Cryo-Letters 15, 53-58(1994).
Published by Cryo-Letters, 7, Wootton Way, Cambridge CB3 9LX, U.K.

CRYOPRESERVATION OF CELL SUSPENSIONS AND EMBRYOGENIC CALLUSES OF *CITRUS* USING A SIMPLIFIED FREEZING PROCESS

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Summary: A simplified cryopreservation process was experimented with a cell suspension of willow leaf mandarin and embryogenic calluses of six varieties of *Citrus*. Its efficiency was comparable to that of the standard freezing protocol developed previously for these materials, which required the use of a programmable freezing apparatus. The cell suspension could be frozen without modifying the original pretreatment conditions (0.15 M sucrose + 5 % DMSO). Embryogenic calluses of five varieties out of the six experimented withstood cryopreservation with the simplified freezing process. Optimal results were obtained by increasing the DMSO concentration to 10 or 15 %.

Key-Words: *Citrus*; cryopreservation; simplified freezing process; cell suspensions; embryogenic calluses.

Introduction

Cryopreservation techniques have been presently developed for a large number of cell suspensions (1). However, most of the processes set up still remain far from being easily applicable. Indeed, freezing procedures generally require the utilization of sophisticated and costly freezing apparatus in order to allow precise monitoring of the freezing rate. Moreover, it is highly recommended to induce (manually or automatically) crystallization in the cryoprotective medium at a determined temperature, in order to reproducively control cell dehydration during prefreezing and thus increase survival (2). A limited amount of research only aimed at developing simplified freezing equipments or procedures: Withers and King (3) designed a low cost freezing apparatus which was successfully employed with several cell lines. Other researchers used domestic freezers for prefreezing samples (4, 5, 6).
A standard cryopreservation process was developed recently for cell suspensions of *Citrus deliciosa* Ten. and embryogenic calluses of several varieties of *Citrus* (7, 8). It required the use of a controlled freezing apparatus in order to achieve cooling of cells at 0.5^{-1}°C min^{-1} from 0 to -40°C. A simple freezing device consisting of a plastic box containing 250 ml of

isopropylic alcohol, developed by Nalge Company (USA) was experimented successfully for the cryopreservation of animal cell lines (Kover, personal communication).
In this paper, we investigated the possibility of using this simple device instead of a programmable freezer for cryopreserving cell suspensions of *Citrus deliciosa* Ten. and embryogenic calluses of four varieties of *Citrus*.

Material and Methods

Plant Material
The embryogenic calluses used in this study originated from nucellar embryos of willow leaf mandarin (*C. deliciosa* Ten.), Chios mandarin (*C. deliciosa* Ten.), Cleopatra mandarin (*C. reshni* Hort. ex Ten.) Shamouti orange (*C. sinensis* Osb.), Hamlin orange (*C. sinensis* (L.) Osb. var. Hamlin) and mexican lime (*C. aurentifolia* Swing.). They were produced at the INRA/CIRAD-FLHOR (Institut National pour la Recherche Agronomique/Centre International de Coopération en Recherche Agronomique pour le Développement - Département Fruits Légumes Horticulture) Research Center of San Giulano (Corsica) according to the method of Ollitrault *et al.* (9). The cell suspension originated from embryogenic calluses of *Citrus deliciosa* Ten. transferred in liquid medium.

Methods

In vitro culture
Calluses were cultured on Murashige and Tucker basal medium (10) supplemented with 0.5 $g.l^{-1}$ malt extract, 0.15 M sucrose and $2g.l^{-1}$ gelrite. They were placed in the dark at 27±1°C. Transfers were performed every 4-5 weeks. For cryopreservation experiments, calluses were sampled 3-4 weeks after the last transfer.
The cell suspension was cultured on rotary shakers (96 rpm) in the same liquid medium. Transfers were performed every 18 days. They were placed in a culture room at 25 ± 1°C, under a light intensity of 30.5 µmol. $m^{-2}.s^{-1}$, with a photoperiod of 12 hours light/12 hours dark. For cryopreservation experiments, cells were harvested at the beginning of their proliferation phase, i.e. 8-10 days after the last transfer.

Cryopreservation

- Cells suspensions
Cells were pretreated for 1 hour at 0°C with 0.15 M sucrose + 5 % dimethylsulfoxide (DMSO). They were then frozen either according to the standard protocol (CFP) or to the simplified process (SFP). CFP comprized freezing at $0.5°C.min^{-1}$ from 0°C to -40°C using a freezing apparatus (Minicool, L'air Liquide, France), followed by immersion in liquid nitrogen. Crystallization in the cryoprotective medium was induced at -8°C by briefly pinching the cryotubes with forceps previously cooled in liquid nitrogen. For freezing according to the SFP, cryotubes were placed on the rack of the freezing device. It was then placed in a deep-freezer thermostated at -80°C. A thermocouple inserted in a control cryotube containing cryoprotective medium only allowed to follow the temperature decrease. When the temperature of -40°C was reached (i.e. after ± 65 min.), cryotubes were either rewarmed immediately (prefreezing controls) or immersed in liquid nitrogen. Depending on the experiments, crystallization was or not induced at -8°C in the cryoprotective medium. After thawing in a 40°C water-bath, cells were plated on filter paper, transferred to fresh medium after 14 and 24 hours, then cultivated for 5 days on solid medium and transferred into liquid medium for proliferation recovery, according to the standard protocol (7).

- Calluses

The efficiency of the two freezing methods was also compared with calluses of four varieties of *Citrus*, Shamouti orange, Chios mandarin, willow leaf mandarin and mexican lime. Two additional varieties (Hamlin orange and Cleopatra mandarin) were frozen using SFP only, without induction of crystallization. In all these experiments, the cryoprotective medium included 0.15 M sucrose and 0 to 15% DMSO. After rapid thawing, calluses were placed on a filter paper disk, transferred onto fresh medium after 1 hour, 24 hrs and then cultivated on standard medium for growth recovery.

Viability and recovery assessment

Viability of cells was assessed immediately after thawing using staining with fluoresceine diacetate (FDA, 11). Survival rate of a sample was estimated by calculating the mean percentage of living cells measured on 20 cell aggregates chosen randomly on a plate observed with a microscope, according to the method of Dussert *et al.* (12). Survival rates were expressed in percentage of the value of untreated control. Growth recovery of cells frozen using the simplified process was followed by measuring the Sedimented Cell Volume (SCV) increase of the cell suspensions during 25 days. One cryotube containing 0.3 ml of SCV was used per condition. Growth recovery of calluses was estimated after one month using a scale reflecting its intensity (- to 5 +). One cryotube containing 300 mg of callus was used per condition. Controls consisted of pretreated, and prefrozen calluses.

Results

Survival of cells prefrozen to -40°C was slightly higher using CFP than SFP (Table 1). However, survival rate of cryopreserved cells was equivalent whatever protocol used. Crystallisation of the cryoprotective medium at -8°C during SFP had no effect on cell survival.

Table 1: Survival rate of prefrozen (-40°C) and cryopreserved (-196°C) willow leaf mandarin cell suspensions using various freezing procedures: controlled freezing with Minicool (CFP), simplified freezing process with induction of crystallization (SFP + ind.) or without induction (SFP - ind.).

	- 40°C	- 196°C
CFP	72	57
SFP + ind	56	49
SFP - ind.	54	54

During recovery in liquid medium after cryopreservation using SFP, cells could be classified in two groups which showed equivalent SCV increase (Fig. 1): untreated and pretreated cells on the one hand, prefrozen and cryopreserved cells on the other. The former group had a higher SCV increase than the latter one. However, proliferation recovery of cryopreserved cells was satisfactory since SCV showed a six-fold increase over 25 days of culture.

Controlled freezing allowed to obtain positive results with all four varieties experimented (Table 2). Increasing the DMSO concentration generally led to an improvement in the proliferation recovery intensity. Whereas proliferation of cryopreserved calluses of Chios mandarin, willow leaf mandarin and Mexican lime was scored 4-5+, recovery of Shamouti orange was lower (2+). After cryopreservation with SFP, recovery was obtained will all varieties except willow leaf mandarin. Increasing the DMSO concentration in the cryoprotective medium improved recovery after freezing. With 15% DMSO, proliferation

recovery of cryopreserved calluses was equivalent with Shamouti orange and Chios mandarin using either CFP or SFP. In the case of Mexican lime, recovery was noted 4+ after CFP and 3+ only after SFP.

Fig. 1: Evolution of settled cell volume (SCV) of a cell suspension of willow leaf mandarin cryopreserved using simplified freezing process. Ctrl: control cells; Pt: pretreated cells; -40: prefrozen cells; LN: cryopreserved cells.

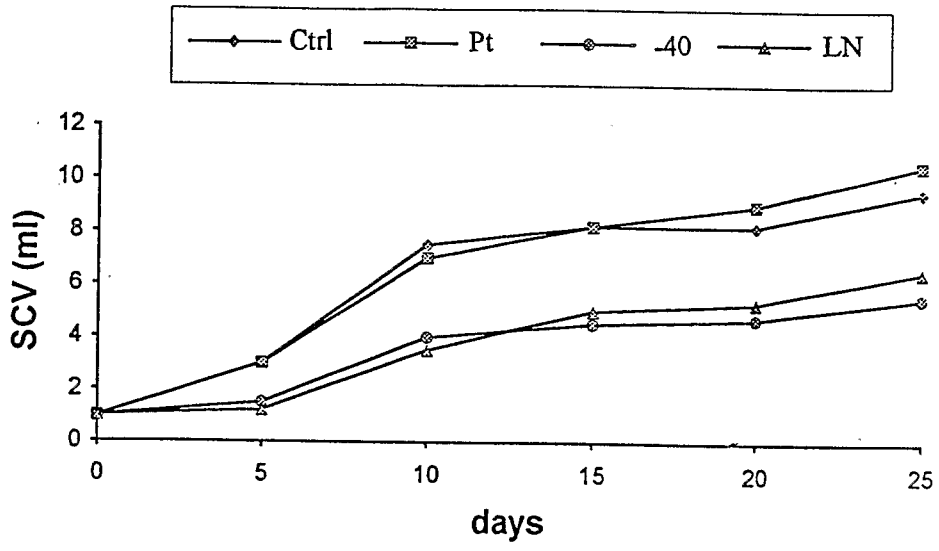

Table 2: Effect of DMSO concentration employed during pretreatment and freezing procedure on the recovery intensity of pretreated (Pt), prefrozen (-40°C) and cryopreserved (LN) embryogenic calluses of Shamouti orange, Chios mandarin, willow leaf mandarin and Mexican lime using a conventional (CFP) or a simplified (SFP) freezing process. Sucrose concentration in the cryoprotective medium was 0.15 M.

		Shamouti orange		Chios mandarin		Willow leaf mandarin		Mexican lime	
DMSO (%)		CFP	SFP	CFP	SFP	CFP	SFP	CFP	SFP
0	Pt	4+	4+	5+	5+	5+	4+	3+	4+
	-40	2+	+	+/-	+/-	+	-	2+	+/-
	LN	+	+	+/-	+/-	+	-	2+	+/-
5	Pt	4+	4+	5+	5+	5+	4+	4+	4+
	-40	+	2+	4+	3+	2+	+/-	2+	+
	LN	2+	+	4+	3+	+	+/-	4+	3+
10	Pt	4+	4+	5+	5+	5+	4+	4+	4+
	-40	2+	2+	3+	5+	5+	2+	4+	2+
	LN	+	+	3+	5+	4+	+/-	4+	+
15	Pt	4+	4+	5+	5+	5+	4+	4+	2+
	-40	3+	2+	5+	5+	5+	3+	4+	+
	LN	2+	2+	5+	5+	5+	+/-	4+	3+

With embryogenic calluses of Hamlin orange and Cleopatra mandarin frozen using SFP, no survival was obtained after prefreezing to -40°C and immersion in liquid nitrogen without DMSO in the cryoprotective medium (Table 3). Proliferation recovery of cryopreserved calluses was noted for both varieties when the pretreatment was performed with 5 to 15% DMSO. Recovery intensity of Hamlin orange calluses was comparable to that noted with calluses of Mexican lime (Table 2) and that of Cleopatra mandarin to that noted with Shamouti orange.

Table 3: Effect of DMSO concentration employed during pretreatment on the recovery intensity of pretreated (Pt), prefrozen (-40) and cryopreserved (LN) embryogenic calluses of Hamlin orange and Cleopatra mandarin (Cleo) using simplified freezing process.

	DMSO (%)											
	0			5			10			15		
	Pt	-40	LN	Pt	-40	LN	Pt	-40	LN	Pt	-40	LN
Hamlin	4+	+/-	+/-	4+	+	3+	4+	2+	3+	4+	+	4+
Cleo	4+	0	0	4+	3+	2+	4+	2+	2+	4+	3+	2+

Discussion/Conclusion

The present study demonstrated that cell suspensions of willow leaf mandarin could be cryopreserved using a simplified freezing process. The results were comparable to those obtained when freezing was performed using a controlled freezing apparatus.

Prefreezing cells to -40°C required 80 min with the freezing apparatus and 65 min with the Nalgene device. Both cooling regimes allowed sufficient cell dehydration since they withstood freezing in liquid nitrogen with high percentages. By contrast, in our previous study concerning the effect of cooling rate with a controlled freezing apparatus, cooling at 1°C.min^{-1} to -40°C (which required 40 min) led to very poor recovery only. In this latter case, cells may have been unsufficiently dehydrated to withstand freezing in liquid nitrogen. During these experiments, when cells were frozen using the simplified freezing process, we noted also that induction of crystallization in the cryoprotective medium did not improve survival. On the contrary, Panis *et al.* (2) observed that induction of crystallization significantly improved the survival rate of embryogenic cell suspensions of banana. In our experimental conditions, the cooling rate may have been sufficiently high to induce immediate crystallization of the whole cryoprotective medium, without necessitating exogenous supply of frigories.

In the case of embryogenic calluses, proliferation recovery could be obtained using CFP with the six varieties experimented but with five only when using SFP. In optimal cryoprotection conditions (10-15% DMSO), proliferation recovery after cryopreservation was equivalent with both freezing processes. Freezing calluses of willow leaf mandarin with CFP gave very good results, whereas no survival could be obtained using SFP in the conditions experimented. This could be due to various factors: willow leaf calluses may more sensitive to freezing than those of the other varieties. Another possibility could be linked with differences in the duration of *in vitro* culture of calluses of the varieties before their cryopreservation. Indeed, the calluses of willow leaf mandarin used in these experiments had been initiated several months before those of the other varieties and could have become more sensitive to cryopreservation, as observed with apices of potato (13). Finaly, modifications of the cryopreservation protocol

such as increase of DMSO concentration during pretreatment or induction of crystallization during freezing may allow to obtain survival with calluses of willow leaf mandarin.
In conclusion, two processes are now available for the cryopreservation of cell suspensions and embryogenic calluses of *Citrus*. Using the simplified freezing process may significantly facilitate the long-term storage of these cultures which represent an important material for the current breeding programmes of *Citrus* using biotechnologies. It may also be usable for the cryopreservation of cell and callus lines of other plant species.

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