Transformation of *Gentiana* spp. cell suspension culture protoplasts by electroporation

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Introduction

Species from *Gentiana* genus, due to the attractive flowers, are priced ornamental plants. In pharmaceutical industry it's a material for pharmacological active compounds isolation (Skrzypczak *et al.* 1993). Esthetic and medicinal value could be increased by genetic transformation. There is not many papers touch this problem. Up to now gentians genome was modified using *Agrobacterium* (Momčilovič *et al.* 2001) or particle bombardment (Hosokawa *et al.* 2000). The aim of our work is transformation of isolated suspension culture protoplasts by electroporation.

Material and methods

Protoplasts were isolated from embryogenic suspension of *Gentiana kurroo* Royle. Cell suspension was digested during 12h with 1.5 % Cellulase RS, 1.5 % Macerozyme R10, 0.5 % Driselase, 0.25 % Hemicellulase, 0.04 % Pectolyase Y23, 0.976 % MES and 9 % mannitol diluted in CPW solution; pH 5.8. After digestion protoplasts were washed in CPW with 9 % mannitol. Protoplasts were re-suspended in electroporation buffer (EB) at density 4×10^5 protoplasts · mL^{-1} and 10-50 µg · mL^{-1} plasmid DNA was added. After incubation on ice protoplasts were exposed to the action of electric pulse. Electroporation was performed using ECM 2001 (BTX) Pulse Generator and application of Cuvette Safety Stand. Two kind of plasmid were used : p7i-UG + PAT + GUS and pCB 3001 + NPT II + GUS + GFP. We tested two electroporation buffers compositions : EB1 and EB2 (Table 1). Pulse length, number of pulses and electric field strength were investigated. After electroporation protoplasts have been incubated at various temperature conditions : at room temperature or on ice. After that electroporated protoplasts were washed in MS medium with 9 % mannitol and after 24h embedded in agarose medium at density 1×10^5 protoplasts · mL^{-1}. Cultures were maintained according to modified method of Fiuk *et al.* (2003). The viability was examined using 0.01 % FDA. Transformation efficiency was determined on the ground of GUS or GFP activity.

Table 1. The composition of electroporation buffers

EB1	EB2
pH 5.8 9 % mannitol 0.1 % MES 5 mM Mg Cl_2 70 mM KCl	pH 5.6 9 % mannitol 0.5 % MES 4 mM Mg Cl_2

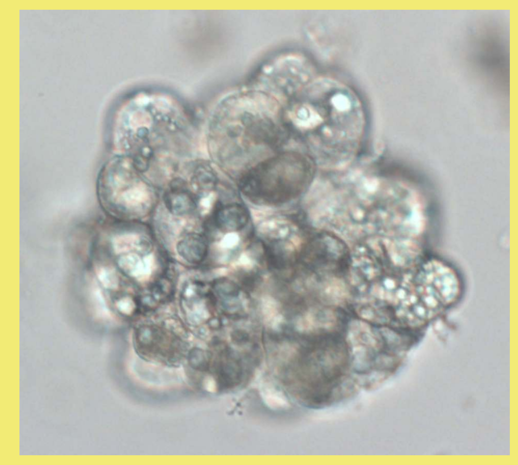

Figure 5. Cell suspension culture of *Gentiana kurroo*

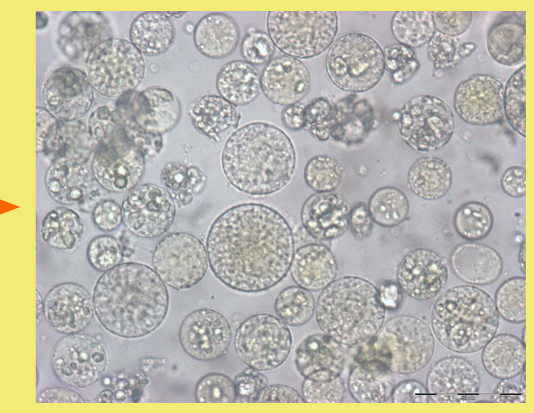

Figure 6. Protoplasts after isolation

Figure 7. First division

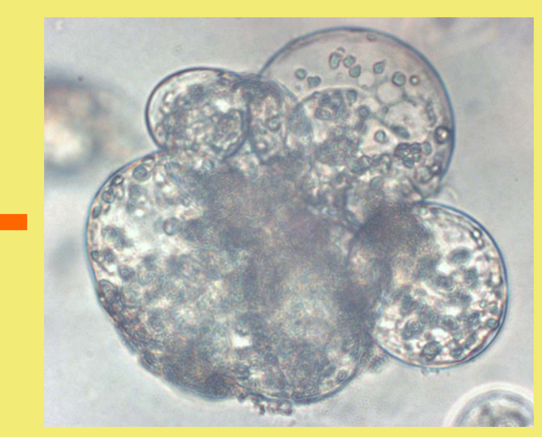

Figure 8. Several cells' protocallus

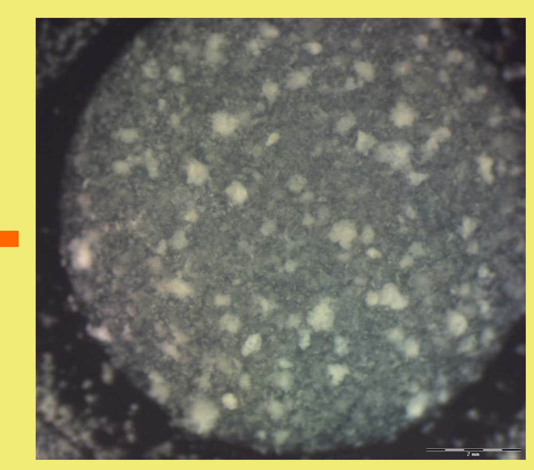

Figure 9. Protocalli in agarose culture

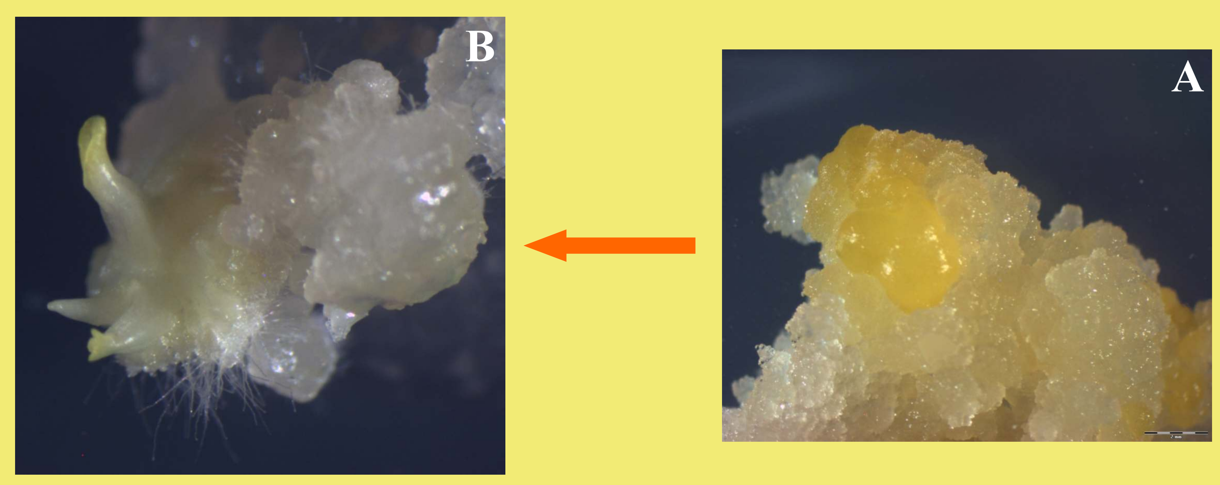

Figure 10. Callus (A) and somatic embyos (B) derived from electroporated protoplasts

Table 2. The viability of protoplast in various electroporation buffers

EB 1	EB 2
80 %	59 %

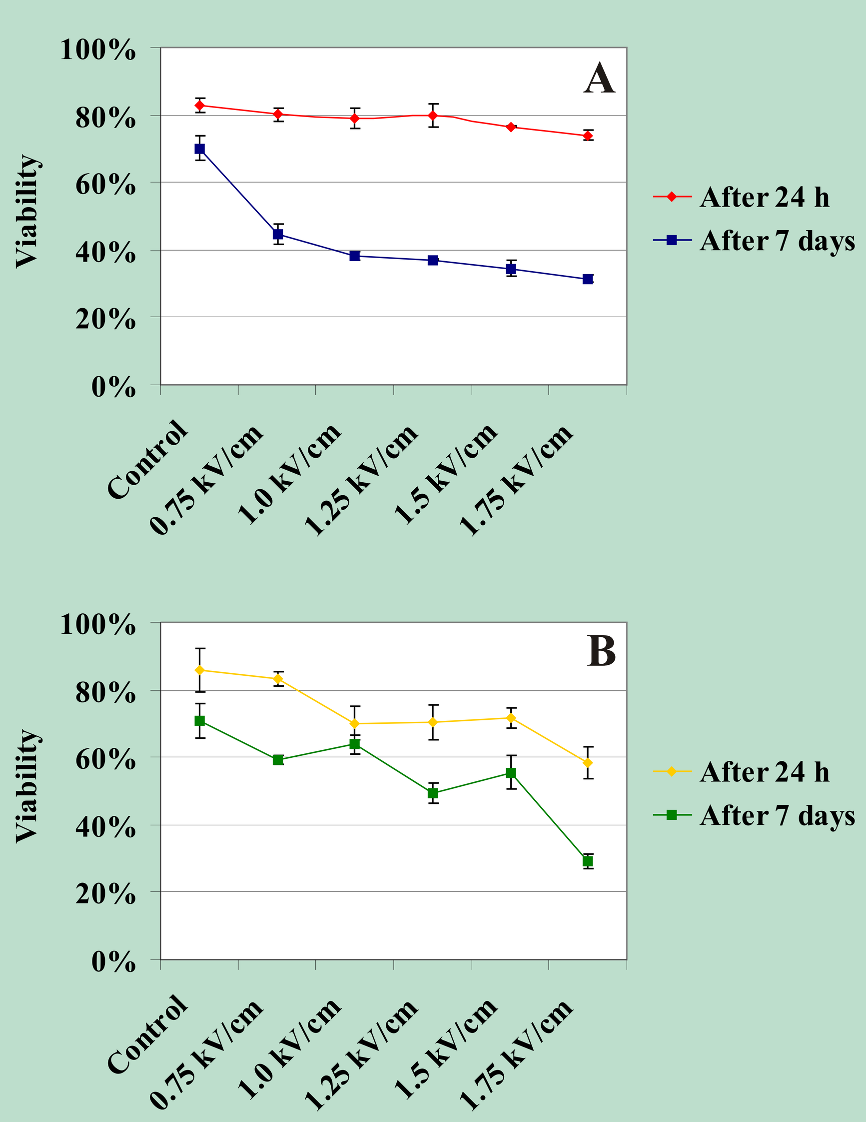

Figure 1. Influence of electric field stregth on protoplast viability after incubation at room temperature (A) or on ice (B)

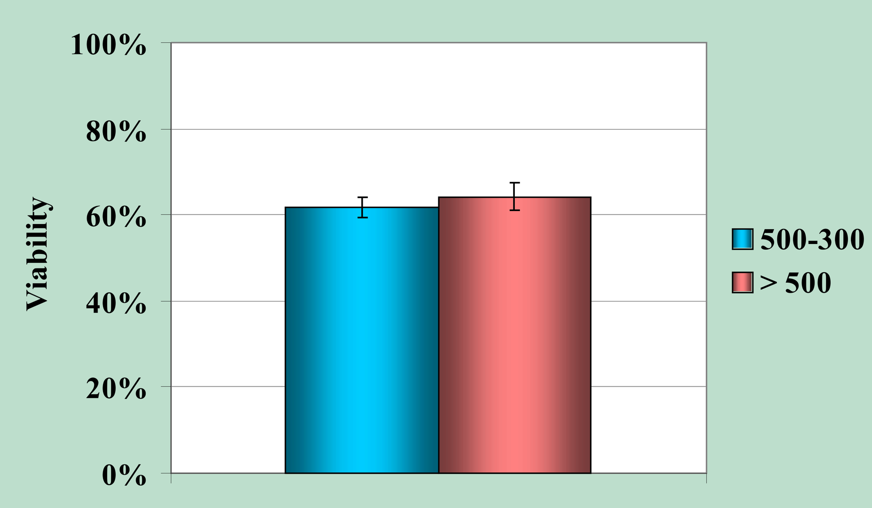

Figure 3. Viability of protoplasts isolated from various cell suspension fractions

Results

The viability of protoplasts re-suspended in EB1 was 20 % higher than in EB2 (Table 2).
The decrease of protoplasts viability one week after electroporation was observed in comparison with results obtained after 24h (by about a half) (Figure 1A).
When protoplasts were incubated at room temperature the increase of electric field strength caused the decrease of the viability (Figure 1A).
Incubation on ice resulted in an enhance in electroporated protoplast viability after 7 days of culture (Figure 1B).
Duplication of pulses number had a negative effect on the viability (Figure 2A).
Pulses 1 and 5 ms caused the decrease of viability after 24 h; one week later electroporated protoplasts died. The viability of protoplasts treated with shorter pulses assessed length run from 6 to 70 % (Figure 2B).
The response of protoplast derived from various cell suspension fractions were not found (Figure 3).
The frequency of protoplasts cell divisions decreased with the electric field strength increasing. Using two highest values the results amounted below 20 % (Figure 4A).
Elongation of pulse length (from 20 to 30 µs) resulted in wide decrease in number of protoplasts cell divisions (by about 37 %) (Figure 4B).
GUS and GFP activity in electroporated protoplasts was not detected. However the regenerants have been growing on 60 mg · mL^{-1} of kanamycin.
Callus and somatic embryo formation was observed.

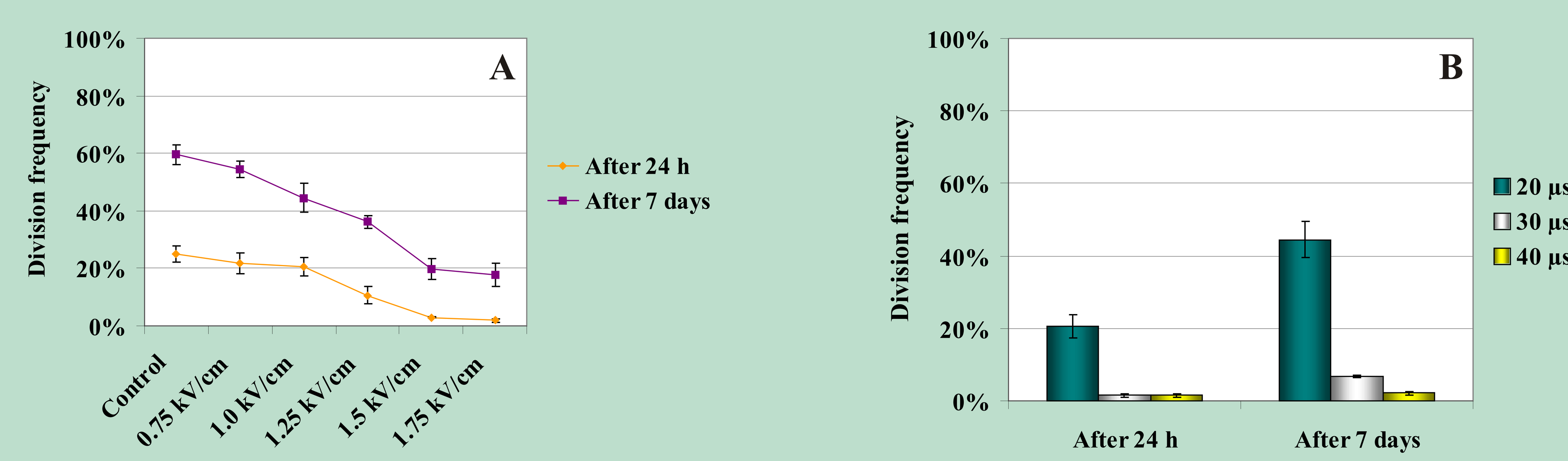

Figure 4. Influence of electric field stregth (A) and pulse length (B) on protoplast viability

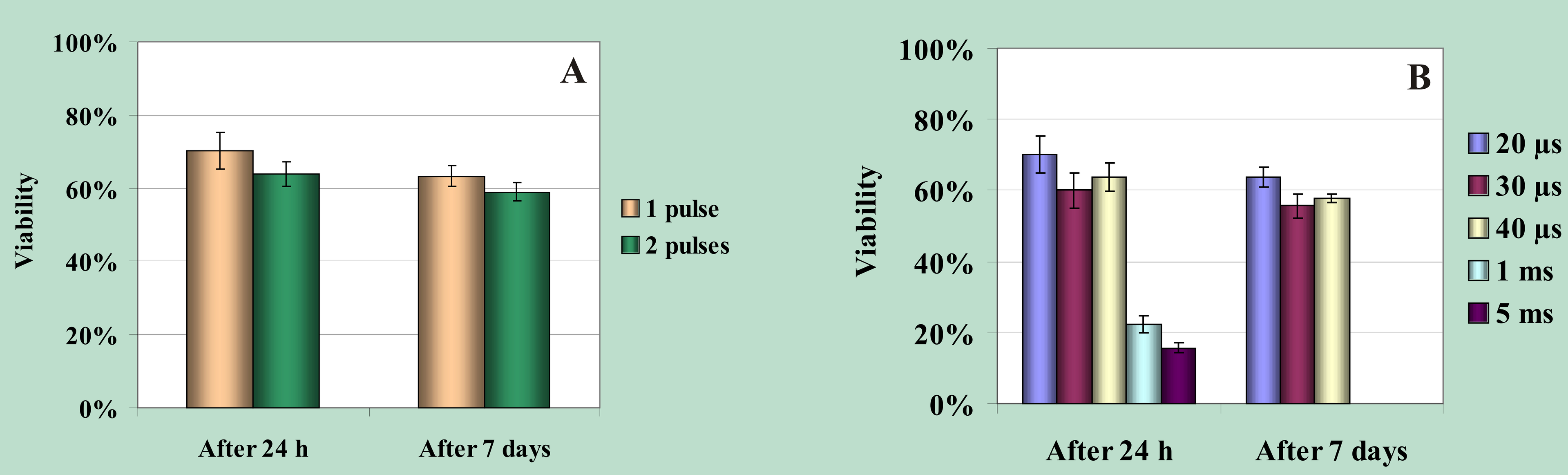

Figure 2. Dependence of a protoplast viability upon the number of pulses (A) and the pulse length (B)

Conclusions

1) EB1 was better than EB2 for electroporation of *Gentiana kurroo* protoplasts.
2) The protoplast viability decreased with electric field strength and pulse length increase.
3) The incubation of protoplasts on ice augmented their viability.
4) Size of cell suspension aggregates have had a little effect on electroporated protoplasts viability.
5) The increase of electric field strength and elongation of pulse length widely decrease of the cell division frequency of protoplasts.
6) GUS and GFP activity was not detected. However the regenerants have been growing on 60 mg · mL^{-1} of kanamycin.

This work was financed by MS&I; Grant 3 PO4C 037 23.