Plant regeneration of *Gentiana kurroo* Royle and *G. tibetica* King from green leaf mesophyll protoplasts

K. Tomiczak, J. J. Rybczyński

Botanical Garden - Center for Biological Diversity, Polish Academy of Sciences, Prawdziwka 2, 02-973 Warsaw, Poland

e-mail: karolina_tomiczak@wp.pl

Introduction

Plant regeneration from protoplasts is one of the essential techniques for plant breeding through genetic transformation and somatic hybridization. For *Gentiana* genus only 3 species have been regenerated from leaf mesophyll protoplasts so far: *Gentiana scabra*, *G. triflora* and *G. triflora* x *G. scabra*. Moreover because plating efficiency and plant regeneration frequency were low, it is necessary to improve technology of *Gentiana* protoplast culture (Takahata Y. et al., 1995).

Aim

The study was undertaken to determine the conditions for *G. kurroo* and *G. tibetica* plant regeneration from leaf mesophyll protoplasts and to analyse obtained plants by FCA and chromosome counting.

Material and Methods

Protoplasts were isolated enzymatically from young leaves of *G. kurroo* and *G. tibetica* and cultured in dark on PCM media with the application of agarose-beads method. After about 10 weeks of culture developed microcalli were transferred on agar-solidified CPM media for further proliferation. For plant regeneration calli were placed on appropriate PRM media **(Tab. 1)** and cultured in growth chamber conditions (21^{0}C, 16-h photoperiod). Regenerated shoots and somatic embryos were transferred to ½ MS medium.

Flow cytometry analysis Nuclear DNA content of 20 regenerated *G. kurroo* plants and 53 of *G. tibetica* were estimated using Partec CCA flow cytometer. Plantlets obtained from seeds were used as controls. *Petunia hybrida* cv PxPc6 (2C = 2.85 pg DNA) and *Pisum sativum* SET (2C = 9.11 pg DNA) served as internal standards.

Chromosome number observation For chromosome counting roots collected from *in vitro* grown regenerants were treated with 2 mM 8-hydroxyquinoline (2 h in 22^{0}C, 2 h in 4^{0}C) and then fixed in ethanol-acetic acid (3:1, v/v) for 24 h. Root tips were hydrolysed in 5 M HCl for 50 min, stained in Schiff's reagent for 2 h and squashed in 45% acetic acid. The chromosomes were counted under immersion and magnification 1000x.

Tab. 1. Composition of media used for protoplast culture, callus proliferation and plant regeneration

Medium	Symbol	Compounds
Protoplast culture	PCM1	MS mod. + 2.0 mg/l NAA+ 0.1 mg/l TDZ
	PCM2	MS mod. + 2.0 mg/l NAA + 1.0 mg/l BAP
Callus proliferation	CPM1	MS + 2.0 mg/l NAA + 0.2 mg/l TDZ
	CPM2	MS + 1.0 mg/l KIN + 5 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	1.0 mg/l KIN + 0.5 mg/l 2,4-D
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

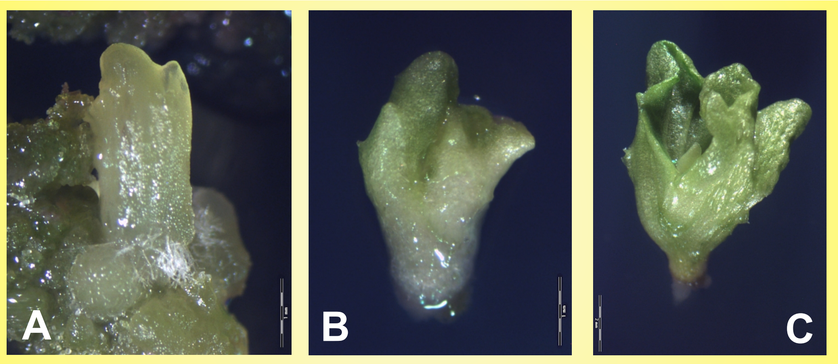


Fig. 1. Somatic embryogenesis of *G. kurroo* after 8 **(A)**, 9 **(B)** and 10 weeks **(C)** on PRM1 medium

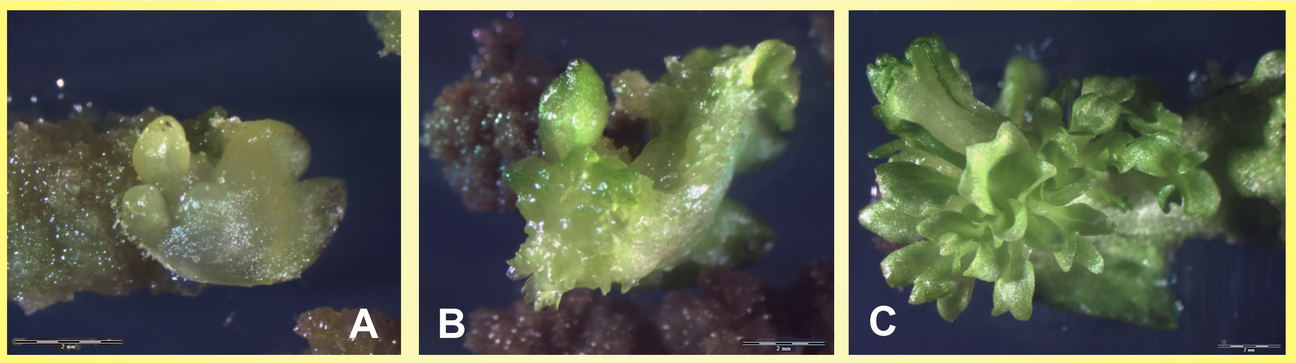


Fig. 2. Shoot organogenesis on *G. kurroo* protoplast-derived callus after 4 **(A)**, 6 **(B)** and 8 weeks **(C)** on PRM2 medium

Fig. 3. *G. kurroo* regenerants

Tab. 2. Optimal media for the highest plant regeneration freguency

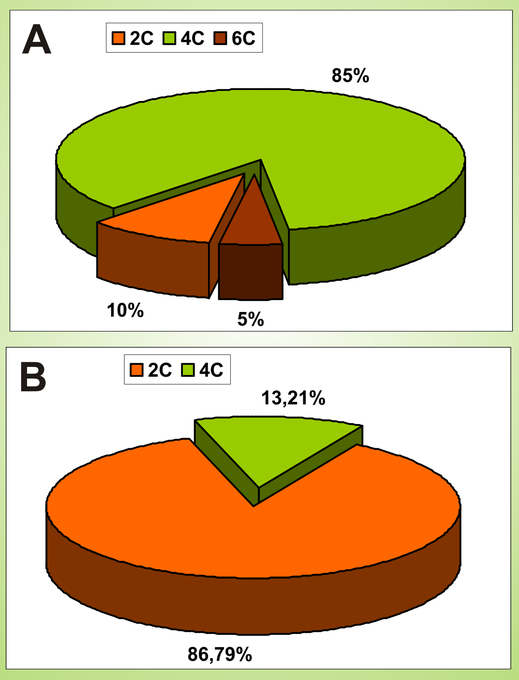
Species	Protoplast culture	Callus proliferation	Plant regeneration
G. kurroo	PCM2	CPM1	PRM2 / PRM3
G. tibetica	PCM2	CPM3	PRM1+PRM3

Tab. 3. Nuclear DNA content, chromosome number and ploidy level of control plants and regenerants of *G. kurroo* and *G. tibetica*

Species		DNA content (pg)	Chromosome number	Ploidy level
G. kurroo	control plants	3.28 ± 0.07	26	2C
	regenerants	3.42 ± 0.04	26	2C
		6.58 ± 0.14	52	4C
		8.75	NT*	6C
G. tibetica	control plants	7.02 ± 0.25	52	2C
	regenerants	7.02 ± 0.34	52	2C
		14.01 ± 0.25	~100	4C

*NT - not tested

Fig. 4. Percentage of 2C, 4C and 6C regenerants of *G. kurroo* **(A)** and *G. tibetica* **(B)**



Results

Total 21 regenerants of *G. kurroo* and 81 of *G. tibetica* were obtained. Regeneration proceeded either by somatic embryogenesis **(Fig. 1)** or organogenesis **(Fig. 2)**. Some embryoids were found to be malformed in morphology **(Fig. 1A)**.

Higher regeneration frequency for both species was obtained for protoplast cultured on PCM2 than on PCM1 medium. Optimal media for callus proliferation were CPM1 for *G. kurroo* and CPM3 for *G. tibetica*. PRM2 and PRM3 media revealed to be the best for regeneration of *G. kurroo* plants, while PRM1 medium in combination with PRM3 provided the higher regeneration frequency for *G. tibetica*.

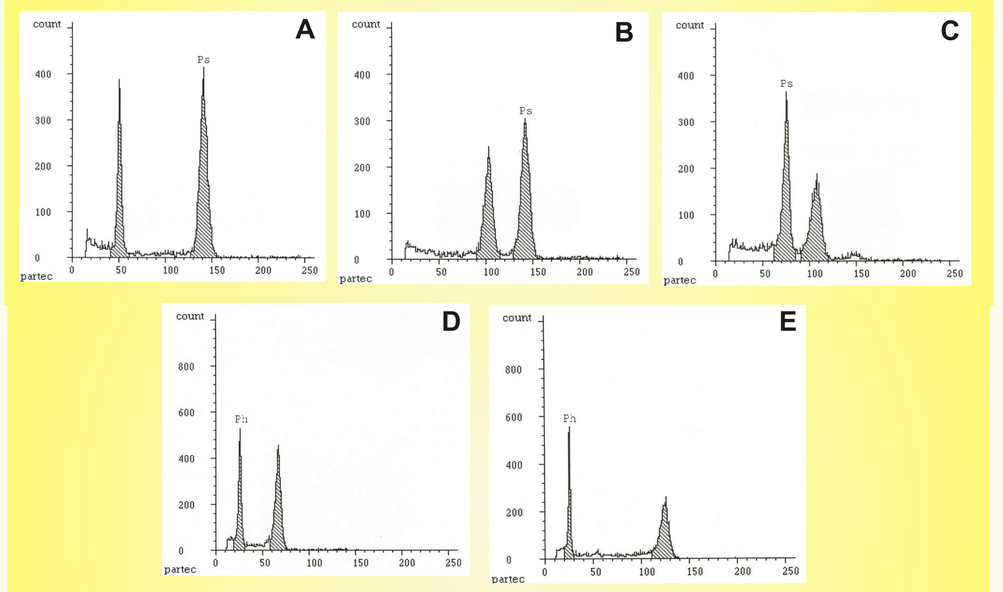


Fig. 5. Nuclear DNA content of 2C **(A)**, 4C **(B)** and 6C **(C)** *G. kurroo* regenerants and 2C **(D)** and 4C **(E)** regenerants of *G. tibetica*. Ps - *Pisum sativum* SET; Ph - *Petunia hybrida* cv PxPc6

Among 20 regenerants of *G. kurroo* analysed by flow cytometry only 10% were diploids with on average 3.42 pg DNA, whereas 85% were tetraploids with 6.58 pg DNA and 5% - hexaploids (8.75 pg DNA). In case of *G. tibetica* 86.8% of 53 tested regenerants were diploids (7.02 pg DNA) and 13.2% - tetraploids (14.01 pg DNA) **(Figs 4, 5)**. Total nuclear DNA content and chromosome number of control plants and regenerants are presented in **Tab. 3**. Unfortunately, it was impossible to count chromosomes of *G. tibetica* 4C regenerants accurately due to their small size and great number **(Fig. 6).**

Plants of various ploidy level differed morphologically. Tetraploids of *G. kurroo* had wider leaves than diploids, whereas hexaploid displayed dwarf stature and thick, wrinkled leaves **(Figs 7A-C)**. Tetraploid plants of *G. tibetica* had also thick, dark-green and wrinkled leaves **(Figs 7D-E)**, as well as shorter, thicker roots than diploids (data not shown).

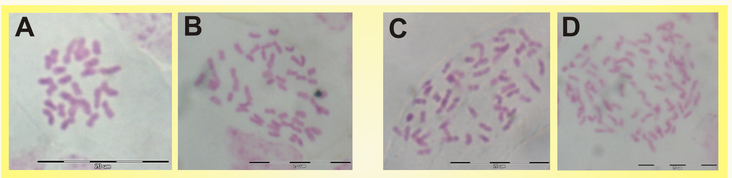


Fig. 6. Chromosomes of 2C **(A)** and 4C **(B)** *G. kurroo* regenerants and of 2C **(C)** and 4C **(D)** regenerants of *G. tibetica* (see **Tab. 3**)

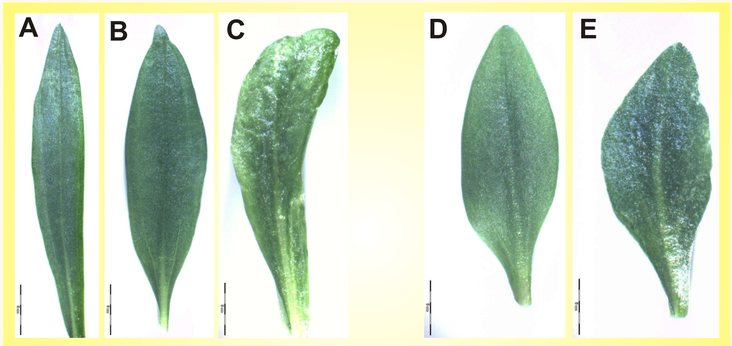


Fig. 7. Leaf morphology of 2C **(A)**, 4C **(B)** and 6C **(C)** *G. kurroo* regenerants and 2C **(D)** and 4C **(E)** regenerants of *G. tibetica*

Conclusions

1. Application of media: PCM2 ➡ CPM1➡ PRM2/PRM3 and PCM2 ➡ CPM3 ➡ PRM1+PRM3 provided the higher regeneration frequency for *G. kurroo* and *G. tibetica*, respectively.
2. Results of FCA and chromosome counting suggest that *G. kurroo* have a higher tendency to polyploidyzation during protoplast culture than *G. tibetica*.
3. Differences in nuclear DNA content of obtained plants could be caused either by spontaneous fusions between freshly isolated protoplasts or by disorders in first cell divisons.

Refrences

Takahata Y., Jomori H., Miyano S., Kunitake H., Mii M., 1995: Regeneration of plants from protoplasts of *Gentiana* species (Gentian). In: Bajaj Y.P.S. (ed) Biotechnology in Agriculture And Forestry Vol. 34. Plant Protoplasts and Genetic Engineering VI. Berlin, Heidelberg, New York, Springer-Verlag, 55-62