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ENDANGERED SPECIES – MODEL PLANTS FOR EXPERIMENTAL BOTANY AND BIOTECHNOLOGY

Jan J. RYBCZYŃSKI, Anna MIKUŁA, Agnieszka FIUK

Departament of Biotechnology, Botanical Garden – Center for Biological Diversity Conservation, Polish Academy of Sciences, Prawdziwka 2 str., 02-973 Warsaw, Poland
e-mail: jjryb@ob.neostrada.pl

SUMMARY

For culture initiation explants originated from various stages of plant development were used. Culture initiation and intensive callus proliferation were carried out with the help of two media. In the presence of 2,4-D and kinetin rich callus tissue proliferation was observed. Cytological, ultrastructural and scanning analysis brought evidences that only some of tissues of initial explant were able to form embryogenic callus. In the case of cotyledon, which possess relatively simple structure, almost each of its cell response by callus formation and somatic embryo differentiation. Embryogenic proliferation by central cylinder gave the best response compare to other tissues of hypocotyl of *Gentiana cruciata* seedling. In some cultures the process of somatic embryogenesis was so intensive, that only callus transfer into liquid medium protected the culture against complete disappearing.

Second used medium possessed 1.0 mg/l dicamba, 0.1 mg/l NAA, 2.0 mg/l BAP and 80 mg/l SA and make our dreams come true for very long term cell suspension cultures of proembryogenic masses (PEMs). Long term culture helped us to develop numerous analysis of the process with special attention paid for collection of evidences of single cell origin of somatic embryo. It was proved that somatic embryos originated from freely suspend single cells or single cells of PEM. Among four studied species *G. tibetica* and *G. kurroo* appeared to handle the highest embryogenic potential. The number of regenerated embryos from implanted on agar medium crossed the hundreds from 100 mg of tissue. Medium supplemented with GA_3 helped to complete development and stimulated the somatic embryo conversion in germlings. The high embryogenic potential of at least two year old suspensions of *G. kurroo* was confirmed by the plant regeneration in protoplast cultures. To protect our cell suspension culture against the loss of their embryogenic potential, the cryopreservation of them in LN_2 was developed.

INTRODUCTION

Techniques of tissue culture which are broadly categorised, as follows: organ culture, callus culture, suspension culture, culture of single cell and protoplast cultures were used for the development of gentian regeneration systems as model plants for experimental botany and biotechnology.

Gentiana taxa includes numerous species originated from various parts of world. In majority, they are growing in high attitude and occupying mountains, being endangered and legally protected by law species. Number of the papers concerning biotechnology of gentians is limited although some of species included in this genus play important role in ethnobotany and pharmacology. Presented paper gives the overview of results received from long term experiments on morphogenic potential of selected gentians. Details of particular experiments, reader can find in our publications listed at the end of the paper. According to our data *Gentiana* species are characterized by very high morphogenic potential, showed as the capability to form somatic embryos in liquid and agar culture systems, from single and multi-cellular explants.

The aim of the paper is to give evidences that species growing widely in nature could served as the model plant for experimental botany and biotechnology.

MATERIALS AND METHODS

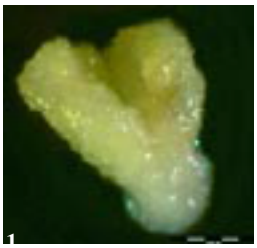
Experiments were carried out with numerous vegetative propagated in our laboratory gentian species: *G. acaulis* L., *G. asclepiadea* L., *G. cruciata* L., *G. kurroo* Royle, *G. lutea* L., *G. pannonica* Scop., *G. punctata* L., *G. purpurea* L., *G. scabra* Bunge, *G. sino-ornata* Balf., *G. tibetica* King ex Hook., *G. triflora var. japonica* Kuns., *G. triflora x purpurea*. In majority, for culture initiation explants originated from various stage of plant development were used (Mikuła and Rybczyński, 2001). Cell suspension cultures were developed on the base of embryogenic character of primary calluses of various explants (Mikuła et al. 2002b).

For different stage of culture, the following media were used: seed germination, callus initiation, maintenance and regeneration, embryo conversion, protoplast culture, plantlet multiplication and plant growth. All mentioned media based on Murashige and Skoog (1962) medium, which was supplemented with various plant growth regulators with different their combinations and concentrations. In the case of protoplast cultures medium modification was connected with the removing of mineral nitrogen source and substituted it with amino acids as the source of organic nitrogen. Media for protoplasts were supplemented with various sugars for the keeping high osmolarity of enzyme mixtures, washing and culture media. Protoplast culture required higher temperature and darkness for initial stages of culture development. Special efforts were required to develop cryopreservation methods of gentiana embryogenic cell suspension. Generally culture were maintained in growth chamber with 16/8hrs (day/night) photoperiod at temperature 20°C. All details of used media and culture conditions were already published elsewhere (Mikuła and Rybczyński 2001, Fiuk et al. 2003, Mikuła et al. 2005a,b).

RESULTS AND DISCUSSION

For culture initiation explants originated from various stages of plant development were used (Figs 1–4). Culture initiation and intensive callus proliferation were carried out with the help of two media. In the presence of 2,4-D and kinetin rich callus tissue proliferation was observed. Cytological, ultrastructural and scanning analysis brought evidences that only some of tissue of initial explant were able to form embryogenic callus tissue (Figs 3 and 4) (Mikuła et al. 2002a, Mikuła et al. 2004). In the case of cotyledon, which possess relatively simple structure, almost each of its cell responsed by callus formation and somatic embryo differentiation. Embryogenic proliferation by central cylinder gave the best response compare to other tissues of hypocotyl of *Gentiana cruciata* seedling (Mikuła et al. 2005). In some cultures the process of somatic embryogenesis was so intensive, that only callus transfer into liquid medium protected the culture against complete disappearing. In the case of leaf explants the response of leaf blade explant was very much species and plant growth hormones concentrations and combinations depending. In these cultures callus proliferation of various morphology

Fig. 1. Callus proliferation of the *G. punctata* zygotic embryo after 4-weeks 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.
Fig. 2. Different type of callus formation on hypocotyl of *G. tibetica* on MS medium after 4-weeks of culture (see above).
Fig. 3. Section of hypocotyl explant of *G. tibetica* producing callus.
Fig. 4. Somatic embryo and callus regeneration on root explant of *G. tibetica* after 7-weeks culture (medium see above).
Fig. 5. 10-months- old cell suspension culture developed from cotyledon callus of *G. cruciata* with the help of MS medium supplemented with 0.5 mg/l 2,4-D + 1.0 mg/l kinetin.
Fig. 6. Fresh specimen of proembryogenic mass in liquid medium of **G. cruciata** (medium see Fig. 5).
Fig. 7. Single cell fraction of embryogenic cell suspension *G. cruciata* (medium see above).
Figs 8 and 9. Two consecutive stages of somatic embryo of *G. tibetica* cell suspension (medium see Fig. 1).
Fig. 10. Somatic embryo conversion into germling on MS medium supplemented with 0.5 mg/l GA_3 + 1.0 mg/l kinetin + 80.0 mg/l adenine sulphate.
Fig. 11. Somatic embryo production of *G. cruciata* in 10 – week – old agar culture of post-freezed suspension.
Fig. 12. Very good developed germling cultured on hormone-free medium.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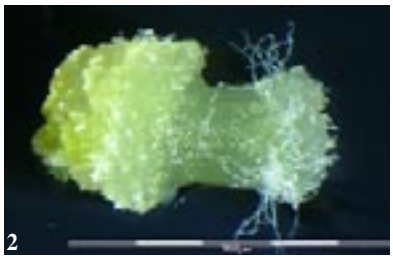
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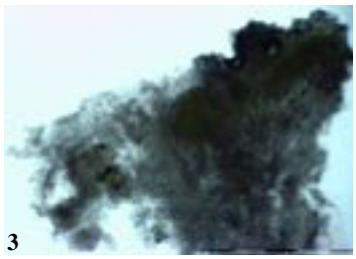
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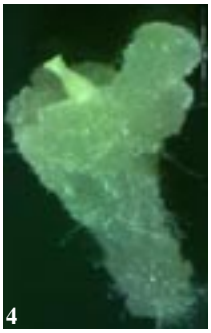
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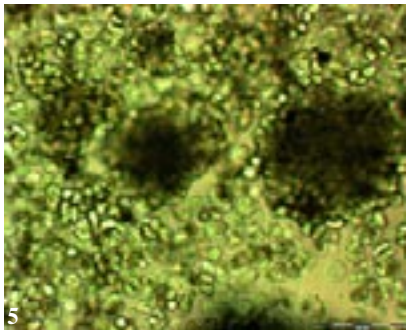
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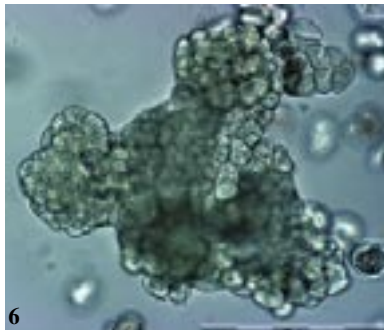
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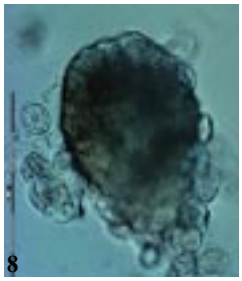
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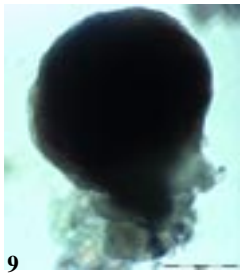
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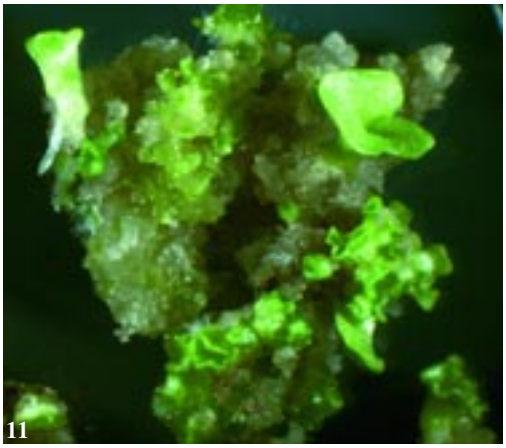
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Table 1. Leaf explant response (%)

Total number of explants	Without response	With response	Type of response		
			Embryos	Roots	Callus*
6989 (100%)	3352 (48%)	3637 (52%)	1243 (17.8%)	721 (10.3%)	3047 (43.6%)

* callus accompanied both embryo and root formation

was observed. Direct and non-direct shoot buds and root regeneration quite often happened. However, cytological analysis revealed that various type of mesophyll cell proliferation leading to somatic embryo regeneration occurred but only for some species. For *G. kurroo* following PGR combinations: 1.0 mg/l NAA + 2.0 mg/l BAP, 2.0 mg/l NAA + 1.0 mg/l CPPU, 1.0 mg/l NAA + 3.0 mg/l TDZ, 1.0 mg/l DIC + 0.25 mg/l zeatin appeared the most effective for embryo production. Table 1 summarized results of experiments concernig leaf explant response.

Medium supplemented with 1.0 mg/l dicamba, 0.1 mg/l NAA, 2.0 mg/l BAP and 80 mg/l SA makes our dreams come true for very long term cell suspension cultures of proembryogenic masses (PEMs) (Figs 5 and 6). Cell suspension cultures were characterized by the aggregate size of cell and type of plastids. Three types of the cell were distinguished: type 1 – small, compactly cling together, intensively dividing, with plastids with non numerous starch grains (pro-embryogenic cells), type 2 – small, with non-numerous plastids with very rich starch within, type 3 – large, with thick cell wall, large amyloplasts and vacuoles. 7-day-long permanent subculture helped to carry on embryogenic character in long term cultures. Cytoplasm of embryogenic suspension could be reconstructed by the use of the increasing of sucrose concentration in culture medium. Four weeks culture resulted in increasing of cytoplasm density, substitution of large vacuoles by numerous small one, the increase of the number of lipid bodies, pararel cistern formation by endoplasmatic reticulum and amyloplasts various in size and shape. At the presence of the highest studied sucrose concentration the frequency of the cell cycle was much reduced, that was expressed by the decrease of fresh mass production. Cells were full of starch grains with very numerous and small vacuoles. Modification of ultrustructure was used to improve the cryopreservation experiments.

Long term culture helped us to develop numerous analysis of the process with special attention paid for collection of evidences of single cell origin of somatic embryo. It was proved that somatic embryos originated from freely suspend single cells or single cells of PEM (Mikuła et al. 1996). Among four studied species *G. tibetica* and *G. kurroo* appeared to handle the highest embryogenic potential. Number of regenerated embryos obtained from cell suspension (Figs 7–9) implanted on agar medium crossed the hundreds from 100 mg of tissue. Medium supplemented with GA_3 helped to complete their development and stimulated the somatic embryo (Fig. 10) conversion in germlings (Mikuła et al. 2002c).

It is very good known and many times proved that extension of culture resulted in decreasing of morphogenetic potential of *in vitro* cultured plant material. This phenomenon depends on many biotic and abiotic factors. In the case of *Gentiana* cultures some of suspension carried on embryogenic capacities by years and others only by months. Cryopreservation appeared the most useful method for conservation of cell suspension viability for long period with the same level of the morphogenic potential.

Cryopreservation is a term of recent derivation and refers to the placing and holding of biological materials at low temperature in a manner such that viability is retained after thawing. Inherent in cryopreservation is the ability to store cells for long periods of time without change or further loss of viability. The preservation of viability is crucial for plant biotechnology, because these materials will be used for future plant cell genetic manipulation. Cryopreservation requires number of treatments of cell culture which affect the changes on ultrustructural level. Sorbitol, one of cryoprotectants, effects dilatations of endoplasmatic

reticulum leading to formation of numerous provacuoles. Vitrification solution composed of a few cryoprotectants induced following changes: hydrolysis of starch in the amyloplasts and in the case of some cells complete degradation of amyloplasts, large inter-starch grain spaces fill with matrix, formation of concentric endoplasmic cisterns and numerous small vacuoles. Successful thawing and plant regeneration from the culture is described by the ultrastructural changes observed during first 48 hrs after tissue defreezing. The most important is to have: very rich rare endoplazmatic reticulum, active and intensively dividing with numerous crests mitochondria, intensive production of dictyosoms by Golgi apparatus, smooth endoplasmatic reticulum with numerous provacuols formed and double biological membranes of all organelles not showing any disturbances. Described above ultrustructure resulted in morphogenic competence of thawing cells. Later, on agar medium cells of suspension aggregates passed series of cell divisions and primary differentiation leading to achieved globular stage of embryo. The culture extension resulted in production of somatic embryos (Fig. 11) which being very green passed stage of conversion, finally giving plantlets (Fig. 12).

In conclusion, on the base at obtained results we would like to put more efforts to better understand the process of somatic embryogenesis on physiological and molecular level.

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