Protoplasts fusion by polyethylene glycol within *Gentiana* spp.

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INTRODUCTION

Somatic hybridization through protoplast fusion is, apart from transformation, one of the basic methods of increasing genetic variability of plants offered to breeders by biotechnology. Two basic fusion methods involve: high-molecular weight polyethylene glycol (PEG) and additional solutions, often with high pH and/or high Ca^{2+} concentration in chemical method and direct and alternating current in electrofusion. So far, for *Gentiana* genus, important for pharmacy and ornamental plants industry, mainly protoplast electrofusion has been used, which provided respectively 2.1-4.1% (Takahata et.al. 1995) and 4.8-6.7% heterokaryons (Fiuk et.al. 2006).

AIM

The aim of this study was to elaborate the conditions of chemical fusion making it possible to obtain heterokaryons for the selected species from *Gentiana* genus.

MATERIAL AND METHODS

Two kinds of protoplasts, white and green, were fused. The source of the white ones was embryogenic cell suspension of *G. kurroo* or *G. cruciata*. Green, mesophyll protoplasts were isolated from the second but still young leaves of *G. cruciata*, *G. tibetica* and *G. kurroo* cultured in axenic conditions. After enzymatic isolation and three-time washing in CPW9 solution (with 9% mannitol) density of both kinds of protoplasts was worked upon to reach $2x10^5$ protoplasts per 1 ml. Next, protoplasts were mixed at 2:1 ratio (white to green) in CPW9 with pH=6.0.

Tab.1 Composition of solutions for fusion of protoplasts at interface (Kao 1988, modified*)

Compound	PEG	Hypotonic high pH
Glucose (g)	-	5.4
$CaCl_2$ $2H_2O$ (mg)	150	294
KH_2PO_4 (mg)	10	-
PEG4000 (g)	30	-
Sucrose (g)	17	-
CAPS (mg)	-	66
H_2O (ml)	100	100
pH	6	10

*For placing protoplasts mixture onto PEG CPW with 9% mannitol was used. PEG and high pH solution were then diluted with MS medium.

The modified method of fusion at the interface according to Kao (1986) was used. 150µl of protoplasts mixture was put onto a 150µl drop of PEG4000 solution (Tab.1) and then two 350µl aliquots of a hypotonic high pH solution (pH=10) were added; the first one after a fixed (studied) time and the second one after 5 min. Finally, 4 ml of modified MS (Murashige and Skoog, 1962) medium was added. After 20 hours the entire mixture was moved into the tubes, centrifuged and rinsed with fresh portion of medium. Next, samples were taken to determine the percentage of heterokaryons and viability (FDA staining). To establish optimal conditions of fusion the time was examined at which protoplasts stayed in PEG solution up to the time when a high pH solution was added, i.e. 5,10 and 15 min.

Microscopic observations were carried out using Vanox-Olympus microscope and computer system of image analysis (analySIS ver. 3.1). Heterokaryons were identified in white light (presence of chloroplasts from mesophyll protoplasts and more distinct bands of cytoplasm as well as amyloplasts from suspension protoplasts.

RESULTS

Preliminary studies conclude that increasing the ratio of suspension protoplasts to mesophyll protoplasts from 1:1 to 2:1 reduced the undesirable process of mesophyll protoplasts aggregation. Moreover, it was found out that using CPW solution with 9% mannitol for putting protoplast mixture onto a drop of PEG, instead of solution with 9% glucose, recommended in the original procedure, decreased protoplast deformation at the very outset of sedimentation.

Fusion efficiency for the combination with *G. kurroo* as a suspension component tended to increase when the time in PEG was longer, and for the combination with *G. cruciata* it was the other way round, however the differences were not significant (Tab.2). Similarly, the frequency of heterokaryon incidence was approximate for the pairs of species under examination and ranged between 1.71 to 3.65%. Using electrofusion for hybridization of the same components enabled obtaining 6.72% of heterokaryons.

Tab.2 The effect of sedimentation time in PEG before adding high pH solution on fusion efficiency [%] for respective pairs of species.

Fusion components-mesophyll+suspension	Sedimentation time in PEG [min.]		
	5	10	15
G.cruciata+G kurroo	2.01 ±0.5	2.62 ±1.4	3.65 ±1.6
G.tibetica+G.kurroo	1.71 ±0.5	2.14 ±1.3	2.42 ±1.2
G.kurroo+G.cruciata	2.58 ±0.8	2.14 ±0.6	2.18 ±0.8

The viability of protoplasts and fusion products for the combination of suspension protoplasts *G.kurroo* with mesophyll *G.tibetica* or *G.cruciata* was quite high (64.9- 73.4%) and was similar for different times in PEG (Tab.3). For the combination of suspension protoplasts *G.cruciata* and mesophyll *G. kurroo* it was considerably lower and approximated that obtained earlier in our electrofusion experiments.

Tab.3 The effect of sedimentation time in PEG before adding high pH solution on viability of protoplasts and fusion products [%] for respective pairs of species.

Fusion components-mesophyll+suspension	Sedimentation time in PEG [min.]		
	5	10	15
G.cruciata+G kurroo	71.29 ±7.1	68.02 ±3.5	64.97 ±2.2
G.tibetica+ G.kurroo	72.81 ±7.8	73.39 ±7.7	69.90 ±6.1
G. kurroo+ G.cruciata	45.31 ±12.1	47.1 ±15.8	49.80 ±7.7

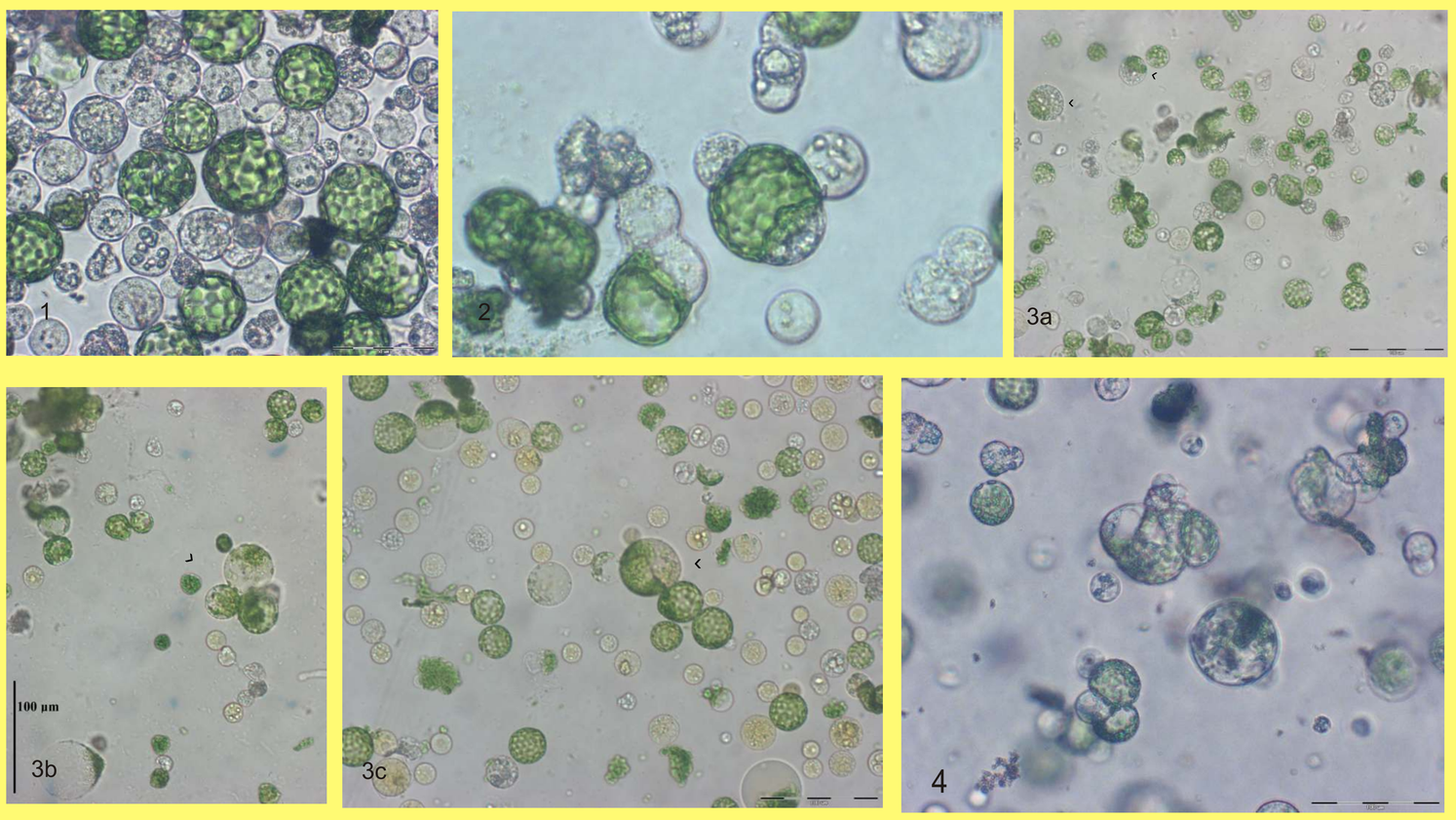

Fig.1-3. Consecutive steps of protoplasts fusion using PEG.**(1)** Mixture of green mesophyll (*G.cruciata*) and cell suspension protoplasts (*G.kurroo*) before placing onto PEG drop. **(2)** Aggregation and fusion of protoplasts in PEG. **(3)** Different looking heterokaryons 20-24 hrs after fusion. **(3a)** Dense cytoplasm and amyloplasts from suspension (*G.kurroo*) and chloroplasts from mesophyll (*G.cruciata*) protoplasts. **(3b)** Distribution of amyloplasts (*G.kurroo*) and chloroplasts (*G.cruciata*) in cytoplasm. **(3c)** Yellowish cytoplasm from suspension protoplast after FDA staining (*G.tibetica* + *G.kurroo*). **Fig.4**. First divisions of putative heterokaryon 7 days after fusion (*G.cruciata*+*G.kurroo*).

CONCLUSIONS

On the basis of the study it seems that:

1. In the range examined sedimentation time of protoplasts in PEG had a small impact on fusion efficiency and viability, however, further research is called for involving e.g. the division ability of fusion products.
2. The combination with protoplasts of *G. cruciata* as suspension component is more sensitive to fusion conditions than the one with *G. kurroo*. However, this can be conducive to heterokaryon selection.
3. The observed fusion efficiencies are slightly lower than those for the same species in the electrofusion examined earlier, however, of significance can be the much-needed improvement of heterokaryon identification method.

References

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