TTC test functionality in evaluation of surviving of *Gentiana* PEM after cryopreservation

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Introduction

The cryopreservation method developed numerous tests helping to describe the survival of low temperature treated plant material. The best test concerns the ability of plant material undergo cell division after freezing when cultures are implanted on agar regeneration medium. There are biochemical tests based on colour reaction of the formazan obtained from the TTC and fluorescence of FDA for estimation of freezing injury. The aim of the paper is the evaluation of usefulness of TTC test for the evaluation of the gentian cell suspension survival after freezing.

Material and Methods

Experiments were carried out on the cell suspension of *Gentiana tibetica* King and *G.kurroo* Royle (Mikuła et al. 2002). The cell suspensions cultured on standard medium (3; 6; 9% sucrose cont.) were treated with different cryoprotectant solutions before freezing: a) control without treatment, b) with 1M sucrose, c) with sorbitol + DMSO, d) with sorbitol + proline, e) vitrification solution. For TTC tests 50 mg of tissue was sampled and treated with 0,5 ml 0,8% TTC solution (in phosphate buffer pH 7.5) for 20 hrs at 25ºC. Formazan (red colour) were extracted with 5 ml of 95% ethanol at 85ºC by 50 min. Absorbance was estimated pectrophotometrically at 485 nm. Presented results show samples viability, expressed as ratio of absorbances after freezing to control x 100%. Each value represents average of 9 repeats of analysis.
Tissue preparation for PEM analysis was carried out as earlier described (Mikuła *et al.* 2002).

Fig. 1. PEM expresses red colour of formazan after TTC test

Conclusions

1. **TTC test might be a good indicator of culture viability**
2. **For reliable results, TTC test should be used at least 24 after thawing**

RESULTS

Without cryoprtectants

The viability of cell suspensions after freezing was strongly correlated with the time of TTC testing and the level of cryoprotection efficiency. The highest values of TTC test were always observed immediatelly after sample rewarming. In those cells the dehydrogenases were active for direct freezing for 1h for all sucrose concentration pretreatments and only for 5 hrs for 9% sucrose. The programmed freezing extanded time of TTC test expression up to 24 hrs for 9% sucrose pretreatment. Both, direct freezing of tissue in LN_2 (Tab. 1A) and programmed freezing (Tab. 1B) without cryoprotectant treatment resulted in a large destruction of organelles of the cells which was proved by the TEM analysis (Fig. 1A, B).

Tab. 1. TTC reduction (% control) by PEM cells after treatments: A) immersion in LN2 B) programmed freezing (20ºC to –40ºC at 1ºC/min; –40ºC to –150ºC at 10ºC/min)

	Sucrose	Time course of formazan production after thawing (hrs)				
		0	1	5	24	48
A	3%	6.46 ±0.6	0.24 ±0.36	0	0	0
	6%	60.38 ±2.3	12.35 ±1.11	0	0	0
	9%	60.38 ±2.3	28.97 ±1.97	6.01 ±0.81	0	0
B	3%	25.12 ±3.12	0.82 ±0.12	0	0	0
	6%	62.56 ±4.87	31.35 ±3.14	2.04 ±0.48	0	0
	9%	72.76 ±1.52	59.92 ±3.71	27.53 ±4.42	14.18 ±1.02	0

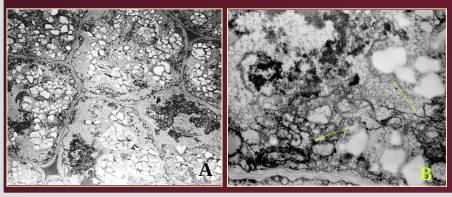

Fig. 2A. General view of destruction of cell protoplast of PEM after freezing
Fig. 2B. Total disorder of membranes

All cells did not survived – TTC test showed activity of dehydrogenase up 24 hrs

With cryoprotectants

1M sucrose treatment presented high level of cryoprotection efficiency at the presence of 6 and 9% sucrose in standard medium directly after freezing, however it decreased according to the passing time from the rewarming - up to 24 hours (Tab. 2).

Tab. 2. TTC reduction (% control) by PEM cells after 1M sucrose treatment

Sucrose	48 hrs after deplasmolysis	Time course of formazan production after thawing (hrs)				
		0	3	5	24	48
3%	80.37 ±2.45	37.59 ±0.61	1.42 ±1.16	0.35 ±0.16	0	0
6%	82.15 ±3.01	77.79 ±2.06	43.37 ±1.01	20.32 ±0.53	6.8 ±0.48	0
9%	82.99 ±2.68	92.00 ±0.89	49.97 ±2.23	25.07 ±0.19	19.82 ±0.88	0

programmed freezing: from 20ºC to –40ºC at 1ºC/min; –40ºC to –150ºC at 10ºC/min

Two-day long sorbitol pretreatment with DMSO or proline resulted in the restoration of the whole culture from the cells which were able to survive the freezing procedure. The dehydrogenase activity in the cells protected by DMSO decreased directly after thawing to 50% of the control and 22% after 24 hrs. Finally it droped down to about 3% (Tab. 3). Ultrastructural changes observed after freezing/rewarming procedure, for example: protoplast plasmolysis and dilatations of membranes (Fig. 3A, B) seem reversible. Some of cells were able to reconstruct their ultrastructure and biological activity. Observed after 48 hrs TTC test results expressed the biological activity of these cells (Tab. 3).

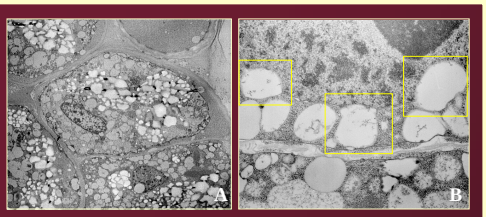

Fig. 3 A. Cells with little bit shrinkted protoplast and 3 B. dilatation of nuclear envelope

Few cells survived - TTC test showed activity only viable cells after 48 hrs

Tab. 3. TTC reduction (% control) by PEM cells after preculture with 0.4M sorbitol and freezed with 0.08M DMSO or 0.25M proline

Cryoprotectant	Time after thawing (hours)		
	0	24	48
***DMSO**	50.43 ±4.21	22.47 ±3.02	2.90 ±0.56
****proline**	12.49 ±2.94	11.76 ±0.54	0.86 ±0.14

programmed freezing: from **20ºC or *0ºC to –40ºC at 1ºC/min; –40ºC to –150ºC at 10ºC/min

Vitrification

Vitrification solution PVS2 protected PEM cells against freezing injury. TTC test confirmed high viability of aggregates directly after thawing and 24 hours (Tab. 4). After 48 hrs freezed samples reached level of control. High production of formazan after 48 hrs may suggest high metabolic activity of studied cell suspension.
Fig 4 A & B presents ultrastructure of cells which succesfully survived LN2 treatment. 5 hrs after thawing cells of PEMs started to divide (Fig. 4 C - arrow). Cell ultrastructure was likely to control after 48 hrs of culture (Fig. 4 D).

Tab. 4. TTC reduction (% control) by PEM cells* after vitrification solution treatment

Cell suspension	Time after thawing (hours)		
	0	24	48
G.tibetica	91.93 ±1.8	83.86 ±2.08	85.68 ±2.35
G.kurroo/H	85.01 ±4.98	77.25 ±3.16	100.94 ±1.3
G.kurroo/L	92.87 ±1.8	87.32 ±3.87	101.86 ±1.48

* immersion in LN2

Fig. 4. Ultrastructural and cytological evidences of cell viability after vitrification. A & B - directly after thawing; C– after 5 hrs; D & E – after 48 hrs in agar culture

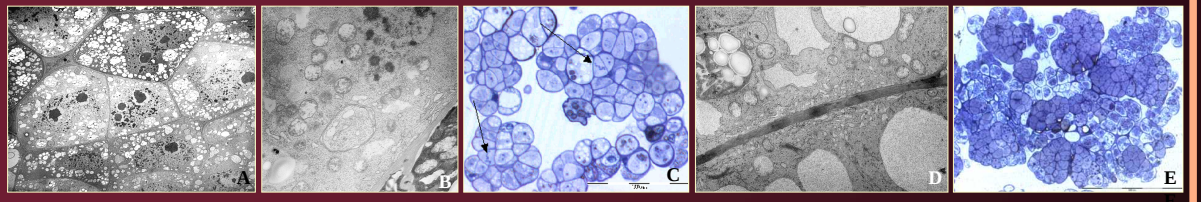

Fig. 5. Consecutive stages of development of vitrificated PEMs A – directly after thawing; B – 5 weeks later and C – 8-week-old culture with newly formed somatic embryos (SE)

High performance in TTC test reflects viability of vitrificated aggregates (Fig. 5)

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