the many available carbon sources, sucrose has

been the most tested carbon source and osmoticum for somatic embryogenesis in plant species (Fuentes *et al.*, 2000). Sucrose concentration of 3% was used as a carbon source for the induction of somatic embryos in carnation (Frey *et al.*, 1992; Nakano *et al.*, 1993; Sankhla *et al.*, 1995; Yantcheva *et al.*, 1998). The effect of different sucrose concentrations in somatic embryogenesis of carnation has yet to be studied. In the present study, the effect of different sucrose concentrations has been investigated on somatic embryogenesis of carnation.

Materials and methods

Plant materials: Immature flower buds of the carnation (*D. caryophyllus*) cultivar 'Nelson' were harvested from greenhouse-grown plants and stored at 4°C for 3-4 weeks. They were then surface-sterilized with 70% ethanol for 30 sec, 2% commercial bleach for 20 min, followed by three times rinses with sterilized distilled water. Sepals and receptacles were removed from the buds and the pieces of approximately 4 mm in length of basal petals part were excised as explant and placed on the solid culture medium. For producing callus, explants were placed on Murashige and Skoog (1962) medium supplemented with 9 μM 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.8 μM of 6-benzyladenine (BA) in combination with various concentrations of sucrose (50, 100, 150, 200, 250 and 300 mM) and sorbitol (145 and 335 mM) alone or with both in the following combinations: 50 mM sucrose + 280mM sorbitol, 100 mM sucrose + 232mM sorbitol, 150 mM sucrose + 149 mM sorbitol, 200 mM sucrose + 109 mM sorbitol and 250 mM sucrose + 54 mM sorbitol. Number of explants producing callus were recorded after 9 weeks of culture. Calli were transferred to growth regulator free MS medium containing various concentrations of sucrose and sorbitol alone or combinations of both to produce somatic embryo. Numbers

of embryos were recorded after 5 weeks time. Somatic embryos were then transferred to the half-strength MS medium containing 3% sucrose for plant regeneration. All cultures were incubated in growth room at 24°C and 16/8 hours photoperiod with 30 μmol m⁻² s⁻¹ illumination provided by cool white fluorescent lamps. The pH of the culture media were adjusted to 5.8 using 1N NaOH before adding 7 g L⁻¹ of gelling agent (Agar-Agar, Merck) to the media and were autoclaved at 121°C for 15 min. The osmolarity of each medium was measured on complete liquid medium using a cryoscopic osmometer (Hermann Roebling, Berlin, Germany) according to the manufacturer's instructions.

Transferring to soil: Agar-based medium was removed from roots of plantlets (approximately 25 mm length) under running water and then transferred to plastic pots containing mixture of sand and compost in the ratio 2:1. Pots were maintained in the growth room (18 ± 2°C, 16-h photoperiod and 25 μmol m⁻² s⁻¹). After four weeks, well established plants were transferred to pots containing garden soil and acclimatized in greenhouse for two weeks prior to field plantation.

Histological examination: For histological investigation, somatic embryos were fixed in FAA (formalin- acetic acid- ethanol, 2:1:17 v/v) for 24 hours, dehydrated in serial grades of alcohol and then embedded in paraffin. Serial sections (7 μm) thick were cut and stained with heamatoxylin.

Statistical analysis: Almost 140-150 explants or 200mg callus were considered for each treatment conducted. The data were presented as mean ± S.E. The ANOVA was performed for analysis of the data obtained for each experiment and the means were tested by LSD test ($P < 0.05$).

Results and discussion

Callus induction: Two types of callus were recognized according to color, texture, and time of callus initiation. Type I calli were soft and yellowish green (Fig. 1A), and on all media, their initiation started at the cut edges of the explants within 2-3 weeks of inoculation. A high-frequency of growth rate (95-100%) was observed on this type of calli. Type I callus were apparently non-embryogenic. Type II calli were creamy-white, compact in structure and appeared nodular (Fig. 1B), and grew slowly. The callus initiation was observed on the edges of the petal explants which grew rather slowly and known as embryogenic.

The high concentrations of sucrose have been found to promote somatic embryo formation in chrysanthemum (May and Trigiano, 1991), cucumber (Lou *et al.*, 1996), iris (Jehan *et al.*, 1994), Spindle tree (Biahoua and Bonneau, 1999) and melon (Nakagawa *et al.*, 2001). In this study, number of explant containing embryogenic calli (type II calli) was significantly enhanced by increasing the sucrose concentration (Table 1) and also in presence of the same osmotic potential provided by sorbitol, the number of explants producing embryogenic calli was significantly ($P < 0.05$) enhanced by increasing the sucrose concentration (Table 1). Explants cultured on media devoid of sucrose never induced callus. In the presence of a constant sucrose concentration, increasing the osmotic potential with sorbitol did not increase the frequency of embryogenic calli (data not shown). This suggests that the role of sucrose in embryogenic calli and high frequency may be related to the source of carbon supply to plant cells.

Table 1. Effect of different sucrose concentrations on embryogenic calli induced from petal of Nelson cultivar of carnation after 9 weeks on MS medium containing 9 μM 2,4-D and 0.8 μM BA*.

Carbon source	Calculated osmotic potential (MPa) at 25 °C**	Number of explants with embryogenic calli ***
Sucrose 50 mM	-0.330	3.66±0.57d
Sucrose 100 mM	-0.512	7.33±0.57c
Sucrose 150 mM	-0.750	15.66±1.15a
Sucrose 200 mM	-0.910	17.66±1.52a
Sucrose 250 mM	-1.025	11.66±1.00b
Sucrose 300 mM	-1.220	6.66±0.57c
Sorbitol 335 mM	-1.220	0.00
Sorbitol 280 mM + Sucrose 50 mM	-1.223	3.00±0.33d
Sorbitol 232 mM + Sucrose 100 mM	-1.225	8.00±1.00c
Sorbitol 149 mM + Sucrose 150 mM	-1.215	16.66±1.00a
Sorbitol 109 mM + Sucrose 200 mM	-1.218	16.00±1.52a
Sorbitol 54 mM + Sucrose 250 mM	-1.217	10.00±0.52b
Sorbitol 149 mM	-0.754	0.00

*30 explants/ replicate, six replicate/ treatment.

** The osmolarity of each medium was measured using a cryoscopic osmometer.

*** Mean ± S.E, means having the same letter in column were not significantly different by LSD test ($P < 0.05$).

Table 2. Effect of different sucrose concentrations on somatic embryo induction from type II calli from petal Nelson cultivar of carnation after 5 weeks on hormone-free MS medium*

Carbon source	Calculated osmotic potential (MPa) at 25 °C**	Number of embryos induced from embryogenic calli***
Sucrose 50 mM	-0.325	40.33 ± 5.33 e
Sucrose 100 mM	-0.510	78.33 ± 7.33 d
Sucrose 150 mM	-0.746	122.33 ± 9.33 c
Sucrose 200 mM	-0.911	186.66 ± 8.00 a
Sucrose 250 mM	-1.010	112.66 ± 11.66 c
Sucrose 300 mM	-1.218	72.66 ± 5.66 d
Sorbitol 335 mM	-1.214	0.00
Sorbitol 280 mM + Sucrose 50 mM	-1.217	141.66 ± 9.8 b
Sorbitol 232 mM + Sucrose 100 mM	-1.220	193.66 ± 11.33 a
Sorbitol 149 mM + Sucrose 150 mM	-1.219	190.66 ± 8.66 a
Sorbitol 109 mM + Sucrose 200 mM	-1.218	184.33 ± 10.00 a
Sorbitol 54 mM + Sucrose 250 mM	-1.215	123.66 ± 7.66 c
Sorbitol 149 mM	-0.750	0.00

*200 mg embryogenic calli / replicate, six replicate/ treatment

**The osmolarity of each medium was measured using a cryoscopic osmometer

***Mean ± S.E, means having the same letter in column were not significantly different by LSD test ($P < 0.05$).

Somatic embryo induction and development: Somatic embryos were induced within 1-2 week from embryogenic calli when transferred to hormone-free medium containing different concentrations of sucrose. Histological studies showed that globular and cotyledonary somatic embryos had no vascular connection to the callus (Fig. 1C and F). After one week, globular embryos were further developed into heart and torpedo-shaped embryos. Cotyledonary embryos were observed on most of the calli (90-95%) 4 weeks after being subcultured (Fig. 1E). No embryo induction occurred on non-embryogenic calli (type I

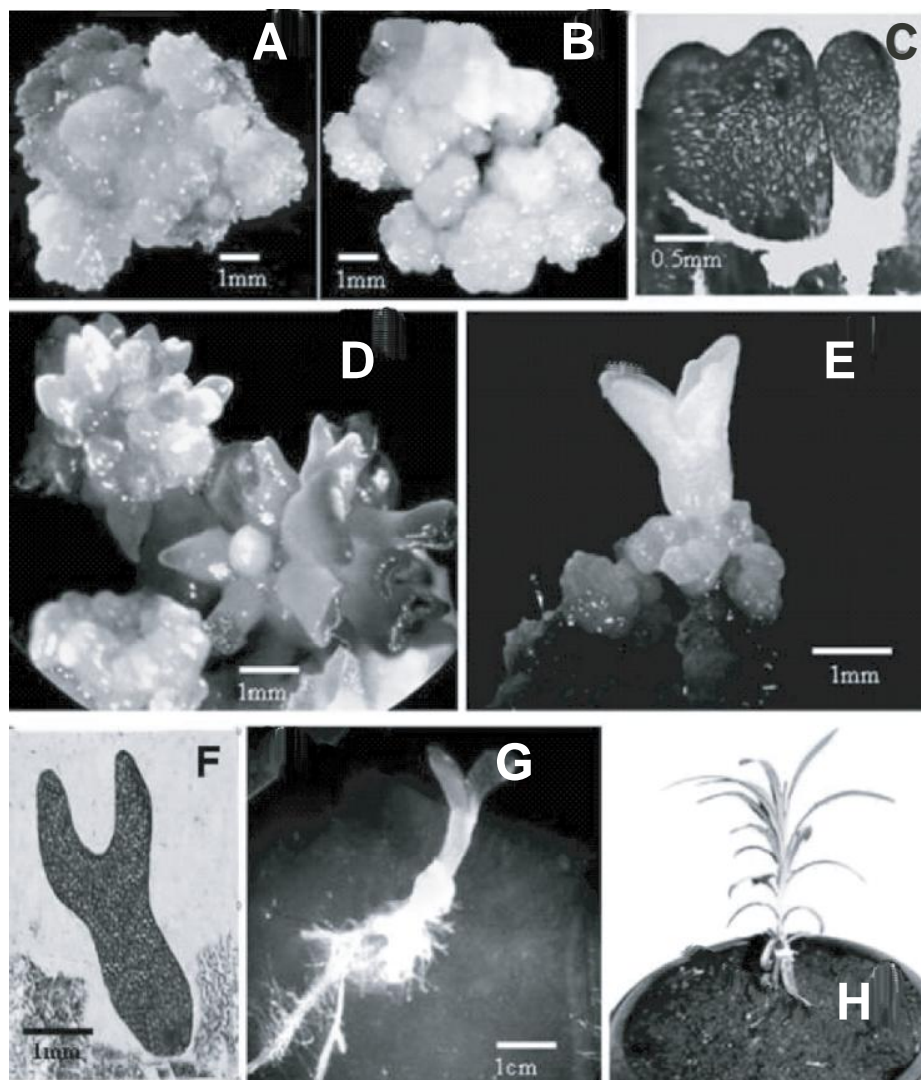


Fig 1. Somatic embryogenesis and plant regeneration in carnation (*Dianthus caryophyllus*). **A:** Type I callus on MS medium containing $9 \mu\text{M}$ 2,4-D and $0.8 \mu\text{M}$ mg L^{-1} BA, 4 weeks after culture. **B:** Type II callus on MS medium containing $9 \mu\text{M}$ mg L^{-1} 2,4-D and $0.8 \mu\text{M}$ mg L^{-1} BA, 9 weeks after culture. **C:** Histological section of somatic embryos in globular and heart stage. **D:** Somatic embryogenesis at different developmental stages on medium containing 150 mM sucrose, 4 weeks after culture. **E:** Cotyledonary embryo on medium containing 200 mM sucrose. **F:** Histological section of somatic embryo in cotyledonary stage. **G:** Plantlet regenerated from a somatic embryo cultured on half-strength MS medium after 2 weeks. **H:** A potted plant in greenhouse.

calli) on medium similar to that used for embryogenic calli.

Table 2 shows the number of embryos on embryogenic calli at different sucrose concentrations. Different sucrose concentrations from 50 to 150 mM significantly increased somatic embryogenesis ($P < 0.05$). With constant osmotic potential medium by sorbitol, number of embryos on embryogenic calli was not enhanced significantly by increasing the sucrose concentration (Table 2). In the presence of a constant sucrose concentration, increasing the osmotic potential with sorbitol led to increase in the frequency of somatic embryos (Table 3). No embryo formed when sorbitol was the sole carbon source. It suggest that the role of sucrose in somatic embryos frequency may be as a result of osmotic potential and carbon supply or their combined action. Our results were consistent with the findings of Litz (1986), May and Trigiano (1991) and Biahoua and Bonneau (1999) who indicated that osmotic potential resulted from higher concentration of sucrose was related to somatic embryogenesis.

The high concentrations of sucrose (250 and 300 mM) reduced

embryogenic calli and somatic embryo formation (Table 1 and 2). This may be due to toxic effects of this sugar and not due to the increase in osmotic potential.

Lower concentrations of sucrose alone (50 and 100 mM) promoted precocious germination and reduced development of normal embryos, but higher concentrations of sucrose alone or in combination with sorbitol promoted the development of normal embryos and prevented precocious germination. Higher concentrations of sucrose were shown to improve maturation of somatic embryos in other plant species (Lu and Thorpe, 1987; Themblay and Themblay, 1991; Ricci *et al.*, 2002). Increasing osmotic activity of the medium due to elevated concentrations of carbohydrate may create the osmotic stress, thus improving the somatic embryogenesis. Therefore, it could be suggested that osmotic effect of the sucrose and sorbitol (in this study) may cause normal development of somatic embryos. The positive effect of high osmolarity may mimic changes in osmolarity that occur in the environment surrounding the embryo within the seed (Merkle *et al.*, 1995).

Table 3. Effect of sorbitol concentrations on the induction of somatic embryos from embryogenic calli in cv. Nelson, 5 weeks after culture on hormone-free MS medium containing 100 mM sucrose

Sorbitol (mM)	Calculated osmotic potential (MPa) at 25 °C**	Number of embryos induced from embryogenic calli***
0	-0.510	40.33 ± 5.33 e
50	-0.800	78.33 ± 7.33 d
100	-0.990	122.33 ± 9.33 c
150	-1.200	186.66 ± 8.00 a
200	-1.350	112.66 ± 11.66 c
250	-1.400	72.66 ± 5.66 d
300	-1.690	0.00 ± 0.00
350	-1.805	141.66 ± 9.8 b

*200 mg embryogenic calli / replicate, six replicate/ treatment

**The osmolarity of each medium was measured using a cryoscopic osmometer

***Mean ± S.E, means having the same letter in column were not significantly different by LSD test ($P \leq 0.05$).

Plant regeneration: After 4 weeks, induced somatic embryos were transferred into half-strength hormone free MS medium where they developed into perfect plantlets within 2 weeks (Fig. 1G). Average germination rate of somatic embryos was about 90 to 95%. As the somatic embryogenesis was asynchronous, plantlets obtained from the same culture media were at different developmental stages. Roots failed to develop, when somatic embryos were not separated from calli cultured on half-strength hormone free MS medium (medium suitable for rooting). A high percentage of rooted plantlets (approximately 95%) were successfully transferred to soil (Fig. 1H) and were developed to normal plants in the greenhouse with an average of 95% survival. All acclimatized plants were transferred to field plots and grew normally in the natural environmental condition.

In conclusion, the results of this study showed that the induction of embryogenic callus on petal explants and production of somatic embryos on embryogenic calli could be obtained by changing sucrose concentration, whether supported by an osmotic potential provided high sucrose concentrations and carbon supply or a combined action of the two. This study also showed that high percentages of somatic embryos could successfully be regenerated into entire normal plants. Establishment of conditions required for the high frequency of regeneration via somatic embryogenesis would facilitate genetic transformation and artificial seed production in carnation.

Reference

Biahoua, A. and L. Bonneau, 1999. Control of *in vitro* somatic embryogenesis of the spindle tree (*Euonymus europaeus* L.) by the sugar type and the osmotic potential of the culture medium. *Plant Cell Rep.*, 19: 185-190

Burich, G., A.P. Mercun, L. Benedtti and A. Giovannini, 1996. Transformation method applicable to ornamental plant. *Pl. Tiss. Cull. Biotech.*, 12: 94-104.

Frey, L., Y. Saranga and J. Bjanik, 1992. Somatic embryogenesis in Carnation. *J. Am. Soc. Hort. Sci.*, 27: 63-65.

Fuentes, S.R.L., M.B.P. Calheiros, J. Manetti-Filho and L.G.E. Vieira, 2000. The effects of silver nitrate and different carbohydrate sources on somatic embryogenesis in *Coffea canephora*. *Pl. Cell Tiss. Org. Cult.*, 60: 5-13.

George, E.F. 1993. *Plant Propagation by Tissue Culture*. Part 1. *The Technology*, 2nd ed. Exegetics Ltd., Edington.

Ghosh, B. and S. Sen, 1994. Plant regeneration from alginate encapsulated somatic embryos of *Asparagus cooperi* Baker. *Plant Cell Rep.*, 12: 381-385.

Gibson, S.I. 2000. Plant sugar-response pathways: Part of a complex regulatory web. *Plant Physiol.*, 124: 1532-1539.

Gray, D.J., D.W. Mcolley and M.E. Compton, 1993. High frequency somatic embryogenesis from quiescent seed cotyledons of *Cucumis melo* cultivars. *J. Am. Soc. Hort. Sci.*, 118: 425-465.

Jehan, H., D. Courtois, C. Ehret, K. Lerch and V. Petiard, 1994. Plant regeneration of *Iris pallida* Lam. and *Iris germanica* L. via somatic embryogenesis from leaves, apices and young flowers. *Plant Cell Rep.*, 13: 671-675.

Kamada, H., K. Kobayashi, T. Kiyosue and H. Harada, 1989. Stress induced somatic embryogenesis in carrot and its application to synthetic seed production. *In Vitro Cell Dev. Biol.*, 25: 1163-1166.

Karhu, S.T. 1997. Sugar use in relation to shoot induction by sorbitol and cytokinin in apple. *J. Am. Soc. Hort. Sci.*, 122: 476-480.

Laux, T. and G. Jugens, 1997. Embryogenesis: new start in life. *Plant Cell*, 9: 989-1000.

Litz, R.E. 1986. Effects of osmotic stress on somatic embryogenesis in *Carica* suspension culture. *J. Am. Soc. Hort. Sci.*, 11: 969-972.

Lu, C.Y. and T.A. Thorpe, 1987. Somatic embryogenesis and plant regeneration in cultured immature embryos of *Picea glauca*. *J. Plant. Physiol.*, 128: 261-265.

Lou, H. and S. Kako, 1995. Role of high sugar concentrations in inducing somatic embryogenesis from cucumber cotyledons. *Scientia Hort.*, 64: 11-20.

Luo, H., P. Obara-Okeyo, M. Tamaki and S. Kako, 1996. Influence of sucrose concentration on *in vitro* morphogenesis in cultured cucumber cotyledon explant. *J. Am. Soc. Hort. Sci.*, 71: 497-502.

May, R.A. and R.N. Trigiano, 1991. Somatic embryogenesis and plant regulation from leaves of *Dendrarthema grandiflora*. *J. Am. Soc. Hort. Sci.*, 116: 366-371.

Merkele, S.A., W.A. Parrott and B.S. Flin, 1995. Morphogenic aspect of somatic embryogenesis. In: *Torpedoed in vitro Embryogenesis in Plant*. Kluwer Academic Publishers, Dordrecht Bosta London, pp 155-203.

Murashige, T. and F. A. Skoog, 1962. Revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 154: 73-479.

Nakagawa, H., T. Saijyo, N. Yamauchi, M. Shigyo, S. Kako and A. Ito, 2001. Effect of sugars and abscisic acid on somatic embryogenesis from melon (*Cucumis melo* L.) expanded cotyledon. *Scientia Hort.*, 90: 85-92.

Nakano, M. and M. Mii, 1993. Antibiotic stimulates somatic embryogenesis without plant growth regulators in several *Dianthus* cultivars. *J. Plant. Physiol.*, 14: 721-725.

Ricci, A.P., F.A.M. Filho, B.M. Januzzi and S.M.S. Piedade, 2002. Somatic embryogenesis in *Citrus sinensis*, *C. reticulata* and *C. nobilis* × *C. deliciosa*. *Scientia Agric.*, 59: 41-46.

Roldan, M., C. Gómez-Mena, L. Ruiz-García, J. Salinas and J.M. Martínez-Zapater, 1999. Sucrose availability on the aerial part of the plant promotes morphogenesis and flowering in *@j alxnorlr* in the dark. *Plant J.*, 20: 581-590.

Sankhla, D., T.D. Vavis, N. Shankla and A. Upadyaya, 1995. *In vitro* regeneration of heat tolerant "German Red" carnation through organogenesis and somatic embryogenesis. *Gartenbouwissenschaft*, 6: 227-233.

Smeeckens, G.S.M. 2000. Sugar-induced signal transduction in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 51: 49-81.

Tremblay, L. and F.M. Tremblay, 1991. Carbohydrates requirement for the development of a black spruce (*Picea mariana* (Mill) B.S.P) and red spruce (*P. rubens* Sarg) somatic embryo. *Plant Cell, Tissue Organ Cult.*, 27: 95-99.

Williams, E.G. and G. Maheswaran, 1986. Somatic embryogenesis: factors influencing coordinated behavior of cell as an embryogenic group. *Ann Bot.*, 57: 443-462.

Yantcheva, A., M. Vlahova and A. Antanassov, 1998. Direct somatic embryogenesis and plant regeneration of carnation (*Dianthus caryophyllus* L.). *Plant Cell Rep.*, 18: 148-153.