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INDUCTION, MAINTENANCE AND PRESERVATION OF EMBRYOGENIC COMPETENCE OF *GENTIANA CRUCIATA* L. CULTURES

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The paper describes structural and ultrastructural changes in primary explants, induction of embryogenesis, somatic embryo development, and four protocols for cryopreservation of cell suspensions. The changes during tissue culture of hypocotyl and cotyledon explants from 10-day-old seedlings and fragments of leaf explant of *Gentiana cruciata* L. were studied. Seedling explants were cultured on MS medium supplemented with 1.0 mg/l dicamba + 0.1 mg/l NAA + 2.00 mg/l BAP + 80.0 mg/l adenine sulphate. The hypocotyl callus tissue was initiated by cell divisions of the vascular cylinder, but in cotyledons only parenchyma cells took part in callus formation. The leaf blade explants usually responded only by proliferation of the wounded surface. The effect of auxins (2,4-D, NAA, DIC) and cytokinins (kinetin, zeatin, BAP) in various concentration and combinations on leaf explant response was examined. Generally, embryos were formed sporadically on media containing NAA (1.64% responding explants) or 2,4-D (0.38%), but were not produced in the presence of dicamba. Production of somatic embryos was more effective from suspension culture than from agar medium. Liquid culture made it possible to maintain the cell suspension's embryogenic competence for 5 years. For preservation of proembryogenic masses, four protocols of cryopreservation were studied: direct cooling, sorbitol/DMSO treatment, vitrification, and encapsulation. Direct cooling and sorbitol/DMSO treatment was unsuccessful. Vitrified tissue required a minimum 3 weeks of culture on solid medium for cell proliferation to reach the proper fresh weight for manipulation. Alginate beads with PEMs were transferred directly to liquid medium for post-freezing culture. Vitrification and encapsulation maintained high viability of post-freezing PEM, but encapsulation ensured faster restoration of *G. cruciata* cell suspension.

Key words: *Gentiana cruciata* L., hypocotyl, leaf explants, dedifferentiation, suspension culture, cryopreservation.

INTRODUCTION

In nature, the cycle of plant reproduction consists in development of zygotic embryos. Their formation begins with divisions of the fertilized egg cell – the zygote. Subsequent orderly progress of cell divisions leads to embryo differentiation. Alternatively, plants may originate from single somatic cells or groups of them. The process of regeneration, called somatic embryogenesis, resembles the zygotic development pathway. Somatic embryogenesis is very frequently employed for vegetative propagation of many species, but the process is still being studied and improved.

Up to now, somatic embryogenesis has been described for only a few species of *Gentiana*. The list includes *G. pneumonanthe* L. (Bach and Pawłowska, 2003), *G. kurroo* Royle (Fiuk et al., 2003), *G. punctata* L. (Mikuła et al., 2004b), *G. pannonica* Scop. (Mikuła et al., 2002a) and *G. tibetica* King (Mikuła and Rybczyński, 2001).

Gentiana cruciata is a protected species of Polish flora. A protocol for effective vegetative propagation of it could help in its conservation. Previous studies indicate that the species possesses high morphogenetic potential in cultures initiated by seedling explants in MS medium supplemented with 2,4-D and kinetin (Kin) (Mikuła et al., 1996; Mikuła and Rybczyński, 2001). Ultrastructural analysis revealed that intensively dividing cells of various tissues of seedling root

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Abbreviations: AS – adenine sulphate; BAP – 6-benzylaminopurine; GA_3 – gibberellic acid; Kin – kinetin - furfurylamonopurine; NAA – α-naphtaleneacetic acid; Dic – dicamba-3,6-dichloro-o-anisic acid; LN – liquid nitrogen; DMSO – dimethylsulfoxide; PEM – proembryogenic mass; Zeat – zeatin – 6-(4-hydroxy–3-methyl–2-enylamino)purine; PGR – plant growth regulator; G.cruc/C – suspension derived from cotyledon callus; G.cruc/H – suspension derived from hypocotyl callus.

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explants showed features of meristematic cells forming non-embryogenic callus (Mikuła et al., 2002b).

In gentian, embryogenic callus was induced in explant cultures maintained on solidified medium. Transfer of the callus from agar to liquid medium ensured better conditions for cell and cell aggregate growth and differentiation. After a period of tissue adaptation, in a proper regime of plant growth regulators the culture started intensive cell proliferation leading to the formation of proembryogenic masses. Prolific embryogenic culture of gentian required 7 days subculture (Mikuła et al., 2002b). Our previous experiments showed that the embryogenic potential of various gentian suspensions fluctuated during a year and decreased during 3–4 years (Mikuła et al., 2002b). Long-term culture usually brings the risk of somaclonal variation (Hao and Deng, 2002) especially in the presence of complex plant growth regulators applied at high concentrations. In addition, maintenance of cultures by successive subculture exposes the suspension to microbial contamination and equipment failure (Swan et al., 1999).

Our research program on the morphogenetic potential of *Gentiana* taxa requires the use of cells carrying the same morphological potential. For this we need uniform experimental material. Cryopreservation has become a tool for long-term storage of biological material, including plant cells, tissues and organs. It is based on reduction and subsequent interruption of the metabolic functions of cells by reducing temperature to -196˚C (Sakai et al., 2002).

So far, protocols for cryopreservation of gentian have been developed only for axillary buds of *G. scabra* (Suzuki et al., 1998) and shoot meristems of *G. scabra, G. trifora, G. trifora* × *G. scabra* and *G. pneumonanthe* (Tanaka et al., 2004). The methods used for introducing the tissues to liquid nitrogen are based on desiccation through preculture with increasing sucrose concentrations and/or application of various cryoprotectants. Vitrification and encapsulation are more frequently applied for recalcitrant plant species (Fang et al., 2004). Experiments on optimization of cryopreservation and estimation of the viability of *G. tibetica* proembryogenic masses have been done (Mikuła et al., 2004a).

This paper presents the results of somatic embryogenesis induction in various types of explants of *Gentiana cruciata*, and protocols for protecting its embryogenic potential. Establishment and cryopreservation of long-term embryogenic cell suspensions were developed as alternative procedures for maintaining its embryogenic potential.

MATERIALS AND METHODS

PLANT MATERIAL

Hypocotyl and cotyledon explants, derived from 10-day-old seedlings of *Gentiana cruciata* L. were used for culture initiation. Seeds were disinfected with Domestos commercial bleach (10% sodium hypochlorite) for 20 min and rinsed 3 times in sterile distilled water, then germinated on 1% agar medium in a plant growth chamber at 22±1˚C with a 16 h photoperiod. Excised hypocotyls were divided into two parts: upper (apical, adjacent to cotyledons) and lower (basal, adjacent to root). Explants of seedlings were placed on MS medium (Murashige and Skoog, 1962) supplemented with 1.0 mg/l DIC, 0.1 mg/l NAA, 2.0 mg/l BAP and 80.0 mg/l AS. Callus tissue initiation and somatic embryo formation were observed in this initial medium (without subcultures).

Leaf explants originating from 2-month-old shoots of axenic cultures were maintained on MS medium without plant growth regulators. Fully unfolded leaves from the first and second whorls were dissected and divided into explants 5 mm long. Explants were cultured in MS medium with auxins (0.5, 1.0, 2.0 mg/l 2,4-D or NAA or Dic) and cytokinins (0.5, 1.0, 2.0 mg/l Kin, Zeat, BAP) in various combinations.

For *G. cruciata* cell suspension culture initiation, 2.0 g of 2-month-old embryogenic calli of hypocotyl and cotyledon explants were transferred to conical flasks with 10 ml liquid medium with the same hormones as in the initial agar medium. After 6 months of culture, 80 ml cell suspension was established by gradually increasing the volume of medium. Suspensions were subcultured every seven days. Culture were maintained under continuous diffused light (3.5 μE m^{-2} s^{-1}) at 22˚C on an Infors rotary shaker at 130 rpm and amplitude 5 cm. For somatic embryo conversion, plant development and vegetative propagation, MS medium supplemented with 0.5 mg/l GA_3, 1.0 mg/l Kin and 80.0 mg/l AS was used.

CRYOPRESERVATION OF PEM

The embryogenic cell suspensions used in the experiments originated from 6-month-old cultures with the sucrose concentration elevated from 3% to 6% and finally to 9%. Sucrose concentrations were increased during the 4 weeks of culture preceding the cryopreservation experiments. Suspensions were subcultured every 7 days. Cell aggregates smaller than 500 μm were used, isolated with stainless steel sieves (Sigma).

These experiments employed different pre- and post-freezing tissue treatments.

1. Direct cooling – tissues from the cultures at 3 different sucrose concentrations were taken directly from culture for programmed cooling experiments. The cooling program went from +20˚C to -20˚C or to -40˚C at 1˚C/min, then further to -150˚C at 10˚C/min and submerging in LN, using a Minicool LC40 programmed freezer.
2. Sorbitol/DMSO treatment – tissue samples (from medium with 6% sucrose) weighing 1.5 g were

placed in 50 ml vials with 20 ml liquid medium. The tissue was treated with 0.2 M sorbitol during the 1st day (0–24 h) and 0.4 M sorbitol on the 2nd (25–48 h). After 48 h the prepared PEM was treated with 0.1 M DMSO (on ice) for 1 h. After addition of cryoprotectant, cryotubes were cooled from 0˚C to -40˚C at 1˚C/min, then further to -150˚C at 10˚C/min, followed by submerging in LN with the same freezer.

3. Vitrification – 1.5 ml sediment tissue (from medium with 6% sucrose) was placed in a 4.5 ml cryovial. The tissue was treated with loading solution (2.0 M glycerol and 0.4 M sucrose) at room temperature for 20 min. Later the tissue was treated with PVS2 vitrification solution containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose (Sakai et al., 1990) for two h at 0˚C, with two changes of fresh solution. Then the tissue was placed directly in liquid nitrogen.
4. Encapsulation – alginate beads with PEM aggregates (~5 mm in diameter) were formed from 3% (w/v) alginic acid (Sigma) made up in liquid calcium-free MS. Fresh mass of tissue was determined before mixing 50:50 (w/v) with alginate. Encapsulated PEMs were dehydrated with 0.3, 0.5, 0.75 and 1.0 M sucrose solution for 48 h at each concentration. After 4 h of desiccation in sterile air, the beads in cryovials were placed directly in LN.

Thawing

For all mentioned combinations of pretreatment, the PEMs were thawed by placing the cryovials in a water bath at +35˚C for 2–3 min. Later, tissue originating from programmed freezing was placed on white filter paper on MS semisolid medium (5 g/l agar). For vitrified tissue, after removing the PVS2, the PEM was immersed in 1.2 M sucrose for 30 min. Later the tissue was placed on semisolid medium for 48 h. Finally the tissue was transferred and maintained on the same medium solidified with 8.0 g/l agar. Encapsulated PEMs were placed on medium with 6% sucrose and 8 g/l agar. After 7 days the beads with PEMs were transferred to standard liquid medium and renewed cell suspensions were developed.

Tissue viability assessment

15 min after thawing and 48 h later, tissue viability was assessed with the TTC test (Towill and Mazur, 1975). 50 mg PEM was immersed in 0.5 ml TTC solution (two independent experiments, 9 replicates each) and incubated at 25˚C for 20 h. After washing 3 times with distilled water, the tissue was submerged in 5 ml 95% ethanol and incubated at 85˚C for 50 min, that is, until total discoloration. Absorption of the red extract was evaluated spectrophotometrically (485 nm) against a blank (Beckman spectrophotometer, model 25). The percentage of formazan content, which later describes viability, was determined and calculated as:

$$\text{Viability of cells} = \frac{\text{absorption in thawed tissue}}{\text{absorption in control (not frozen tissue)}} \times 100\%$$

TABLE 1. Effectiveness of somatic embryogenesis (SE) induction in *G. cruciata* seedling explants

Explant type		% of explants with SE	% of explants with abundant SE
Cotyledon		23.30 ± 8.9	5.6 ± 3.6
Hypocotyl	apical part	34.44 ± 7.8	5.6 ± 3.6
	basal part	5.56 ± 3.46	3.3 ± 4.6
Root		0	0

± SD – standard deviation, data are averages of 90 seedlings (of three independent experiments).

SLIDE PREPARATION

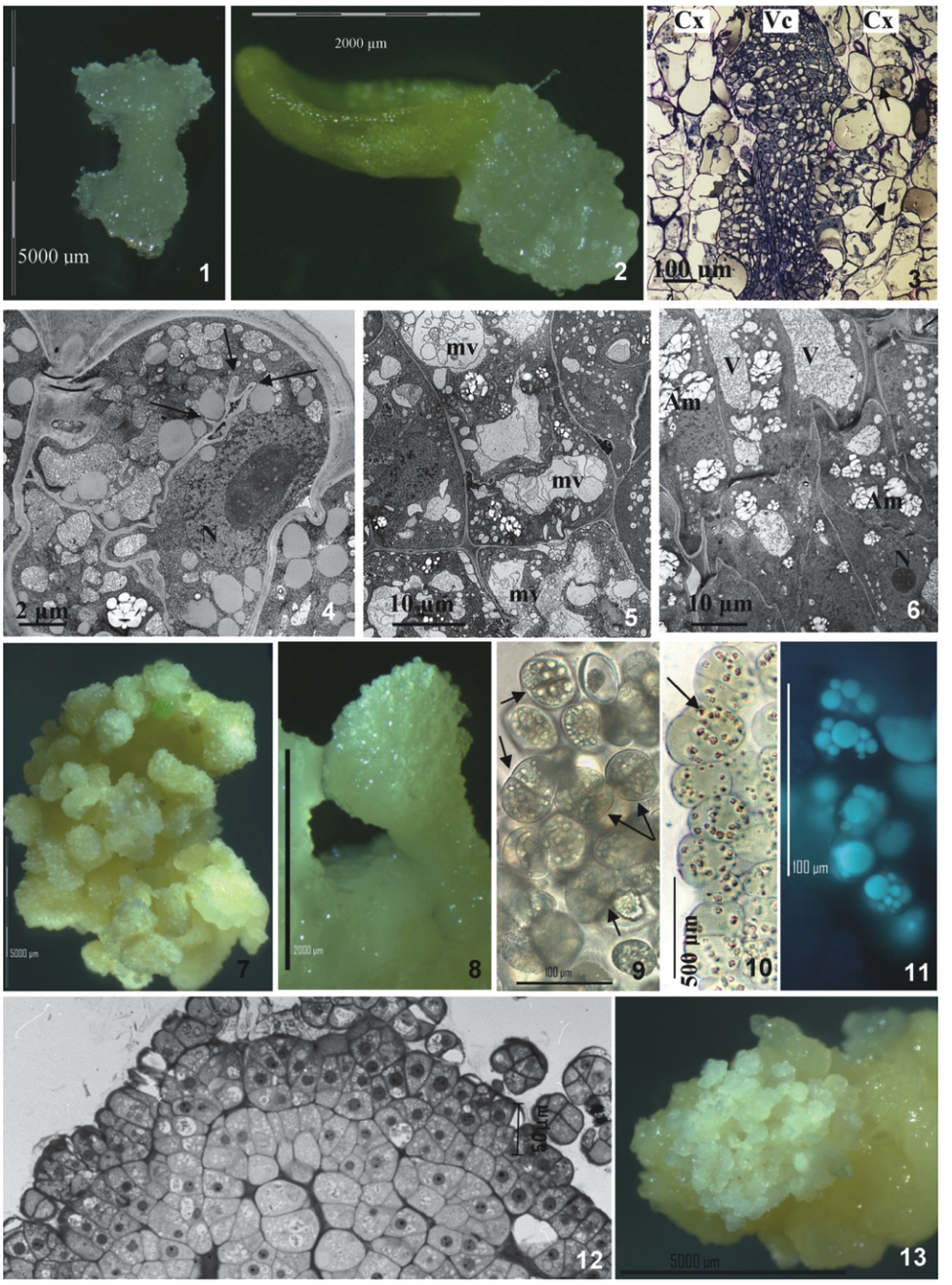
Plant material samples were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h, followed by 2% OsO_4 for 2 h at 4˚C. They were dehydrated in a graded series of ethanol and propylene oxide and embedded in a mixture of Epon and Spurr resins. Semithin (1–2 μm) and ultrathin (60 nm) sections were cut with an LKB III 8800 ultramicrotome (Sweden). Semithin sections were stained with 0.1% toluidine blue in 1% borax and analyzed with a Navox-Olympus light microscope. Ultrathin sections were stained in a solution of 70% uranyl acetate and lead citrate (Reynolds, 1963) for 30 min each. Specimens were observed in a JEOL JEM 100C transmission electron microscope.

RESULTS

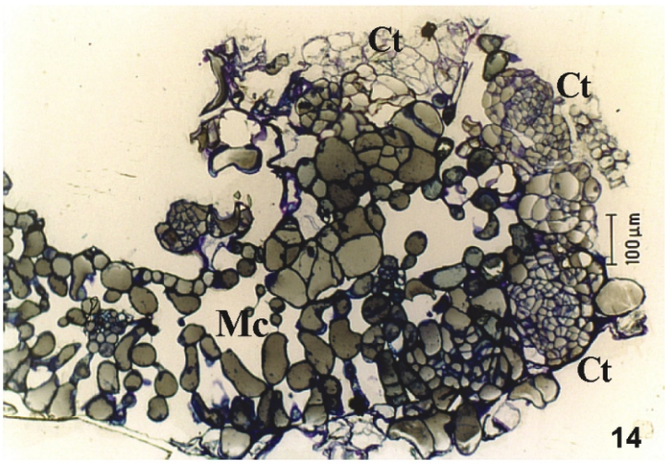
INITIATION OF EMBRYOGENIC CULTURES

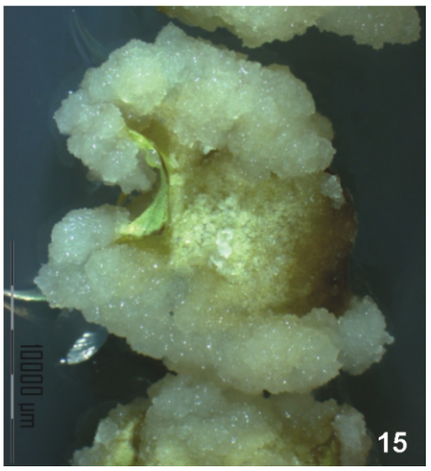
Among the explants of 90 seedlings of *G. cruciata* taken for the experiments, somatic embryogenesis was induced in 23% of the cotyledons and 34% of the hypocotyl apices (Tab. 1). Somatic embryos formed sporadically at the bases of hypocotyls. Root explants did not show embryogenic activity. Only 3.6–5.6% of the explants showed very high embryogenic response.

The time required for the response of explants varied, and was typical for each explant. After 11 days of culture, hypocotyl tissues were evidently dedifferentiated, but cell divisions in the cotyledon explants had only begun. In hypocotyls sectioned into two explants (upper, adjacent to cotyledon; lower, adjacent to root), callus was more intensively formed by the top of the upper explant (Fig. 1). In cotyledons, the response



Figs. 1–13. Initiation of embryogenic cultures. **Fig. 1.** Initial response of hypocotyl explant on MS medium supplemented with 1.0 mg/l of dicamba, 0.1 mg/l NAA, 2.0 mg/l BAP and 80.0 mg/l of adenine sulphate after 3 weeks. **Fig. 2 .** Initial response of cotyledon explant only near cut edge after 4 weeks. **Fig. 3.** Vacuolization and degradation of primary cortex cells (Cx) and expansion of cells in vascular cylinder (Vc) on 11th day of culture. arrows – discontinuous cell wall. **Fig. 4.** More details of discontinuous cell wall (arrows) formation (ultrathin section). N – nucleus. **Fig. 5.** Endoderm cells of hypocotyl explant with signs of degradation. mv – multivesicular structures. **Fig. 6.** Vascular cylinder cells of hypocotyl explant with very dense cytoplasm and prominent nucleus. Am – amyloplast; N – nucleus; V - vacuole. **Fig. 7.** Callus tissue on the hypocotyl explant after 6 weeks of culture. **Fig. 8.** Callus with very small nodules. **Fig. 9.** Cell division (arrows) of embryogenic cells on the surface of proliferating tissue. **Fig. 10.** Semithin section of dividing embryogenic cells of callus with amyloplasts (arrow). **Fig. 11.** Individual cells on surface of callus possessing natural fluorescence under UV light (400–440 nm) **Fig. 12.** Meristematic cells in most of the surface layer of callus. **Fig. 13.** Differentiation of somatic embryos in initial MS medium after 7 weeks of culture.





Figs. 14–15. Leaf explant response on MS medium supplemented with 0.5 mg/l kinetin and 3.0 mg/l 2,4-D. **Fig. 14.** Cross section of leaf explant, revealing callus formation. Ct – callus tissue; Mc – mesophyll cells. **Fig. 15.** Callus proliferation on cut surface of leaf explant after 4 weeks of culture.

usually occurred only in the vicinity of the cut edge or mechanically damaged surface (Fig. 2).

In hypocotyl explants, epidermis and cortex cells were the quickest to divide. Their ultrastructure revealed numerous disturbances of karyo- and cytokinesis, leading to incomplete formation of the cell wall (Figs. 3, 4) and multinucleate cells. These abnormalities led to inhibition of further divisions and vacuolation of cortex and epidermis cells (Fig. 3). Finally, mitotic aberrations led to degradation of cortex cells (Fig. 5) and their detachment from the callus mass. While defective divisions of primary cortex were observed as early as the 2nd day of culture, the first divisions of the axial cylinder cells occurred about the 6th day. They were normal, and gradually led to enlargement of the vascular cylinder of the hypocotyl (Figs. 3, 6), formation of callus tissue (Figs. 7, 8), formation of zones typical for induction of somatic embryogenesis, with embryogenic cells located on its outer layers (Figs. 9–12), and finally formation of somatic embryos (Fig. 13). Individual cells located on the surface of the callus had natural fluorescence under UV light (400–440 nm) (Fig. 11).

In cotyledon cells, dedifferentiation occurred close to the cut or damaged surface. The remaining part of the cotyledon usually showed no response. Cotyledon parenchyma cells underwent isolation, followed by initiation of divisions. The dividing cells were the beginning of tissue callus formation. Often the explants producing callus were gradually surrounded with a watery, sticky areola formed from a single dispersed cell suspended in dense matrix. The embryogenic character of the tissue was identified at ~6–7 weeks of culture.

Initial callus proliferation occurred on the cut surfaces of leaf explants (Figs. 14, 15), and the majority of the leaf blade surface did not show a response (Fig. 15). The percentage of responding explants was low, ~15–32% (Tab. 2). The percentage of explants regenerating somatic embryos was comparatively low, 1.64% and 0.38% in the presence of NAA and 2,4-D, respectively. The mean number of somatic embryos ranged from 5

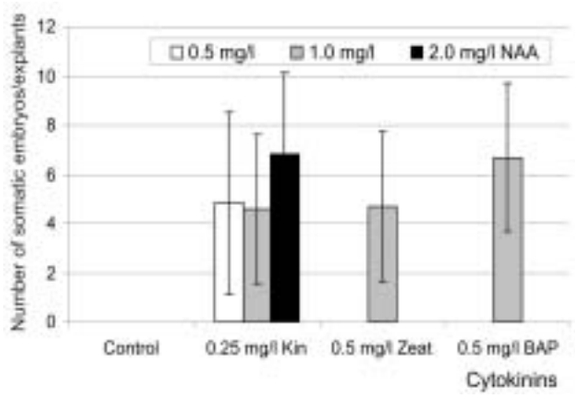


Fig. 16. Mean number of embryos produced per leaf explant in the presence of NAA with the best combinations of cytokinins.

TABLE 2. Percentage of responding leaf explants in the presence of the best cytokinin concentration

Cytokinins	NAA		
	0.5 mg/l	1.0 mg/l	2.0 mg/l
Control	0	0	0
Kin (0.25 mg/l)	14.7	21.52	10.71
Zeat (0.5 mg/l)	0	17.1	0
BAP (0.5 mg/l)	0	32.56	0

TABLE 3. Cell suspension viability (%) after cryopreservation of cultures maintained in the presence of 3%, 6%, 9% sucrose in two programs of cooling: from +20˚C to -20˚C or to -40˚C (at 1˚C/min then further to -150˚C at 10˚C/min followed by submerging in LN) without cryoprotection. Values represent means ± standard deviation of two independent experiments, 9 replicates each

PEM	Viability (%)					
	Directly after thawing					48 h after thawing
	3% sucrose		6% sucrose		9% sucrose	
	to -20˚C	to -40˚C	to -20˚C	to -40˚C	to -40˚C	
G.cruc/C*	0.97 ± 0.72	11.91 ± 0.89	1.15 ± 0.58	27.97 ± 0.79	61.05 ± 0.70	0
G.cruc/H**	4.83 ± 1.01	21.80 ± 0.94	5.47 ± 0.91	32.21 ± 1.56	64.99 ± 1.45	0

*G.cruc/C – suspension derived from cotyledon callus; **G.cruc/H – suspension derived from hypocotyl callus

to 7 per explant (Fig. 16). In these cultures, embryos were formed on media supplemented with NAA or 2,4-D, but were not produced in the presence of dicamba. The best PGR concentrations appeared at 0.5 mg/l BAP and 1.0 mg/l NAA.

MAINTENANCE OF EMBRYOGENIC COMPETENCE

In liquid medium, small pieces of embryogenic callus originated from cotyledon and hypocotyl cultures developed into intensively growing proembryogenic masses (PEM) (Fig. 17). In these conditions, embryo differentiation reached only the globular stage (Fig. 18). Embryos in the late globular stage proliferated and their structures loosened. They disintegrated into single cells and aggregates of different sizes. Such a culture produced new embryo structures within 6 weeks. Competent cells could be found among the outer layer of cells of PEMs and cells floating freely in the medium. The progress of somatic embryo development required culture implantation on agar medium (Figs. 19, 20), and usually took 6 weeks to reach the cotyledonary stage. Mature embryos possessed green cotyledons growing as one (Figs. 20, 21). During embryogenesis, red fluorescence of chlorophyll (induced by UV 330–385 nm) helped to distinguish particular parts of the embryo: cotyledons, shortened hypocotyl and radicle (Fig. 21). Proper development of primary leaves confirmed the lack of morphological disorders in shoot apex formation (Fig. 22). After 8 weeks of culture, 100 mg of PEM implanted on agar medium produced ~200 embryos at the cotyledonary stage. Embryo-to-plantlet conversion was initiated by elongation of the cotyledons and root. Primary leaves emerged from surrounding cotyledons after the next 2 to 4 weeks on MS medium supplemented with 0.5 mg/l GA_3, 1.0 mg/l Kin and 80.0 mg/l AS. The effectiveness of embryo-to-plant conversion was scored at 79–95% for independent experiments carried out over two years.

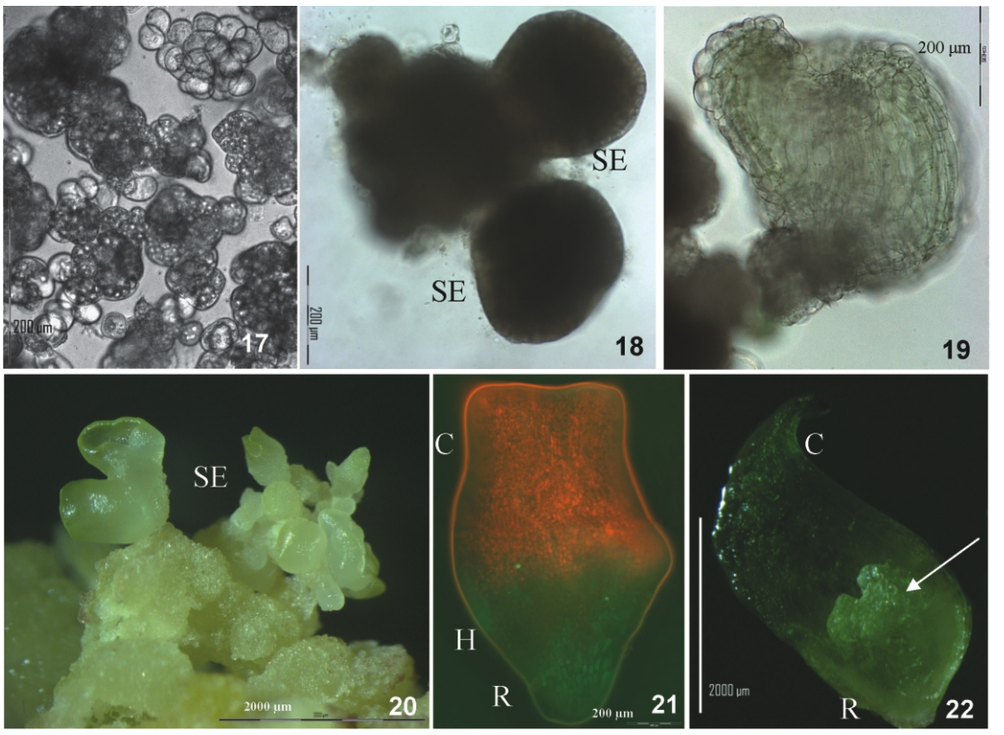
TABLE 4. Cell suspension viability (%) cultured in the presence of 6% sucrose after cryopreservation in three protocols of freezing: sorbitol/DMSO, vitrification and encapsulation. Values represent means ± standard deviation of two independent experiments, 9 replicates each

PEM	Viability (%) after thawing	
	directly	48 h
	Sorbitol/DMSO	
G.cruc/C*	47.56 ± 1.64	2.71 ± 0.42
G.cruc/H**	51.33 ± 0.96	2.51 ± 0.36
	Vitrification	
G.cruc/C*	85.21 ± 10.30	91.04 ± 9.75
G.cruc/H**	82.46 ± 7.65	85.68 ± 11.62
	Encapsulation	
G.cruc/C*	60.59 ± 10.01	64.79 ± 7.57

*G.cruc/C – suspension derived from cotyledon callus;
**G.cruc/H – suspension derived from hypocotyl callus.

PRESERVATION OF EMBRYOGENIC COMPETENCE

Among the studied freezing programs, slow reduction of the temperature of PEMs from +20˚C to -40˚C, at 1˚C/min gave a higher level of survival than reduction to -20˚C (Tab. 3). The viability of frozen tissue without cryoprotectants was highly dependent on the sucrose concentration of the precultured medium. With increasing sucrose concentration from 3% to 6% to 9%, The viability of PEM increased from 1–12% to 28% to 61%, respectively, as measured by the TTC test directly after thawing. However, TTC tests conducted 48 h after thawing and later culture showed death of tissue (Tab. 3). PEM treated with 0.4 M sorbitol and additionally protected with 0.1 M DMSO showed a survival rate of ~50% (Tab. 4) when checked directly after unfreezing. The culture extension showed a drastic decrease of specimen survival to 2.5% after 48 h of culture. Later the cells stopped dividing. Real improvement of survi-



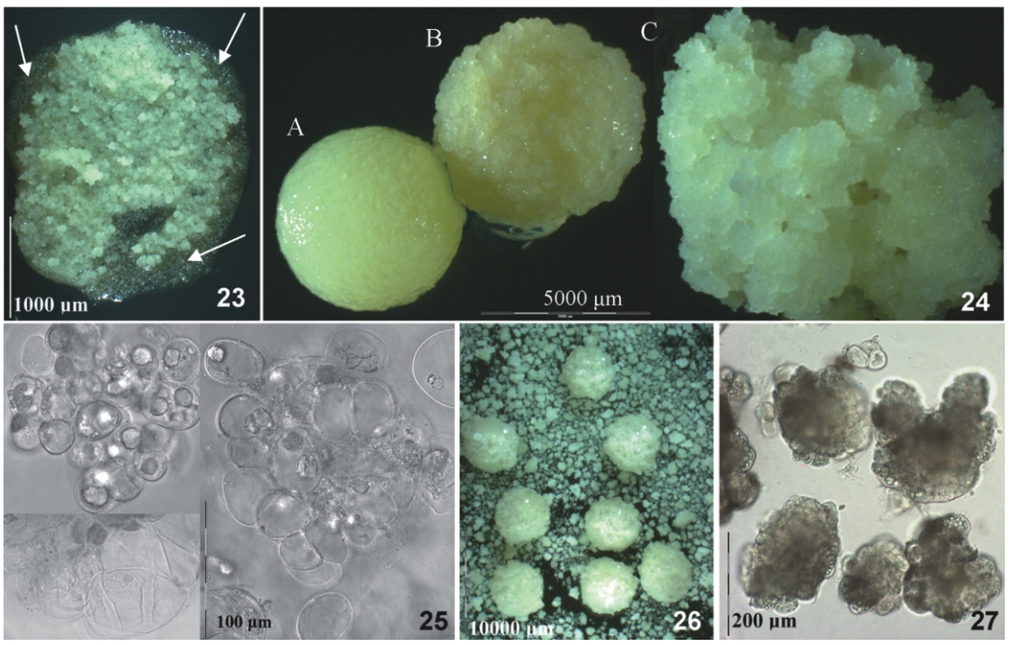
Figs. 17–22. Maintenance of embryogenic competence. **Fig. 17.** General view of liquid cell suspension cultures in MS medium supplemented with 1.0 mg/l dicamba, 0.1 mg/l NAA, 2.0 mg/l BAP and 80.0 mg/l adenine sulphate. **Fig. 18.** Globular embryo (SE) production in auxin-cytokinin environment of liquid medium. **Fig. 19.** Heart-shaped stage embryo formed by proembryogenic mass on agar medium supplemented with 0.5 mg/l GA_3, 1.0 mg/l Kin and 80.0 mg/l AS. **Fig. 20.** Callus with numerous somatic embryos (SE) in cotyledonary stage, 5 weeks after implantation of proembryogenic mass on agar medium. **Fig. 21.** Natural fluorescence of embryo in cotyledonary stage induced by UV light (330–385 nm). Red autofluorescence of chlorophyll in cotyledon (C) and hypocotyl (H), and green autofluorescence in root (R). **Fig. 22.** Longitudinal section of somatic embryo with cotyledon (C), shoot apex (arrow) and radicle (R).

val of cryopreserved tissue was achieved when the tissue was encapsulated or treated with vitrification solution. In the first 48 h period, ~65% and ~85% cells survived, respectively (Tab. 4). In post-freezing culture of tissue after vitrification, the death of small aggregates directly adjacent to the solid medium was observed (Fig. 23). The remaining intensively dividing cells increased their fresh weight by ~47% during 7 days of culture. The tissue required a minimum 3 weeks of post-freezing culture on solid medium. Figure 24 shows very intensive overgrowth of cell aggregates originated from alginate beads. High morphological variation of cells was noted in all cultures on agar medium. The cells varied in size and the degree of vacuolization (Fig. 25). The cells of cultures initiated from alginate beads with PEMs transferred directly to liquid medium in post-thawing culture (Fig. 26) presented features similar to those of the initial cell cultures (Fig. 27). Vitrification and encapsulation maintained high viability in post-freezing PEM, but encapsulation ensured faster restoration of *G. cruciata* cell suspensions.

DISCUSSION

The already described morphogenetic potential of gentian, while characteristic (Momčilović et al., 1997; Mikuła and Rybczyński, 2001), is also strongly dependant on the explant (Hosokawa et al., 1996). In many plant species, the influence of the type and developmental stage of the explant on the formation of callus and somatic embryos has been described (Street, 1978; Williams and Maheswaran, 1986). In gentian, among seedling explants, the root showed the least ability to form embryogenic callus tissue in *G. pannonica*, and no such ability in *G. cruciata* and *G. tibetica* cultures (Mikuła and Rybczyński, 2001). Cotyledons and hypocotyls of *G. cruciata*, *G. pannonica* and *G. tibetica*, and roots of *G. pannonica* seedlings, were sources of embryogenic callus tissue and somatic embryos.

Callus tissue may be formed on the whole explant or on the cut edge only. In *G. cruciata* seedling explants in the presence of 2,4-D + kinetin (Mikuła and Rybczyński, 2001) or dicamba + NAA+ BAP + AS, the



Figs. 23–27. Preservation of embryogenic competence. **Fig. 23.** Growth of proembryogenic mass (frozen by vitrification) after 7 days in post-freezing culture. Arrows show crown of small dead aggregates directly adjacent to the medium. **Fig. 24.** Intensive proliferation of cells in post-freezing culture of alginate beads of PEM: (**a**) Directly after thawing, (**b**) After 2 and (**c**) 6 weeks of culture (MS + 0.5 mg/l 2,4-D + 1.0 mg/l Kin). **Fig. 25.** Large morphological differences between cells in post-freezing culture after 6 weeks of culture on solid medium (originating from vitrification experiments). **Fig. 26.** Cell aggregates from post-freezing culture of alginate beads in liquid medium after 3 weeks. **Fig. 27.** Restored cell suspension with embryogenic structures after 6 weeks of liquid culture.

earliest and most abundant formation of callus was observed mainly on the cut edge. This phenomenon is a typical reaction of wounded tissue. Wounding increases the activity of many enzymes and causes the accumulation of endogenous auxin (IAA), which leads to cicatrization of the wound, or, in culture, to proliferation of callus characterized by morphogenic potential. Too strong an influence of exogenous hormones in culture together with endogenous hormones may lead to mitotic aberrations (Menéndez-Yuffá et al., 2000) and complete destruction of some explant tissues.

It is commonly thought that all types of cells forming the tissues of a given explant, except for highly specialized ones, can be used for initiation of culture (Warren, 1992). However, not all types of cells respond identically to given culture conditions; sometimes only some types give the expected response in culture. In *G. punctata* zygotic embryo, primary cortex cells in the subcotyledonary part of the embryo became strongly vacuolated and formed thickenings. In its hypocotyl and root parts, dividing cells of that tissue gave rise to callus (Mikuła et al., 2004b). While callus in *G. cruciata* root explants was formed by cortex and pericycle cells (Mikuła et al., 2002b), hypocotyl explants of that species produced it only from cells of the axial cylinder.

Gentian leaf explants are not often used for induction of somatic embryogenesis. In our experiments, plantlet-derived leaves had relatively delicate structure, with weakly developed palisade parenchyma proliferating on non-embryogenic callus tissue only on the cut ends. However, leaf explants of *Gentiana pneumonanthe* produced a great amount of embryogenic callus on media containing 2,4-D or picloram (Bach and Pawłowska, 2003).

Callus cell differentiation of hypocotyls, callus proliferation and induction of somatic embryogenesis is a series of phenomena often reported in the literature (Michaux-Ferrčire and Carron, 1989; Canhoto et al., 1999; Verdeil et al., 2001). A set of morphological changes accompanying the acquisition of embryogenic competence by callus was previously described for zygotic embryo explants of *G. punctata* (Mikuła et al., 2004b). These changes are typical of embryogenic callus of gentians, consisting in the formation of meristematic zones constructed by cells with a rich accumulation of starch in amyloplasts. The formation of somatic embryos of *G. cruciata*, as in many other plants (Canhoto et al., 1996; Cruz et al., 1990; Pierson et al., 1983; Puigderrajols et al., 2001; Verdeil et al., 2001), began from single cells isolated from the surface meristematic layer. This physical and physiological

isolation of a single competent cell by a thick wall lacking plasmodesmata is crucial for formation of the proembryo (Yeung, 1995).

The yield of somatic embryos in *G. cruciata* was low in comparison with previously investigated species. In agar culture, primary explants produced 2–3 times fewer somatic embryos than in *G. pannonica* or *G. tibetica* (Mikuła and Rybczyński, 2001). Among the studied seedlings of *G. cruciata*, explants of only 5 seedlings presented a higher morphogenetic potential to form somatic embryos. Thus there is high variation of regeneration potential between seedlings.

The embryogenic potential of primary explants will be reflected in the productivity of the suspension initiated from them. This suggestion is supported by earlier results from experiments on suspension cultures of *G. pannonica* differing in origin (Mikuła et al., 2002a). In comparison with cotyledons and root, hypocotyl showed the highest ability to form embryos indirectly, both in agar (Mikuła et al., 2001) and in liquid culture (Mikuła et al., 2002b).

Studies carried out on cell suspensions of *G. cruciata*, *G. pannonica*, *G. tibetica* and *G. kurroo* showed that all of them exhibit auxin-independent cycles of tissue development in liquid medium stimulating long-term proliferation of cells. In such conditions of culture, somatic embryos were massively produced, reaching globular stage (Mikuła et al., 1996, 2001, 2002a). Additionally, in young (2–3 years old) cultures of *G. tibetica* and *G. pannonica*, heart-stage embryos were formed, and in *G. kurroo* the process of somatic embryogenesis reached even the cotyledonary stage (data not published).

This is the first report on cryopreservation of embryogenic cell suspension of *G. cruciata*. Of four studied protocols, only two appeared successful. Plant cells in culture in vitro may acquire freeze-tolerance upon preculture in the presence of sucrose, sorbitol, mannitol, proline or ABA (Göldner et al., 1991; Swan et al., 1999). In our experiments, preculture of gentian PEMs with sucrose or sorbitol did not help develop a proper system of proembryogenic mass cryopreservation with the application of programmed freezing. Osmotic dehydration by PVS2, which enables cells to survive at -196˚C by vitrification, has significant advantages over freeze-induced dehydration, in that it dehydrates cytosol more effectively, more uniformly, faster and less injuriously at non-freezing temperature (Sakai et al., 2002). Vitrification of *G. cruciata* cell suspension resulted in high viability (~85% surviving). Similarly, encapsulation ensured high viability of the investigated gentian PEM, and other species such as *Catharanthus roseus* (Bachiri et al., 1995), *Ipomea batatas* (Bhatti et al., 1997) and *Vitis vinifera* (Wang et al., 2002). Very similar results were achieved with other cell suspensions of *G. tibetica* (9-year-old) and *G. kurroo* (2-year-old) (Mikuła et al., 2004a). In the case of axillary buds (*G. scabra*), viability reached 78–90% (Suzuki et al., 1998), and in shoot tips from 43% (*G. trifora*) to 93% (*G. scabra*) (Tanaka et al., 2004). In *G. cruciata*, both encapsulation and vitrification methods yielded a high level of quick regeneration in post-freezing culture, with the same embryogenic potential as the control culture.

We emphasize that the *G. cruciata* explants originated from various stages of plant development were characterized by different morphogenetic potential, expressed in differences in somatic embryo formation. The developed cultures, especially those in liquid media, maintained their embryogenic ability even after treatment with ultra-low temperature (-196˚C) with the use of vitrification and encapsulation. Both systems make it possible to maintain high viability of post-freezing PEM, but encapsulation is superior because it ensures quick restoration of cell suspensions.

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