Leaf mesophyll protoplast culture of *Gentiana* spp. genus

K. Tomiczak, A. Fiuk, J. J. Rybczyński

Botanical Garden - Center for Biological Diversity, Polish Academy of Science,
Prawdziwka 2, 02-973 Warsaw, Poland
karolina_tomiczak@wp.pl

Introduction

A protoplast-to-plants regeneration system allows to use such genetic energeering techniques like somatic hybridization by protoplast fusion or direct transformation by DNA uptake. Previous attempts of green leaf mesophyll protoplasts culture in *Gentiana* were carried out only with *G. acaulis*, *G. scabra*, *G. triflora* and *G. scabra x G. triflora* and plant regeneration of the last three was achived. The aim of our experiments was to work out conditions of protoplast isolation and culture for *G. cruciata*, *G. kurroo*, *G. septemfida-lagodechiana* and *G. tibetica*.

Material and Methods

Young leaves were harvested from axenic culture of four *Gentiana* species obtained from seeds. After removal of lower epidermis leaves were incubated in enzyme solution for 3 - 4 hours in the dark at 26^{0}C on a rotary shaker (60 rpm). The enzyme mixture consisted of 1.0 % Cellulase Onozuka R-10 and 0.5 % Macerozyme R-10 (both from Yakult Honsha Co., LTD) dissolved in CPW solution containing 9.0 % mannitol and 5mM MES, pH 5.8. Released protoplasts were filtred through a 45-µm nylon sieve and rinsed three times with WS (*G. tibetica*) (Table 1) or CPW (other gentians) solution supplemented with 9.0 % mannitol. Viability of protoplasts was estimated by FDA staining.

Purified protoplast were cultured in PCM media (NH_4NO_3-free modified MS medium containing 0.3 % glutamine, 3.0 % glucose and 9.0 % mannitol) (Table 1) at a density of 0.5 - 1.0 x 10^5 cells/ml. Culture was carried out with application of agarose-bead method at 21^{0}C or 26^{0}C in the dark. Cell wall regeneration was monitored by protoplasts staining with calcofluor. The efficiency of cell divisions was calculated on 7th day of culture. After 4 weeks the osmotic pressure of media was gradually reduced to stimulate cell colony formation.

When protoplast-derived microcalli reached about 1- 2 mm in diameter, agarose beads were transferred to agar-solidified CPM media (Table 1) for calli proliferation and cultured in the dark at 26^{0}C. After 1 month calli were placed on PRM media (Table 1) in growth chamber conditions (21^{0}C, 16-h photoperiod) to induce shoot regeneration. The shoots were excised from the callus tissue and transferred to ½ MS medium for rooting and further developement.

Fig. 1. Axenic culture of *G. cruciata*

Table 1. Composition of washing solution and media used for protoplast culture, callus proliferation and plant regeneration

Media	Symbol	Media compounds
1. Washing solution	WS	1mM $CaCl_2$ x $2H_2O$ + 5mM MES, pH 5.8
2. Protoplast culture	PCM1	MS modified + 2.0 mg/l NAA+ 0.1 mg/l TDZ
	PCM2	MS modified + 2.0 mg/l NAA + 1.0 mg/l BAP
3. Callus proliferation	CPM1	MS + 2.0 mg/l NAA + 0.2 mg/l TDZ
	CPM2	MS + 2.0 mg/l NAA + 5.0 mg/l TDZ
	CPM3	MS + 2.0 mg/l BAP + 1.0 mg/l DIC + 0.1 mg/l NAA + 80 mg/l SA
	CPM4	MS + 1.0 mg/l KIN + 0.5 mg/l 2,4-D
4. Plant regeneration	PRM1	MS + 0.1 mg/l NAA + 8.0 mg/l TDZ
	PRM2	MS + 0.1 mg/l NAA + 6.0 mg/l BAP
	PRM3	MS + 1.0 mg/l KIN + 0.5 mg/l GA_3 + 80 mg/l SA

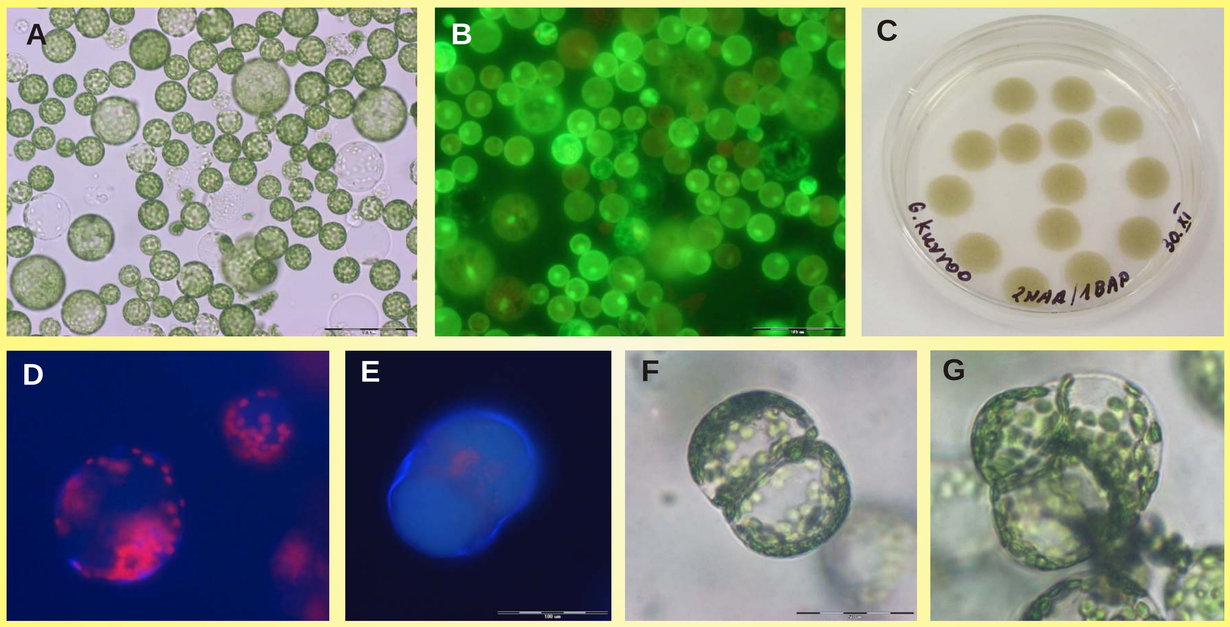


Fig. 2(A-G). A. Freshly isolated leaf mesophyll protoplasts of *G. tibetica*; **B.** Viability of protoplasts (FDA staining); **C.** Agarose-bead culture of *G. kurroo* protoplasts; **D-E.** Cell wall regeneration after 2 **(D)** and 5 days **(E)** of culture (calcofluor staining); **F-G.** First **(F)** and second **(G)** division of protoplast-derived cell

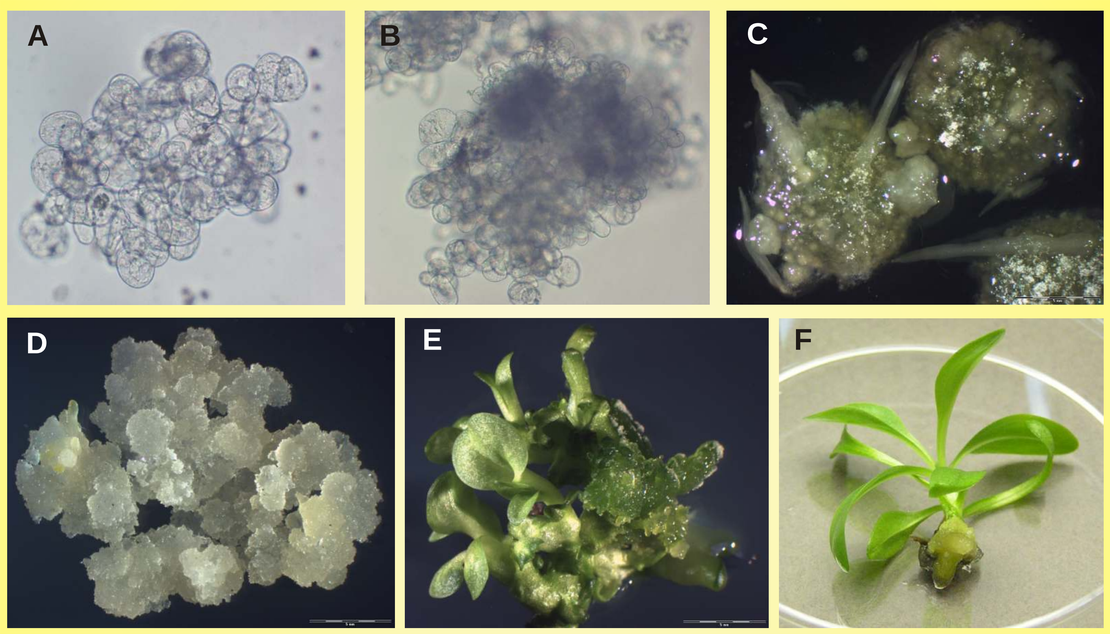


Fig. 4(A-F). A. Protoplast-derived cell colony formation after 6 weeks of culture on PCM1; **B.** Protoplast-derived microcalli after 8 weeks; **C.** Visible small calli in agarose beads after 10 weeks; **D.** Callus tissue after 1 month of culture on CPM1; **E.** Shoot diferentiation from protoplast-derived callus of *G. tibetica* after 3 months of culture on PRM3; **F.** Regenerated plant of *G. kurroo*

Table 2. Plating efficiency of protoplasts (%) after 7 days of culture

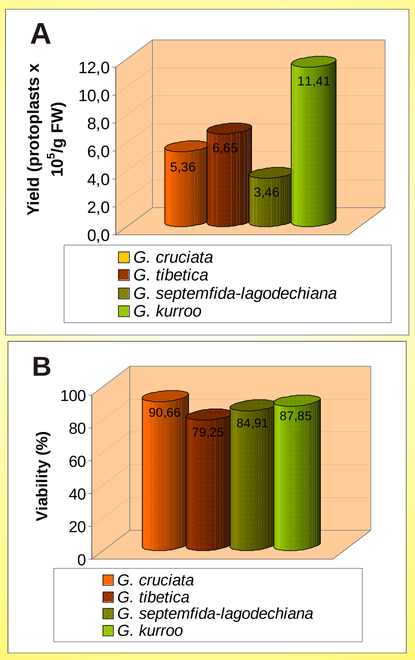
Species	PCM1		PCM2	
	26^{0}C	21^{0}C	26^{0}C	21^{0}C
G. cruciata	4.83 ± 1.45	2.00 ± 0.32	3.45 ± 0.71	1.20 ± 0.34
G. tibetica	5.46 ± 0.73	2.18 ± 0.68	3.01 ± 0.58	1.72 ± 0.27
G. septemfida-lagodechiana	6.25 ± 1.74	2.98 ± 0.67	5.01 ± 1.92	2.81 ± 0.94
G. kurroo	6.13 ± 1.60	3.46 ± 0.87	3.50 ± 0.42	2.31 ± 0.67

Table 3. Protoplast-derived callus formation on various CPM media

Species	Protoplast culture medium	Callus proliferation medium			
		CPM 1	CPM 2	CPM 3	CPM 4
G. tibetica	PCM1	++++	NT*	+++++	+++
	PCM2	+++	NT*	++++	+
G. kurroo	PCM1	+++++	++++	++++	+++
	PCM2	++++	+++	+++	++

NT* - not tested

Fig. 4(A-B). Yield **(A)** and viability **(B)** of green leaf mesophyll protoplasts of various gentians



Results

Green leaf mesophyll protoplasts were easily isolated for all *Gentiana* species. The average yield of protoplasts varied from about 3.46 x 10^5/g FW for *G. septemfida-lagodechiana* to 1.14 x 10^6/g FW for *G. kurroo* (Fig. A). The best viability was observed for *G. cruciata* protoplasts (over 90%) and the worst - for *G. tibetica* (about 79%) (Fig. B). Regeneration of cell wall and first divisions were observed within 3- 5 days of culture. The best conditions for protoplast divisions were ensured at presence of PCM1 medium and temperature 26^{0}C. The highest frequency of cell division after 7 days of culture was performed by protoplasts of *G. septemfida-lagodechiana* (Table 2).

In spite of weekly addition of the fresh medium, protoplasts of *G. cruciata* and *G. septemfida-lagodechiana* turned brown and died within 10 - 14 days of culture. Protoplast-derived cells of *G. kurroo* and *G. tibetica* continued to divide and formed multicellular aggregates after 6 - 8 weeks on either PCM1 or PCM2 medium. Callus proliferation was achived on all tested media with the best results for CPM1(*G. kurroo*) and CPM3 (*G. tibetica*) (Table 3).

Using PRM media shoots regeneration was achived and finally 68 plantlets of *G. tibetica* and 12 of *G. kurroo* has been obtained, so far.

Conclusions

1). Tested conditions of protoplast isolation and culture allowed to obtain intact, viable and able to divide leaf mesophyll protoplasts of four *Gentiana* species.
2). *G. kurroo* and *G. tibetica* plants could be regenerated from green mesophyll protoplasts via organogenesis in described manner.
3). Further investigations are needed to work out the protoplast-to-plants regeneration system for *G. cruciata* and *G. septemfida-lagodechiana*.

This work was financed by MS&I; Grant 3PO4C03723