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RECURRENT SOMATIC EMBRYOGENESIS IN LONG-TERM CULTURES OF *GENTIANA LUTEA* L. AS A SOURCE FOR SYNTHETIC SEED PRODUCTION FOR MEDIUM-TERM PRESERVATION

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Abstract - Our aim was to establish an efficient and reproducible system for producing synthetic seeds from recurrent somatic embryogenesis in long-term cultures of *Gentiana lutea* L. This species is a vulnerable medicinal plant, protected both at the national and international levels, and is included in different Red Lists and Books. *In vitro* culture, as an alternative to classical methods of preservation, allows for the cyclic multiplication of plant material and short-, medium- and long-term preservation of tissue collections. Biotechnological approaches allow for maintenance of the plant material in a confined space and protection against biotic and abiotic factors. Somatic embryogenesis (SE) is the most efficient way to regenerate plants, ensuring material for preservation and fundamental research. In our experiment, recurrent somatic embryogenesis was developed in long-term cultures in the presence of sugar alcohols (mannitol, sorbitol) and in the absence of growth factors. This process proceeded at a high rate, with adventive somatic embryos being generated in a continuous process, followed by maturation, germination and development into plants. To follow the somatic embryogenesis process, histological samples were made. We used these embryogenic cultures for synthetic seed production and medium-term conservation. The viability of somatic embryos after moderate osmotic stress treatment was tested using TTC. Our methodology relied on the induction of somatic embryogenesis in the presence of auxins in the first cycle of *in vitro* cultures, long-term high embryogenic culture maintenance in the presence of sugar alcohols and synthetic seed production.

Key words: *in vitro* conservation; *Gentiana lutea*; recurrent somatic embryogenesis; synthetic seeds

INTRODUCTION

Taking into account the toxicity and adverse effects of synthetic drugs, interest in the utilization of medicinal plants has increased. Plant-based drugs relying on traditional medicine have been revived throughout the world. Owing to the overexploitation associated with habitat destruction and global climate change, many medicinal plants are nowadays threatened (Thuiller, 2005).

Although plants are mainly conserved *in situ*, *ex situ* approaches can be efficiently used to complement *in situ* methods and in some cases can contribute to the preservation of some endangered species (Benson, 1999; Holobiuc, 2005; Sarasan et al., 2006; Rybczynski and Mikula, 2006; Reed et al., 2011; Engelmann, 2011). An efficient conservation methodology involves maintenance of living plant material at different time intervals (Wenyuan, et al., 2005). *In vitro* tissue cultures *per se* ensure short-term preservation; also, the plant material can be medium-term or long-term preserved. *In vitro* tissue cultures allow for plant regeneration through two main developmental processes: organogenesis and somatic embryogenesis (ES). ES is the ideal procedure for clonal mass propagation and for the preservation of plant

material medium-term, long-term or for synthetic seed production.

Somatic embryos are more genetically stable compared to shoots; the development is not compatible with major genetic alterations owing to several important genes involved in this process, vegetative meristems being more tolerant to genetic alterations (Ozias-Akins and Vasil, 1988). Synthetic seeds are considered as encapsulated buds, meristems and somatic embryos that functionally mimic seeds and can develop into seedlings (Bapat, 1988; Janick and Kitto, 1986). Synthetic seeds can be used for the propagation of *in vitro*-produced propagules and for medium- or long-term conservation purposes (Gray et al., 1995; Benson, 2009). Direct delivery of encapsulated material preserves many subcultures so that plants can be obtained by eliminating the difficult stage of acclimatization of *in vitro* grown plants. To mimic natural seeds, somatic embryos are encapsulated in a nutrient gel containing essential salts and optional carbon sources; plant hormones and antimicrobial agents can be added.

Gentiana lutea L. is a perennial plant that grows on alpine pastures in Europe and Asia at 1000-2500 m altitude. It is registered as a rare and vulnerable medicinal plant species both at the national and European levels. Thus, the harvesting of this species is forbidden, the plant having the status of a protected species with commercial restrictions. In "The Critical List of Vascular Plants from Romania" (Oprea, 2005) the species is considered critically endangered.

For this species, we developed a protocol of continuous recurrent somatic embryogenesis induction under moderate osmotic stress conditions as a tool for its propagation, synthetic seed production and conservation. Somatic embryogenesis was proven and characterized at the histological level.

MATERIALS AND METHODS

In vitro initiation of the aseptic primary cultures was previously described for this species (Holobiuc and Blindu, 2006). Fragments of hypocotyls and roots were used for primary culture initiation from the aseptic germinated seedlings obtained after 50-60 days.

Somatic embryogenesis induction.

MS medium variants (Murashige and Skoog, 1962) were supplemented with Gamborg's vitamin mixture (Gamborg, 1968), 30 g/l sucrose and different growth factors, previously shown to induce good results in *Gentiana lutea*, were used as follows: M1= MS + 2 mg/l 2,4-D(2,4-dichlorphenoxyacetic acid); M2= MS + 2,4,5-T(2,4,5-trichlorphenoxyacetic acid; M3= MS+ 1 mg/l 2,4-D, 1 mg/l IBA (indole butyric acid), 0.25 mg/l kinetin, M4= MS+1 mg/l 2,4,5-T, mg/l, 1mg/l IBA, 0.25 mg/l kinetin. For the second cycle of culture, the same M1-M4 media were used and another different two variants: M5= MS+ 30 g/l sucrose and 30 g/l sorbitol, M6=MS+30 g/l sucrose and 30 g/l mannitol. The tissues cultures were kept at 25°C, with 16/8 h day photoperiod at 80µmol/m^2/s illumination. Subculturing was performed at 4-6 weeks.

The efficiency of regeneration was determined using two parameters: the mean number of somatic embryos/explants and the germination rate of somatic embryos (SE) into plants (expressed as germinated SE/total SE X100) were scored after the first culture cycle on growth regulators added in the media (M1-M4), and in the second culture cycle, using the somatic embryos as an explant source cultured on the same media (M1-M4), and on hormone-free media with added sugar alcohols (M5, M6)

The embryogenic response (the mean number of embryos/explants for 3 replicates x 5 explants/Petri dish) and the rate of germination of the embryos were registered after 2 months of culture and were expressed as mean values ± SD.

Statistical analysis was carried out using Prism-Demo software; one way analysis of variance (ANOVA) was used. Values <0.05 were considered as significant. Tukey's multiple comparison test was also used.

For long-term maintenance, the regenerative cultures were exposed for two weeks to higher mannitol concentrations (6 and 9%) before use in synthetic seed preparation.

We used the TTC viability test (Towill and Mazur, 1975) to assay the SE viability after the exposure to mannitol. Somatic embryos were maintained for 20 h in 0.8% TTC prepared in phosphate buffer at pH 7.5. The red-colored SE were scored and reported viable as the percentage of the total of SE.

The somatic embryogenesis process was monitored with a Stereomicroscope at 1.6 X, and a DOC-UVAL microscope with 5 mm diameter for observing freshly prepared samples and also for histological samples.

Light microscopy

Fresh samples were analyzed after immersion in a distilled water drop containing 0.01% methylene blue to obtain a better contrast. To prove the recurrent somatic embryogenesis process, histological studies were made. The histological analysis of somatic embryogenesis consisted of several steps: fixation in 3.7% formaldehyde, dehydration in ethyl alcohol 2 times for 15 min in 70º, 85º, 96º series, 3 immersions for 6 h in butyl alcohol and finally immersion of the samples in benzene twice for 15 min, followed by inclusion of paraffin at 60ºC for 2 h. The pieces embedded in paraffin were sectioned using a LKB microtome at 7 μm thickness. The samples were treated as follows: immersion in xylene for 30 min, transfer to alcohol (96º-15º) for 15 min each, washing and staining in Mayer-Hemalaun for 10 min, washing in running tap water and staining with 1% eosin, and finally washed in running tap water.

Digital shots were taken using a microscope adapted CANON photo apparatus with 3.2 zoom.

The production of synthetic seeds

Somatic embryos about 1-2 mm in height that were detached or in small aggregates were used. SE were first washed in a modified MS liquid medium without $CaCl_2$ and growth factors. Next, they were included in the same medium; 3% sodium alginate was added and sterilized by autoclaving; the pH was adjusted at 5.8. The mixture of hydrogel containing alginate and explants was shaken. Using a micropipette, the material was gently dropped onto sterile MS medium containing 100 mM $CaCl_2$ to make hydrogel capsules. For complexing Ca^{2+} ions to alginate, the mixture was left for about 30 min and washed with MS liquid medium. The synthetic seeds in hydrogel capsules were subsequently cultured on MS ½-reduced salt medium and maintained at 10ºC in an incubator at 1000 lux illumination and a 16/8 h day photoperiod. The rate of survival of plant material from the synthetic seeds was scored.

RESULTS AND DISCUSSION

The use of *in vitro* methods for conservation involves the establishment of reproducible methods not only for induction but also for the maintenance of regenerative cultures.

Somatic embryogenesis is not routine for most species, requiring a change in the fate of a vegetative (somatic) cell. It generally occurs as an effect of hormone signals that determine in a single cell or a cell group the dedifferentiation and the capacity to actively divide. In the case of direct somatic embryogenesis, the differentiated tissues of the explants change their status, the embryogenic competence being induced through growth factor signals. Most common treatments require the initiation of cell division and the establishment of a new polarity in somatic cells; 2,4-D and also other auxins such as 2,4,5-T or Picloram are effective.

In the case of *Gentiana lutea,* when IAA, NAA and IBA were applied alone they were inefficient (Holobiuc and Blindu, 2006). 2,4-D alone induced the formation of a non-embryogenic friable callus, while 2,4,5-T (2mg/l) or in combination with 2,4-D or 2,4,5-T and IBA (1 mg/l) and kinetin (0.2 mg/l) allowed for the development of embryogenic aggregates.

In our study, the mean number of embryos/explants in the first cycle of the culture was lower and the scored germination rate was also reduced (Fig.1 A and B). The majority of embryos did not develop into plants; just a very low rate of germinated embryos (2-3/explant) was registered in the first culture cycle (Fig.3).

The germinated embryos exhibited both shoot and root development. In the second culture, using the obtained embryogenic tissue explants, the rate of embryo/explant production increased significantly. Embryo production of M1-M4 cultures supplemented with auxins was higher (Fig. 2A).

Despite the testing of media variants at the beginning of the experiments, for the development of embryos (including hormone-free medium or with gibberelic acid or abscisic acid- ABA), only variants containing the sugar alcohols, mannitol and sorbitol (3%) in variants M5, M6, were shown to be capable of sustaining both the embryo development and the process of secondary embryogenesis (Fig.4).

At later stages of SE, polarity emerged and the developed radicular and apical meristems were finally transformed into small plants. Without the addition of growth factors, secondary (recurrent) somatic embryogenesis on the first developed structures occurred. A direct recurring SE process in *Gentiana lutea* was induced by osmotic stress, which acted as an inducer of secondary somatic embryo development.

The germination of somatic embryos registered in the first cycle on M1-M4 media variants was lower in the second culture cycle (Fig. 1B), especially in the case when sugar alcohols were present and when this parameter increased over 80% (Fig.2B).

When the embryogenic aggregates were maintained on variants M5 and M6 supplemented with an osmolyte, they continued to undergo SE. When the somatic embryos at later stages (torpedo, cotyledonary) were cultured separately on the same media, they developed into plants with well-developed roots (in the absence of growth regulators). Similarly, Corredoirra et al. (2003) used either 6% sucrose or 3-6% maltose to promote the maturation and germination of somatic chestnut embryos.

There are some studies on *Gentiana lutea* concerning *in vitro* multiplication that mainly reveal the possibility of regeneration through organogenesis or shoot induction (direct and indirect), using various combinations of plant growth regulators, albeit with a predominance of cytokinins (BAP, Zeatin, 2-IP) combined with NAA and IAA (Wesolowska et al., 1985; Lamproye et al., 1987; Viola and Franz, 1989; Skrzypczak et al, 1993; Momčilović et al., 1997; Mencović et al., 2000; Petrova et al., 2006). The last authors described morphogenesis in *Gentiana lutea* in MS medium supplemented with Zeatin and AIA (in a 10:1 ratio) where the mean regeneration rate was about 4.5 regenerants/explants.

In *Gentiana punctata* L., a rare species, closely related to *G. lutea*, Butiuc-Keul et al. (2005) reported on the regeneration through organogenesis. Here, plant multiplication was quite low (2.6 plantlets/explants) and rooting occurred with difficulty. Vinterhalter and Vinterhalter (1998) studied *G. punctata in vitro*. The authors obtained good results concerning indirect morphogenesis.

Gentiana lutea L. has already been tested in Romania with regard to its *in vitro* conservation (Holobiuc and Blindu, 2006, Holobiuc et al., 2008) and *different pathways of* development, such as callusogenesis, morphogenesis and direct and indirect embryogenesis. Analysis of fresh samples from embryogenic tissue revealed all the characteristic stages of somatic embryogenesis, such as globular, heart, torpedo (Fig.5), also proving recurrent (secondary) embryogenesis on the previously formed embryos (Fig. 6). The development of successive multiplication cycles in the same culture medium containing mannitol or sorbitol (3%), allowed for medium-term preservation for over 5 years and the acquisition of a large number of individuals if they were transferred on ½ MS medium supplemented with 0.01 mg/l-naphthylacetic acid (NAA) for plant growth.

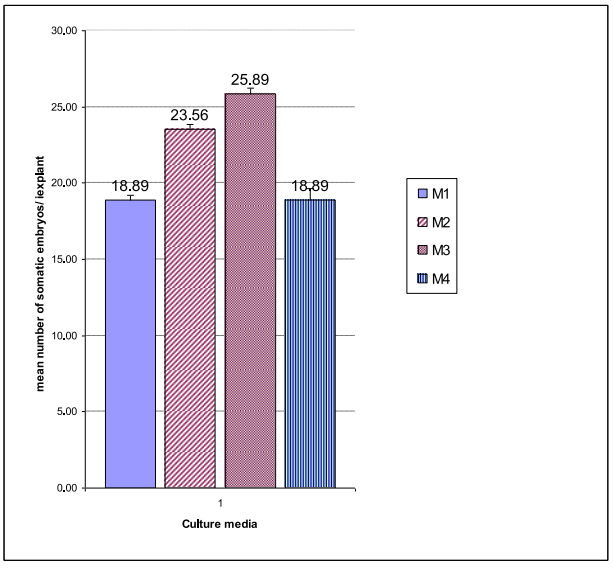

Fig. 1A. Mean number of somatic embryos/explants (mean values +SD) in the first culture cycle; non-significant at p<0.05(0.0646).

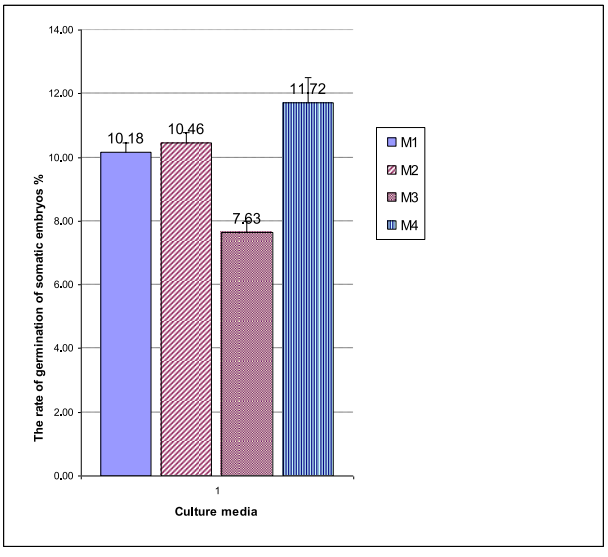

Fig. 1B. The rate of somatic embryo germination in the first cycle of culture in *Gentiana lutea* L (mean values+ SD); non-significant at p<0.05(0.1576).

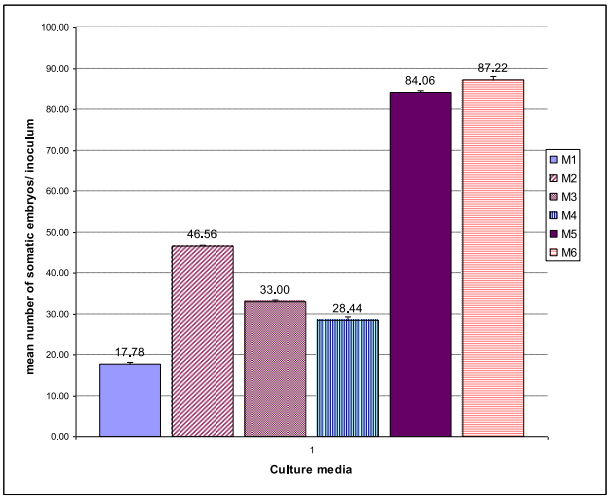

Fig. 2A. Mean number of somatic embryos/explants (mean values +SD) in the second culture cycle; significant at p<0.05(0.0447); M5, M6 mean values are highly significant compared to other variants.

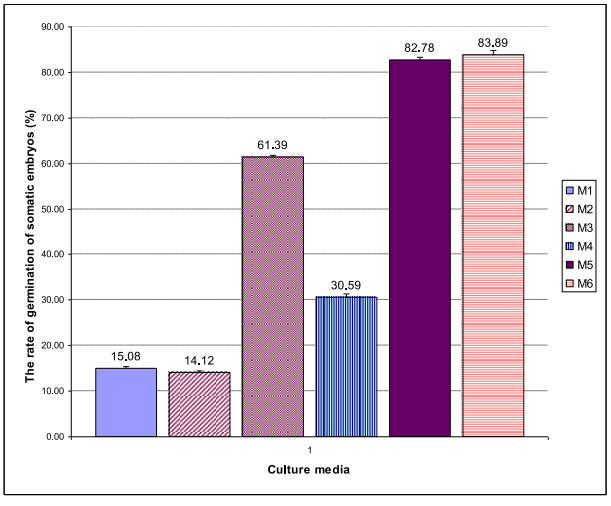

Fig. 2B. The rate of somatic embryo germination in the second cycle of *Gentiana lutea* L culture (mean values+ SD); M5, M6 are highly significant at p<0.05 (0.7223) compared to other variants.

Several authors have already reported similar results concerning the effect of different stress factors generally associated with the hormones as contributors and/or stimulators of somatic embryogenesis in different species, but during short periods of application, such as heat shock, osmotic stress, heavy metals, thermal shock (reviewed by Jimenez et al., 2001). Different stress factors can activate genes involved in the somatic embryogenesis process Karami and Saidi, 2009).

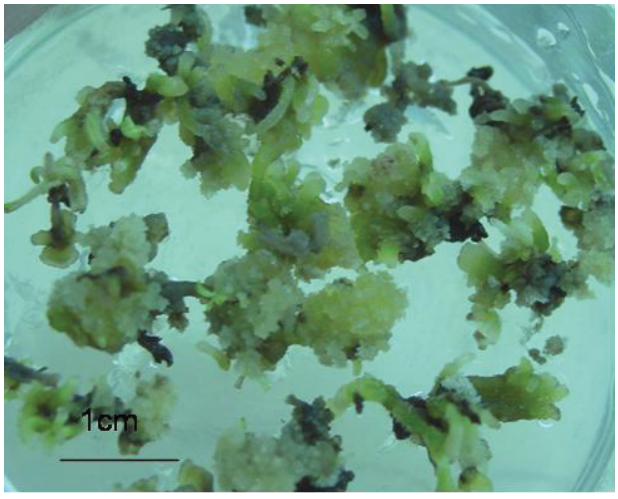

Fig. 3. Somatic embryogenesis induced in the first culture cycle in an auxin containing medium (with 2, 4, 5-T).

Fig. 4. Somatic embryogenesis on a mannitol supplemented medium after 2 months of culture in the second cycle.

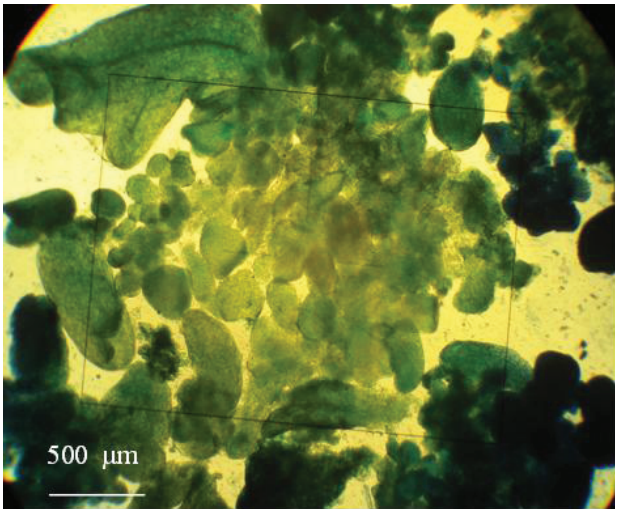

Fig. 5. Different stages of somatic embryogenesis induced in a mannitol-containing medium.

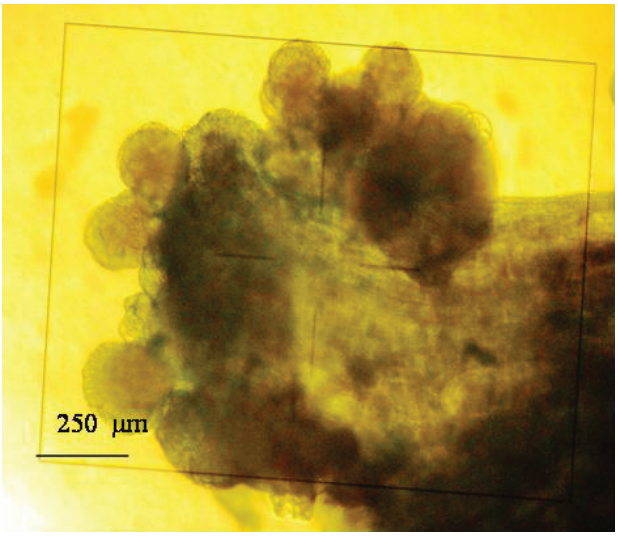

Fig. 6. Globular recurrent somatic embryos that developed at the apical end of an earlier developed embryo in the second culture cycle in the presence of mannitol.

Ikeda-Iwai et al. (2003) induced ES, starting from apical meristems through a short-term treatment (6-9 h) with high levels of mannitol (0.7M), but without eliminating the application of 2, 4-D. In this case, mannitol acted as an inducer of dedifferentiation; the presence of auxin was compulsory. In *Daucus carrota*, SE was induced through the culture of apical meristems in media supplemented with different stress factors, such as sucrose (0.7M), NaCl (0.3 M), CdCl (0.6 mM) (Harada et al., 1990; Kyosue et al., 1990; Kamada et al., 1993). Similar to our results, in *Brassica napus*, starting from immature zygotic embryos as the explant source, Kon and Loh (2000) described a secondary somatic embryogenesis process in the absence of growth factors in a culture medium with low pH. Patnaik et al. (2005) described the effect of water stress and heavy metals on the induction of SE in wheat. The positive effect of osmotic stress in SE was found by other authors, especially in studies concerning the matu-

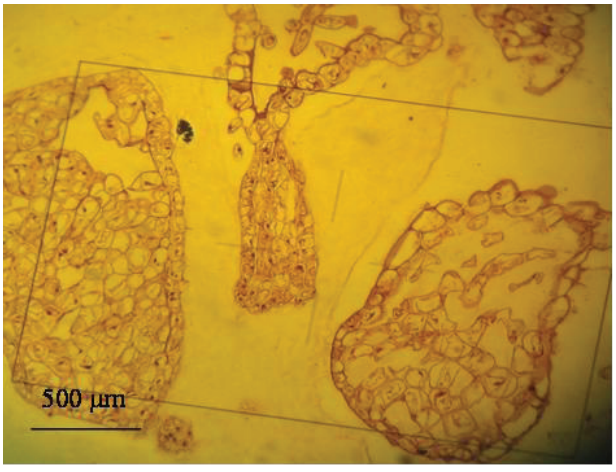

Fig. 7. Histological sample of somatic embryogenesis in *G. lutea* revealed a recurrent process of somatic embryogenesis with suspensor-like structures.

Fig. 8. Synthetic seed production using embryogenic *G. lutea* aggregates as the source of plant material.

ration of SE in woody species and gymnosperms. Usually ES induction proceeds in the presence of growth factors (Corredoira et al., 2003; Belmonte et al., 2005). The increase of the endogenous level of ABA in embryos may be a mechanism of SE induction by moderate osmotic stress (Robinson and Barritt, 1990). It is also possible that osmotic factors have a role in the synthesis of specific proteins (Xu et al., 1990).

In a previous experiment that examined the osmotic effect of different compounds on the promotion of somatic embryogenesis, among PEG, sucrose, mannitol and sorbitol, only the two last ones sustained normal embryogenesis (Holobiuc et al., 2008).

The analysis of histological samples cultured on the mannitol-containing medium revealed different typical stages of ES (Fig.7). The origin of recurrent somatic embryos was the epidermal cell layer of the previously formed somatic embryos. In some cases, the development of "suspensor-like" structures similar to zygotic embryos was observed (Fig.7).

Adventive somatic embryos that developed in the ES process were used for synthetic seed production (Fig.8).

The survival rate for the somatic embryos treated with a high level of mannitol (6%) as tested using TTC, was 90%; with 9% mannitol it was 75%.

Synthetic seeds can be preserved at 10°C in the growing room for several months.

Gentiana lutea plants can be regenerated both directly from somatic embryos from standard cultures, but also from synthetic seeds.

By combining the benefits of a vegetative propagation system with the capability of medium-term storage, our study shows the utility of recurrent somatic embryogenesis as a source for synthetic seeds production in *Gentiana lutea*.

CONCLUSIONS

In the second culture cycle of *Gentiana lutea*, using already formed somatic embryos as explants, somatic embryogenesis was significantly improved, especially using culture media containing mannitol and sorbitol. Histological investigations performed on samples maintained in the presence of mannitol showed that recurrent somatic embryogenesis originated from the epidermal cell layer of the already de-

veloped embryos. The embryogenic mass produced in this system is optimal for use in synthetic seed production, and synthetic seeds can be maintained medium-term for several months at 10^0C, without additional treatments.

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