



SOMATIC EMBRYOGENESIS IN *GENTIANA PNEUMONANTHE* L.

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A method for micropropagation of the protected species *Gentiana pneumonanthe* L. by somatic embryogenesis (SE) was elaborated. For SE induction, secondary explants of leaves and apical meristems from in vitro cultures were successfully used. Tests of callus induction were carried out on ten media containing BA (0.04–8.0 μ M) and Picloram or 2,4-D (4.0–8.0 μ M). The greatest amounts of embryogenic callus developed on media containing 2,4-D and also when the incubation period in darkness was prolonged. Embryos matured on media with reduced content of auxins [Picloram or 2,4-D (0.8 μ M or 0.08 μ M) and BA (0.8 μ M)] and germinated on hormone-free media. Cytometric analysis of the callus and plants obtained from somatic embryos showed that DNA content differed from that of the mother plants, but acclimatized marsh gentians contained the same amount of DNA as donor plants.

Key words: *Gentiana pneumonanthe* L., in vitro, somatic embryogenesis, zygotic embryos, nature protection, nuclear DNA amount, flow cytometry.

INTRODUCTION

Regeneration of plants by somatic embryogenesis is an important method of obtaining plants by large-scale vegetative propagation when generative reproduction is not possible, and of producing somatic seeds or breeding plants with new traits (Zimmermann, 1993).

Marsh gentian (*Gentiana pneumonanthe* L.), legally protected in Poland, is a component of plant communities in marsh areas of the Molinion type. The plant is the host of the myrmecophilous lepidopteran *Maculinaalcon*, a butterfly listed as a declining species in the RED BOOK (Thomas et al., 1989). Marsh gentian is a perennial with flowers of an interesting blue color, suitable for flower beds and naturalistic garden arrangements, and can be used as a valuable cut flower.

In this work we developed a laboratory method for reproduction of marsh gentian by somatic embryogenesis. After acclimatization in the greenhouse and planting in the field, plants regenerated from somatic embryos can become valuable material for gardeners or florists. They can also be used in

active protection of the genus *Gentiana* and of the butterfly *Maculinaalcon*.

MATERIALS AND METHODS

CULTURE INITIATION AND MAINTENANCE

Leaf blades (Fig. 1a) cut along the main rib, and apical meristems with the first pair of leaves, were sampled from juvenile marsh gentian plants regenerated in vitro, using the method described by Pawłowska and Bach (2003). The explants were placed on basic medium according to Murashige and Skoog (1962) with the content of macro- and microelements reduced by half, pH 5.8, without growth regulators (control) or enriched with 2,4-D or Picloram and BA in various combinations (Tab. 1). Incubation proceeded in darkness at 23–25°C from 0 (control) to 8 weeks, after which the explants were kept in light. The explants were transferred to fresh media every three weeks. There were five replicates, each with 10 explants, of every combination of media. For statistical analyses, differences between mean values derived from the data



Fig. 1. Somatic embryogenesis of *Gentiana pneumonanthe* L. (a) Leaf explant – culture initiation, (b) Leaf on 25th of culture – pre-embryos, (c) Proliferation of embryogenic callus on medium with 2,4-D, (d) Somatic embryos at globular stage, (e) Embryogenic callus induced by Picloram – leaf, (f) Embryogenic callus induced by Picloram – apical meristem, (g) Mature somatic embryos, (h) Conversion of embryos to plants. Bars in a, b, f = 1 mm; in c, d, e, g, h = 5 mm.

TABLE 1. Effect of type of explant and of the medium on induction of embryogenic callus in marsh gentian

Plant growth regulators (μM)			Embryogenic response of explants (%)	
Picloram	2,4-D	BA	Leaf	Apical meristem
–	–	–	0 a	0 a
–	–	8	0 a	0 a
–	–	4	0 a	0 a
8	–	8	20 bcde	18 bcd
8	–	0.08	24 de	25 de
4	–	4	24 de	23 cde
4	–	0.04	21 cde	15 bc
–	8	8	28 e	37 f
–	8	0.08	26 de	25 de
–	4	4	28 e	27 de
–	4	0.04	27 de	25 de

Mean values marked with the same letters do not significantly differ.

were verified with Duncan's test. Statistical significance was assumed at $\alpha = 0.05$.

MATURATION AND EMBRYO CONVERSION

Regenerated somatic embryos were stimulated to maturation on MS medium with Picloram or 2,4-D ($0.8 \mu\text{M}$ or $0.08 \mu\text{M}$) and BA ($0.8 \mu\text{M}$) added. Mature somatic embryos germinated on hormone-free 50% or 100% MS media. Maturation and germination of somatic embryos occurred in growth chamber conditions ($23\text{--}25^\circ\text{C}$, 80% relative humidity, 16 h day, PPFD – Photosynthetic Photon Flux Density, $30 \mu\text{M m}^{-2}\text{s}^{-1}$) for one month.

CYTOLOGICAL AND MORPHOLOGICAL ANALYSIS OF EXPLANTS

To document somatic embryogenesis (SE initiation, proliferation of embryogenic callus, maturation of somatic embryos), samples were taken every three to five days for analyses. Slides were made by Richardson et al.'s (1960) method. Tissues were fixed with Carnoy solution. After thorough dehydration in 100% ethyl alcohol and acetone the tissue was sealed in Epon 812-epoxy resin. Sections $1 \mu\text{m}$ thick were stained with Azur II and toluidine blue. O'Brien and McCully's (1981) paraffin method was used for large explants. Slides from different kinds of callus obtained at this stage were also stained with acetocarmine.

FLOW CYTOMETRY

Embryogenic callus and regenerated plants of marsh gentian were analyzed for DNA content with

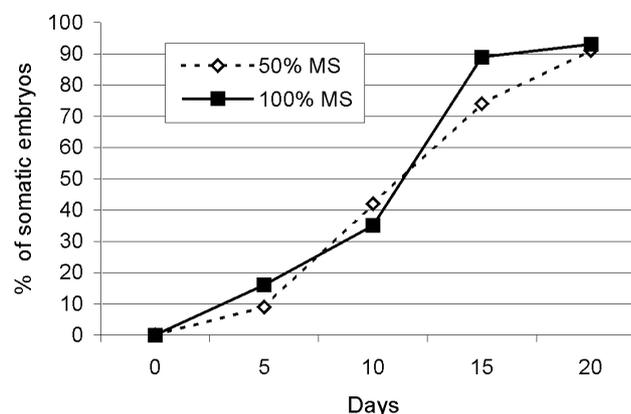


Fig. 2. Effect of medium on conversion of marsh gentian somatic embryos to plants.

a PARTEC CA II flow cytometer. Plant materials were prepared by a two-stage procedure, using Partec solution for isolation of cell nuclei and DAPI labelling. DNA content was estimated from a minimum 5000 cells per specimen. Ten plant tissue samples per specimen were tested.

RESULTS

INDUCTION OF SOMATIC EMBRYOGENESIS

In marsh gentian, somatic embryogenesis was induced in leaf explants (Fig. 1a) and apical meristems (Fig. 1f) taken from plants cultured in vitro. Embryogenic callus formed on all media containing the auxins 2,4-D or Picloram. Initially, after 7–10 days of culture the explants were covered with a thin crystalline callus. After 3–4 weeks of culture the first visible somatic pre-embryos were observed (Fig. 1b,e).

More explants formed embryogenic callus on media containing 2,4-D, and also when the period of explant incubation in darkness was extended. Induction of callus was more efficient with 2,4-D (25–37% regenerated explants) than with Picloram (15–25% regenerated explants) (Tab. 1). The kind of explant (apical meristem or leaf) did not affect induction of embryogenic callus.

Embryogenic callus proliferated in darkness on media for induction of SE; the increase in the percentage of explants forming callus was highest on media containing $8 \mu\text{M}$ 2,4-D.

The color and structure of callus varied depending on the SE-inducing auxin. Callus induced by 2,4-D was

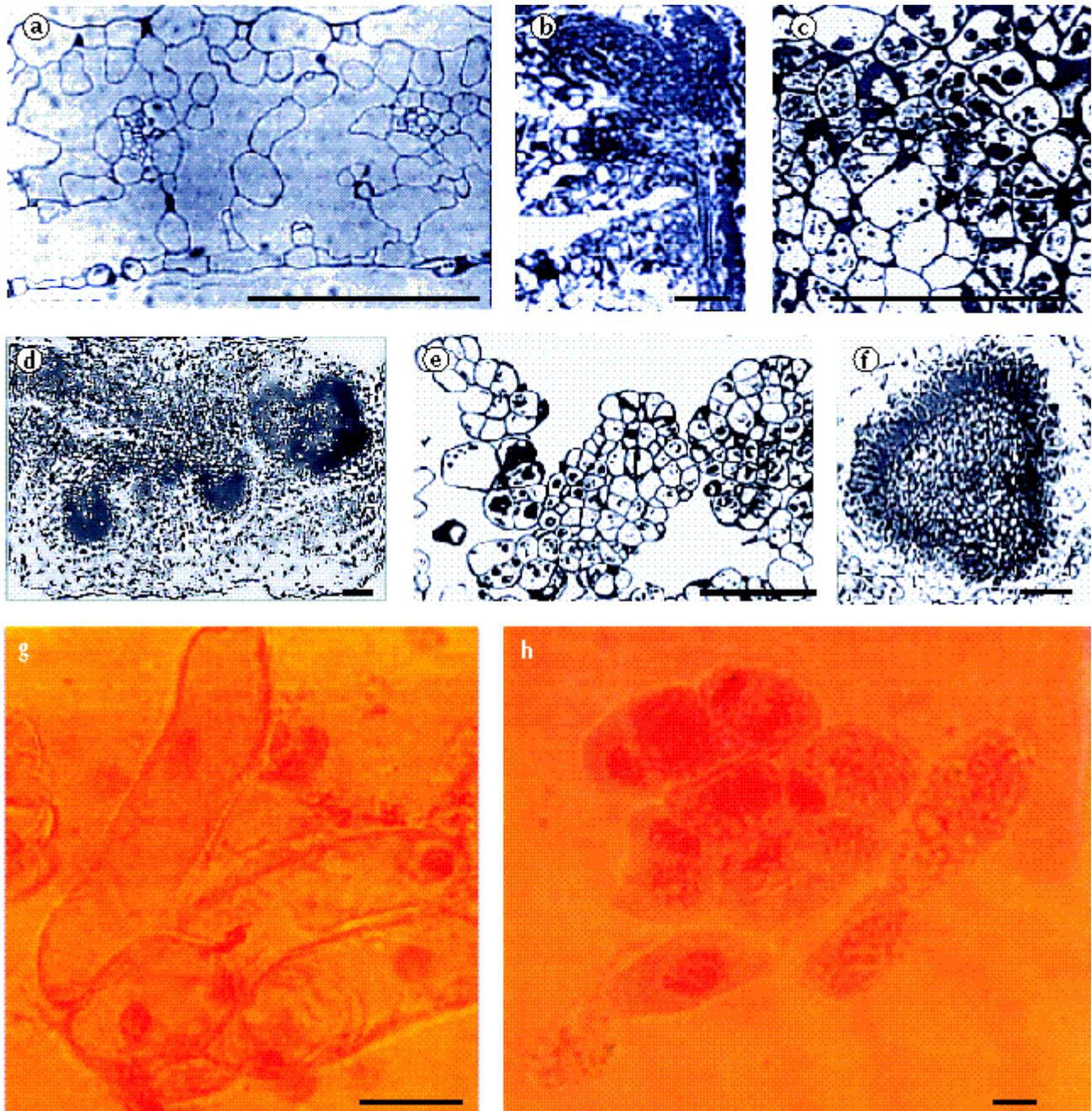


Fig. 3. Histological observation. (a) Cross section of marsh gentian (control leaf), (b) Division of cells surrounding vascular bundles (longitudinal section), (c) Intensely dividing cells of yellow callus, (d) Meristematic centers and further developmental stages of somatic embryos, (e) Pro-embryogenic tissue on medium with high auxin content, (f) Somatic embryo at heart-shaped stage, (g) Cells of white callus, (h) Cells of yellow callus. g and h – material stained with acetocarmine. Bars in a–f = 100 μm ; in g,h = 10 μm .

more yellow and compact (Fig. 1c), and yellow-brown and looser with Picloram (Fig. 1e). Callus cultures required fresh media every three weeks, browning and decaying if kept on the same medium for longer than four weeks. Three-week subculture is required to continue growth of callus.

GROWTH AND DEVELOPMENT OF EMBRYO

Somatic embryos did not differentiate in control explants on hormone-free media, on media supplemented with cytokinins, on those without auxins

TABLE 2. Effect of medium on percentage of explants developing somatic embryos from embryogenic callus

Plant growth regulators (μM)			% of explants
Picloram	0.80	BA 0.08	9.0 a
Picloram	0.08	BA 0.08	7.0 a
2,4-D	0.80	BA 0.08	89.0 b
2,4-D	0.08	BA 0.08	75.0 b

TABLE 3. Effect of length of incubation time and of explant type on induction of somatic embryogenesis in marsh gentian

Time of incubation (weeks)	% of explants forming embryogenic callus	
	Leaf	Apical meristem
0	0 a	0 a
1	0 a	0 a
2	0 a	0 a
3	10 b	11 b
4	29 c	21 c
5	61 ef	63 f
6	69 ef	77 e
7	33 d	38 d

(Tab. 1), or when incubation in darkness proceeded for less than three weeks (Tab. 3).

Somatic embryos matured on media with drastically reduced auxin content (0.8 μM or 0.08 μM 2,4-D or Picloram) and 0.8 μM BA (Tab. 2; Fig. 1d,g). Embryos developed in three to four weeks. If after this period the somatic embryos were not transferred to medium without auxins, they degenerated and embryogenic callus developed on them. The appropriate dose of auxins could again induce embryogenic centers.

Callus induced by 2,4-D was capable of differentiating into somatic embryos (Fig. 1h). When the 2,4-D concentration was 0.8 μM (a tenth that of the SE-inducing medium), 89% of the transferred explants formed somatic embryos. On medium with Picloram only 7–9% of the explants responded (Tab. 2).

Somatic embryos that formed on medium with 2,4-D converted almost immediately after they were transferred to hormone-free medium. On both MS media (50% and 100%), 15% of the somatic embryos germinated after 5 days, and 92% after three weeks (Fig. 2).

In the development of somatic embryos, there were frequent anomalies such as lack of cotyledons, more than two cotyledons, abnormal cotyledon development, deformed embryos or cotyledons, and accretions.

HISTOLOGICAL OBSERVATIONS

The leaf of the donor plant presents typical bifacial structure (Fig. 3a). The lower and upper sides of the leaf differ morphologically and anatomically. The one-layer epidermis envelops the leaf on both sides. The cells of the epidermis are elongated, and the stomatal apparatus occurs on the lower side of the leaf blade (hypostomatic leaf). Palisade parenchyma lies under the epidermis of the upper side of the leaf blade; its elongated cells are perpendicular to the leaf surface. Between the parenchyma and the lower epidermis is spongy parenchyma with irregularly shaped cells. The parenchymal tissue is poorly differentiated into palisade and spongy layers, and has many intercellular spaces. Passing through the parenchyma are bundles of vascular tissue, with xylem toward the center and phloem toward the outside of the bundle. A layer of rounded cells forms a sheath enveloping the bundles (Fig. 3g).

The first histological changes were observed in specimens from 8-day-old cultures. The changes consisted in intensive division of cells surrounding the vascular bundles. Meristematic centers formed later (14-day cultures) (Fig. 3b).

Embryogenic callus of various appearance formed: yellow, white or crystalline. Yellow embryogenic callus was accompanied by non-embryogenic crystalline and white callus. In both the acetocarmine and paraffin preparations and the epoxy resin preparations, the cells of white callus were larger and strongly vacuolated, and their nuclei were localized near the cell wall, with weakly stained nucleoli. In contrast, yellow callus was built of much smaller, ball-shaped cells with dense cytoplasm, a central large nucleus and abundant storage material (Fig. 3a,c,h); in 8-day cultures, fairly large amounts of storage substances were found in the intensively divided cells of yellow callus. Various developmental stages were observed in the same specimens. Apart from meristematic centers giving rise to future embryogenic structures, very advanced somatic embryos were recorded (Fig. 3d,e). Preparations from 28-day cultures had embryos in the heart-shaped stage (Figs. 1g, 3f). Further developmental stages were observed on media with reduced auxin content (Fig. 1h).

FLOW CYTOMETRY

Flow cytometry was used to monitor the genetic stability of embryogenic callus cultures and of marsh gentian plants converted from somatic em-

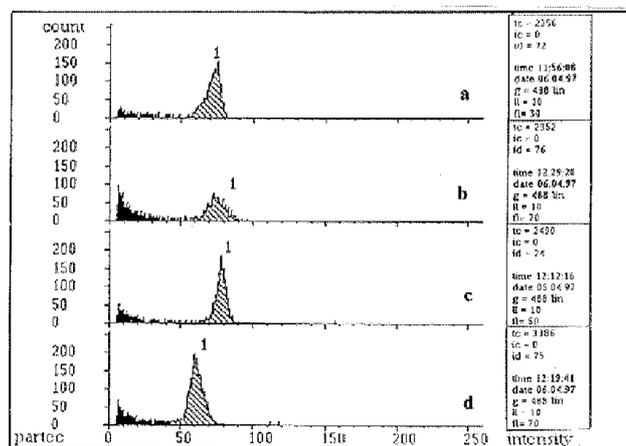


Fig. 4. Analysis of DNA content in cells of marsh gentian. (a) Control plant, (b) Embryogenic callus induced by 2,4-D, (c) Embryogenic callus induced by Picloram, (d) White crystalline callus.

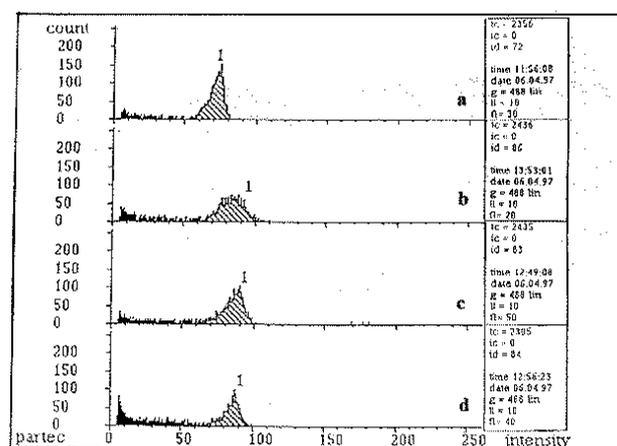


Fig. 5. Analysis of DNA content in cells of marsh gentian. (a) Control plants, (b,c) Plants regenerated from somatic embryos: Emblings (b) and plants from greenhouse after acclimatization (c), (d) Plants after planting in soil.

bryos. Abnormalities of DNA content were detected at an early stage of callus formation *in vitro* (Fig. 4). The amount of DNA in plants converted from embryos and in *ex vitro* plants was similar to gentians from the wild (Fig. 5). No haploids were found in the investigated samples.

DISCUSSION

Somatic embryogenesis is one of the most intensively investigated phenomena in plant biotechnology (Zimmermann, 1993). Only a small amount of plant material is needed to initiate embryogenic cultures, and there is much evidence that it is much more efficient than micropropagation by organogenesis. In dicotyledonous plants, various primary and secondary explants are used in SE initiation (Ammirato, 1987; Bach et al., 1994; Mikula and Rybczyński, 2001). In our cultures of marsh gentian, leaf segments and meristems (secondary explants) proved very useful for induction of embryogenesis. Our results with gentian explants agree with those of earlier experiments with other plants (Zimmermann, 1993). To obtain embryogenic calluses of *G. cruciata*, *G. pannonica* and *G. tibetica*, Mikula et al. (1996) used parts of seedlings or zygotic embryos isolated from seeds, placing them on media enriched with 2,4-D (0.5 mg/l) and kinetin (1 mg/l). Tabira (1994) described the development of embryogenic callus in *G. triflora* effected by 2,4-D. In the present experiment, 2,4-D induced embryogenic callus in marsh

gentian. Somatic embryos grew and developed under the following sequence of media: medium with drastically reduced auxin content, hormone-free medium, and medium with a small amount of BA added. Similarly, Mikula et al. (1997, 2001) used MS medium without 2,4-D but enriched with kinetin (1 mg/l) and GA₃ for maturation of somatic embryos of *G. cruciata* and *G. tibetica*. In media for inducing embryos, cytokinins are commonly used in addition to auxins for 65% of cultivated plants and for 21% of the remaining species (Ammirato, 1983). In a study of the role of cytokinins in maturing *Picea abies* embryos, Mo and Arnold (1991) also found that BA added at the first stage of SE assisted the maturation of somatic embryos.

Anatomical studies of leaf cultured *in vitro* showed poor differentiation of parenchyma into palisade and spongy layers, and many more intercellular spaces than in leaf *in vivo*.

In our experiments, the somatic embryos apparently originated from parenchymal tissue close to the vascular bundles. This kind of embryo formation suggests direct embryogenesis, that is, the formation of adventitious embryos directly from the explant, the phase of callus tissue being omitted (Stefaniak, 1994). Embryogenic structures most frequently form in cells in the zone of the callus tissue growing out of an explant (Schwendiman et al., 1988; Michaux-Ferriere and Carron, 1989). Many cases of the development of embryogenic tissue around vascular bundles have been confirmed experimentally (Guierdoni and Demarly, 1988;

Schwendiman et al., 1988). The formation of meristematic centers around vascular bundles suggests that this is where cells are most rapidly and abundantly supplied with growth regulators from medium. They play the role of stimuli for the start of division and thus for the formation of somatic embryos. Yoshida and Komae's (1994) results confirmed the high sensitivity of vascular bundles to the effect of auxins; embryoidal centers formed close to them.

In 8- and 14-day leaf cultures, histological analysis showed many starch grains in the cells surrounding vascular bundles. Starch is regarded as an indicator of the development of tissues in the direction of somatic embryos (Thomas, 1972; Fransz and Schel, 1991; Konieczny, 2000). Somatic and zygotic embryos accumulate similar storage proteins (Crouch, 1982). Accumulation of lipids is associated with increasing acetyl-Co A carboxylase activity, suggesting that lipid accumulation also is an early indicator of in vitro development of tissues into somatic embryos (Turnham and Northcote, 1982).

In older (21-day) explants, in addition to meristematic centers there were globular stages and also more advanced stages of embryos, frequently even with differentiated organs. In explants not transferred to media without auxins, breakdown of globular-phase somatic embryos was observed, manifested in detachment of epidermal cells followed by loosening structure and then breakdown. In thus-developed embryogenic callus, somatic embryos formed again, preceded by the appearance of new vacuoles in already vacuolated cells, and of abundant storage materials (Mikuła et al., 2001).

To maintain embryogenic tissue in the undifferentiated phase it is not indispensable to have exogenous auxin in the medium (according to the model of the auxin cycle). This was shown by the SE monitoring system developed for cyclamen as a model plant (Winkelmann et al., 1998). Low pH affected the tissues and inhibited differentiation (Jay et al., 1994). In this case there was proliferation of pro-embryogenic mass (PEM). This tissue responded by producing embryos on media with a stable, higher pH value. In general, changes in the organization of cells observed in vitro are rapid. In tissue cultures it takes several days for somatic embryos to form (Fransz and Schel, 1991). In our experiments it took 21 days on average.

In the studied cultures, embryogenic callus was accompanied by white callus of loose structure, built of large vacuolated cells with small cell nuclei. The development of white callus was much slower, and

on the investigated media it could not produce embryos, supporting Winkelmann et al.'s (1998) observations of the high metabolic activity of embryogenic callus.

Flow cytometry showed differences in DNA content between the cells of mother plants and those of callus and the plants regenerated from it. There were no haploids among the regenerated plants. These results support Roux et al.'s (2001) findings from *Musa* cultures. In vitro culture of gentian plants can induce changes in their morphology; permanent genetic changes are fairly rare, and most off-types are sporadic and reversible (Kononowicz and Janick, 1988)

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