Cryopreservation of Plant Cell Cultures

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1. Introduction

Plant cell cultures are widely used for studies of growth, metabolism, and differentiation. They are an important tool in the biotechnological production of precious secondary metabolites. Safe and maintenance-free deposition is mandatory because the special properties of any cell line may be lost by somaclonal variation, technical or human failure, infections, etc. Further, their maintenance by subculture is very costly. Finally, patenting requires deposition in a culture collection.

We worked out some relatively simple and cheap procedures for long term preservation in liquid nitrogen. These are based both on two-step freezing (in which the specimen is progressively dehydrated when ice crystals grow in the medium during slow cooling) and on vitrification (absence of ice crystals due to rapid freezing in highly concentrated vitrifying solutions). For recalcitrant strains alternative storage methods have been studied.

In order to monitor (genetic) drift, easy an rapid screening methods were developed. These include measurement of growth (non invasive), metabolism (HPLC, NMR), and DNA-content (flow cytometry).

In a more fundamental approach, we study the molecular changes within cells that accompany the acquisition of cryotolerance of a model system (tobacco cells).

2. Standard protocol with controlled rate freezing

The basic protocol (see also Withers and King (1)), is described in Tab. I and makes use of a programmable freezer (BV-6, Cryoson, Middenbeemster,

TABLE I
TWO-STEP FREEZING (CONTROLLED RATE)

Cell material:	In rapid growth phase
Preculture:	0.33 M mannitol for 3 days
Cryoprotection:	1 M sucrose + 0.5 M DMSO + 0.5 M glycerol (SDG) for 1 hour at 0°C,
Cooling	1°C. min^{-1} to -35°C, 30 min at -35°C, Immerse vial into LN[a]
Storage	In storage dewar; in or over LN[a]
Thawing:	Rapidly, in a water bath at 40°C
Recovery:	Pour on 2 filter paper discs on top of medium with 0.75% agarose and 0.5% activated charcoal. After 3 d, transfer upper disk to fresh plate

[a] liquid nitrogen

the Netherlands). For details, we refer to (2). It was applied to over 70 different cell suspension cultures, differing widely in growth rate, cell size and morphology, cluster size, etc. From Tab. II, A and B it appears that, usually, this protocol is successful, but some species and cell lines are recalcitrant. Both on a species and on a cell line basis the success ratio appears to be around 70%. All cultures receive some damage during the procedure, as appeared from (slightly) retarded regrowth after cryopreservation (compare the last two columns of Tab. II A). However, after a few subcultures in liquid medium, all appeared to have fully recovered and to have retained relevant properties. We usually tested growth rate, cell size and morphology, ploidy level, plus the special characteristics of each cell line. Thus, we found no significant changes in rice in its regeneration ability, in *Catharanthus* lines in ajmalicine and serpentine productivity, and in some *N. tabacum* lines in their degree of G2 arrest in the stationary phase. Most of the 'recalcitrant' lines (Tab. II B) were poorly growing ones, but some were well within the range of the tolerant ones.

TABLE II
RESULTS OF THE STANDARD TWO-STEP CONTROLLED RATE FREEZING PROCEDURE

A. Successful		Regrowth[a]	
	number of lines	untreated controls	after cryostorage
DICOTS			
Catharanthus roseus	7	++(+)	+(+)
ibid., transformed	1	++	+++
Tabernaemontana divaricata	2	+++	±
Tabernaemontana oriëntalis	1	+(+)	+
Nicotiana tabacum cv White burley	4	+++	++(+)
ibid., transformed	2	+++	++
cv Bright Yellow 2	2	+++	+++
ibid., transformed	5	++	++
Daucus carota	3	+++	+++
Petunia hybrida	2	+++	++
MONOCOTS			
Hordeum vulgare	4	+++	++
ibid., transformed	2	+++	++
Oryza sativa cv japonica	10	++(+)	++(+)
ibid., transformed	3	++	+(+)
cv indica	5	++(+)	++(+)

[a] Regrowth was on 60 mm agarose plates and was judged by macroscopic observation +++ = 2 to 4 d; ++ = 4 to 10 d; + = 11 to 17 d; ± over 17 d

B. Not successful	number of lines
Catharanthus roseus	2
Nicotiana tabacum cv Samsun	2
cv White B, transf.	3
Oryza sativa cv *japonica*, transf.	2
cv *indica*	1
Tabernaemontana longiflora	1
Cinchona ledgeriana	2
Cinchona robusta	2
Quillaja spec	2

3. Modifications of the controlled rate freezing protocol

In order to reduce the number of recalcitrant lines (Tab. II B), many variations have been tried with respect to preculture, composition of cryoprotectant cocktail, way of application of cryoprotectants, cooling rates, suppression of the freezing isotherm (see next chapter), inoculation density during recovery, composition of recovery media, renewal of recovery medium, etc. We could recover one of the *Catharanthus* lines from two-step freezing by application of an elaborate preculture. Further, none of the modifications changed any recalcitrant line into a tolerant one. Almost all of the modifications have been tested on *Cinchona robusta*, but, apart from one single plate with a fully recovering cell cluster, all results were negative. Some modifications resulted in a higher recovery than the standard procedure, with one line but not with another. Sometimes equal but usually better recovery was obtained for several species by raising inoculation density on the recovery plate. This was achieved either by gentle centrifugation after thawing, or by putting the post-thaw suspension within a steel ring with a diameter of 10 to 12 mm on top of the plate.

4. A simplified standard two-step protocol

For many cell lines, we found the exact cooling curve to be not critical for the over-all performance of the two-step freezing procedure. Moreover, during programmed freezing, the moment at which ice crystals appear varied widely, resulting in temperature jump of 2 to 12°C within the vials. Also this phenomenon appeared to have little or no effect, as was found for several mammalian cell types (3). Therefore, we tested the performance of the simple Nalgene Cryo 1°C Freezing Container 'Mr Frosty' (Nalge Cy, Sevenoaks, GB). It accommodates 18 vials and can be filled with ethanol (replace each time). When put at -80°C in a laboratory freezer for 1.5 to 2 hours the cooling rate is roughly 1°C. min^{-1} over the first part of the trajectory, and the end temperature comes close to -80°C. As a second modification we replaced the DMSO in the cryoprotectant cocktail with the less toxic propane-1,2-diol. Finally, centrifugation of the thawn cells (see previous chapter) has recently been included in our simplified standard protocol. For further details, see (2)).

Up to now, this protocol has been tested with some 20 different cell lines out of Tab. II, including some of the recalcitrant ones. We found no differences relative to controlled rate freezing, but, of course, some cell cultures may appear more demanding.

5. Vitrification

The vitrification approach differs mainly from the two-step method in the high concentration of dehydrating cryoprotectants and the rapid freezing. Thus ice crystals are avoided also in the medium, which then forms a glass (i.e., vitrifies). Both methods, of course, aim at vitrification of the cell contents. Our vitrification procedure comprises the same preculture phase as the two-step procedure. The protocol is summarized in Tab. III, and, for details, we refer to (4).

TABLE III
VITRIFICATION

Cell material:	In rapid growth phase.
Preculture:	0.33 M mannitol for 1 day. Cool 1 ml packed cells.
Cryoprotection:	Transfer cells gradually to; 3.25 M glycerol + 2.5 M ethane diol +1.9 M DMSO + 0.4 M sucrose
Cooling:	Fill 0,5 ml straws; immerse in LN
Storage:	In LN
Thawing:	In water bath 40°C. Empty into 1.2 M sucrose; leave 20 min
Recovery:	Centrifuge; discard supernatant; Plate on filter paper disks on top of medium with 0.75% agarose. After 2 days, transfer disk to fresh plate.

Out of the 'tolerant' lines of Tab. III A, four (*C. roseus*, *D. carota*, *H. vulgare*, *N. tabacum*) were subjected to the vitrification protocol. Survival was equal to or better than survival after two-step freezing. In addition, the *C. roseus* line, that survived a modified two-step freezing protocol (see before) also recovered somewhat better from vitrification. The second 'recalcitrant' *C. roseus* line from Tab. III B, that did not survive several two-step procedures, rapidly resumed growth after vitrification. However, the highly recalcitrant *C. robusta* also did not survive this treatment. So, the vitrification procedure appears significantly better than two-step freezing, but it is not (yet) the final solution for the cryopreservation of plant cell suspensions.

6. Alternative storage

We systematically studied the possibilities to store plant cell suspension cultures at low temperature as standing culture, with or without extra sugar

as medium additive. Of course, the performance of cryo-sensitive (recalcitrant) lines would be of interest. For each culture, 43 ml of rapidly growing culture was inoculated into membrane-sealed screw cap bottles (no. 658175, Greiner Gmbh, Frickenhausen, Germany), which were put at 10°C in the dark. For details, see (5).

Three cultures thus appeared to survive for many weeks totally maintenance-free (Tab. IV). For the highly cryo-recalcitrant *Cinchona* the gain was, however, very limited. The cryo-tolerant rice not only survived for 16 weeks, but also demonstrated quantitatively good regeneration into whole plants. However, many regenerants suffered from serious somaclonal variation. We consider this method to be of limited value.

TABLE IV
RESULTS OF STANDING CULTURE

		Regrowth after			
Species	Medium sugar	4 wk	8 wk	16 wk	30 wk
Oryza sativa	2% sucrose	+	+	+[a]	–
	6% sucrose	+	+	+[a]	–
Nicotiana tabacum	3% sucrose	+	+	+	–
	6% sucrose	+	+	+	+
Cinchona robusta	2% glucose	+	+	±	–
	6% glucose	+	+	–	–

[a] good regeneration, but 20% of plants albino

7. Rapid screening of the growth rate of plant cell cultures

The growth of plant cells in any standard shake flask can be monitored non-invasively by placing the flask on a simple fixed angle standard with a calibrated ruler. After the cells have settled (in minutes) the volume of the cells can be read relative to the culture volume. With time, a growth curve is obtained that accurately follows the fresh weight (6). Thus, any substantial change in growth characteristics can be detected objectively without much effort.

8. Rapid screening of the DNA content

The DNA-content of cells can be measured after chopping with a razor blade in the presence of a fluorescent DNA stain like DAPI. A simple sieve then suffices before flow-cytometry. Thousands of nuclei can thus be analyzed within minutes (Fig. 1). We use the relatively cheap and simple table top flow-cytometer CAII (Partec, Münster, Germany). For details, we refer to (7, p. 255).

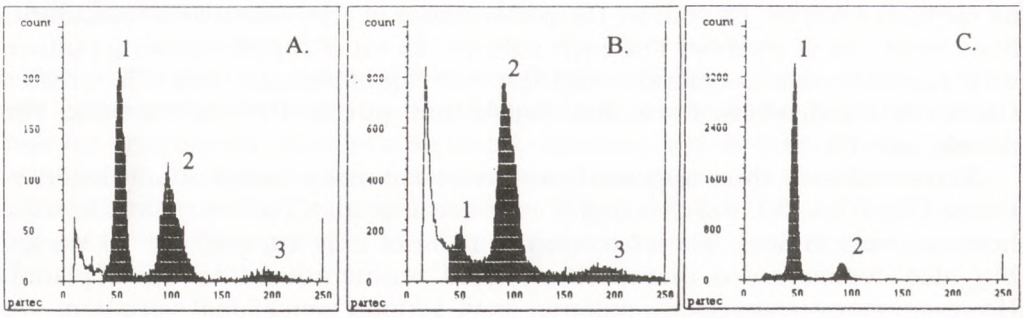

Fig. 1. Flow cytometric analysis of plant cell suspension cultures.

A. A stationary tobacco culture with almost 50% G2-arrested cells plus some 10% 'tetraploid' ones.

B. A rice culture (peak 2 and 3) chopped together with a small amount of diploid leaf material (peak 1). The cell suspension appears 'tetraploid'.

C. A stationary culture of a diploid rice line.

Plant cells tend to endoreduplicate, viz., they may skip mitosis and directly shift from the G2 phase of the cell cycle, via the G1, through the S-phase. As a result, the amount of DNA per chromosome (and, thus, per cell) is doubled, yielding tetraploid cells. Fig 1. A. shows a stationary tobacco culture revealing an unusually high proportion of G2-arrested cells (almost 50%) in combination with the onset of endoreduplication (6% of the cells is 8C, so, more than 10% is 'tetraploid'). Especially for cultures isolated from callus, chromosomal aberrations can be detected. Figs. 1. B an C show an aneuploid and a clearly diploid cell line of rice respectively. We regularly check our cultures, discard ones like in Fig. 1. A, and replace them (with cryopreserved ones).

9. Rapid screening of metabolism

Profiles of intracellular sugars and amino acids can be easily obtained by ^1H NMR after homogenization of lyophilized cells, centrifugation, lyophilization of the supernatant, dissolution in D_2O, and centrifugation (8). We use them in fundamental studies (see next chapter) and in order to monitor changes.

10. Mechanisms of induction of cryo-tolerance

Presently, we are analysing which factors in the preculture treatment are relevant for the induction of cryo-tolerance in a model system, and what intracellular changes occur concomitantly. We use tobacco suspension cells

that do not survive cryopreservation protocols unless they are pretreated with, e.g., 0.33 M mannitol for 1 or more days.

The minimal effective mannitol preculture appeared to be shorter for vitrification (1 d) than for two-step freezing (3 d) (9). During the induction period, the cells synthesise, a.o., proline. By differential RNA display (difference between induced and non-induced cells at the mRNA level), two clones have been isolated. Kinetics of induction of, on the one hand, proline, and, on the other hand, the transcripts correlate rather well with the induction of cryo-tolerance in the tobacco cells. Sequence analysis of the corresponding genes has been almost completed. Their putative role in the acquisition of cryotolerance is under investigation.

11. Conclusions

Most plant cell suspension cultures can be cryopreserved simply and cheap. The vitrification approach is better. Standing culture is of limited value. Cultures can be easily monitored for (somaclonal) instability by a simple growth test, by flow cytometric analysis of cell DNA content, and by ^{1}H NMR.

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Summary

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Key words:
cryopreservation, plant cells, suspension culture.

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