Advantages of Cryopreservation of Cell Suspensions and Embryogenic Calli for *Citrus* Breeding Programmes

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SUMMARY

Cryopreservation of willow leaf mandarin cell suspensions and embryogenic calli of six *Citrus* varieties was performed using two freezing techniques, either a standard process utilizing a programmable freezer or a simplified freezing process. Comparable results were obtained with both tested techniques.
For cell suspensions, the best results were obtained by pretreatment with 0.15 M sucrose and 5% DMSO followed by prefreezing to – 40°C. *In vitro* plantlets morphologically similar to unfrozen controls were produced from cryopreserved suspensions. It was found that a 10-month storage period at – 196°C did not modify cell survival. For calli, increasing the DMSO concentration to 10% or 15% improved growth recovery after freezing. Cryopreservation will allow optimal management of plant material in *Citrus* breeding programmes using biotechnological techniques.

KEYWORDS

Citrus, freezing, cell culture, embryonic development, plant breeding, somatic embryos.

Intérêt de la cryoconservation de suspensions cellulaires et de cals embryogènes dans les schémas d'amélioration génétique des agrumes.

RÉSUMÉ

La cryoconservation d'une suspension cellulaire de mandarine commune et de cals embryogènes de six variétés de *Citrus* est réalisée grâce à deux techniques de congélation, soit un procédé standard utilisant un congélateur programmable, soit un procédé simplifié.
Les résultats sont comparables avec les deux techniques expérimentées. Dans le cas des suspensions cellulaires, un prétraitement avec 0,15 M de saccharose et 5 % de DMSO suivi d'un prérefroidissement jusqu'à – 40 °C donne les meilleurs résultats.
Des plantules *in vitro* morphologiquement semblables aux témoins non congelés sont produites à partir de suspensions cryoconservées. Le taux de survie des cellules n'est pas modifié par une durée de stockage de 10 mois à – 196 °C. Pour les cals, une augmentation de la concentration en DMSO à 10 ou 15 % améliore la reprise de croissance après congélation.
La cryoconservation permettra d'optimiser la gestion du matériel végétal utilisé dans les programmes d'amélioration génétique des agrumes par les biotechnologies.

MOTS CLÉS

Citrus, congélation, culture de cellule, développement embryonnaire, amélioration des plantes, embryon somatique.

Interés de la crioconservación de suspensiones celulares y de callos embriogénicos en el esquema del mejoramiento genetico de los cítricos.

RESUMEN

La crioconservación de suspensiones celulares de la mandarina común y de callos embriogénicos de 6 variedades de *Citrus*, fue realizada gracias a dos técnicas de congelación : el procedimiento estandard utilisando un congelador programable y el procedimiento simplificado. Los resultados fueron comparables con las dos técnicas utilizadas.
En el caso de las suspensiones celulares, un pretratamiento con 0,15 M de sacarosa y 5% de DMSO, seguido de un preenfriamiento hasta – 40 °C proporcionó los mejores resultados. Plántulas in vitro, semejantes morfológicamente al testigo no congelado, fueron producidas a partir de las suspensiones crioconservadas. La tasa de sobrevivencia de células almacenadas durante 10 meses a – 196 °C no fue modificada. En los callos, el aumento en la concentración de DMSO de 10 a 15% mejoró la recuperación del crecimiento después de la congelación.
La crioconservación permitirá la optimización del manejo de material vegetal y el uso de la biotecnología en los programas de mejoramiento genético de los cítricos.

PALABRAS CLAVE

Citrus, congelación, cultivo de células, desarrollo embrionario, fitomejoramiento, embrion somático.

introduction

Genetic improvement of *Citrus* was long hampered by the complexity of genetic structures of cultivars. They present generally high allelic and structural heterozygosity which, along with frequent gametic sterility, considerably limit improvement programmes using sexual reproduction (Ollitrault and Favre, 1992).

The development of somatic embryogenesis and protoplast culture techniques could thus be promising for plant breeding involving gene transfer or somatic fusion. Induction of embryogenic calli from nucellar tissue and subsequent stabilization is a long and difficult process. The development of a collection of calli derived from elite cultivars and rootstocks is a considerable management problem since cultures have to be transferred monthly. Moreover, the risks of somaclonal variation increase with the duration of *in vitro* culture. Cryopreservation (conservation at – 196°C, the temperature of liquid nitrogen) is the only suitable current method for long-term storage of plant material without modifications and with limited maintenance.

Cryopreservation has been applied to more than 70 plant species, including 20 tree species (Withers, 1992; Engelmann, 1993). In *Citrus*, very little work has been done with various types of materials. Bajaj (1984) cryopreserved ovules of *Citrus sp.*. In other studies, *Citrus sinensis* (Washington navel) somatic embryos withstood freezing, but there was a low survival rate, and then developed into plantlets which were transferred *in vivo* (Marin and Duran-Vila, 1988). More recently, Japanese researchers developed cryopreservation techniques for *Citrus sinensis* var. *brasiliensis* cell suspensions, using either a standard freezing process (Kobayashi *et al.*, 1990) or a vitrification process (Sakai *et al.*, 1990, 1991). Cryopreservation of *Poncirus trifoliata* (L.) Raf. was achieved by Radhamani and Chandel (1992). Finally, as part of a joint study between ORSTOM (Laboratoire de Ressources Génétiques et Amélioration des Plantes Tropicales, Montpellier) and CIRAD, cryopreservation of *Citrus deliciosa* Ten. cell suspensions and embryogenic calli obtained from several *Citrus* varieties was carried out using a standard or a simplified freezing process (Aguilar *et al.*, 1993; Engelmann *et al.*, 1994).

In this paper, we present the main results obtained during development of cryopreservation techniques for cell suspensions and embryogenic calli, along with some additional results.

materials and methods

plant material

The calli used in this study were derived from nucellar tissues of ovules of willow leaf and Chios mandarin (*Citrus deliciosa* Ten.), Cleopatra mandarin (*C. reshni* Hort. ex Ten.), Mexican lime (*C. aurantifolia* Chr. Sw.), Shamouti orange (*C. sinensis* Osb.) and Hamlin orange (*C. sinensis* L. Osb. var. Hamlin). They were produced at the INRA/IRFA San Guliano station (Corsica), according to the method of Ollitrault *et al.* (1992). The cell suspension contained calli from willow leaf mandarin in liquid medium.

methods

in vitro culture

Calli were cultivated on MT medium (Murashige and Tucker, 1969) supplemented with 0.5 g/l malt extract and 0.15 M sucrose, solidified with 2 g/l gelrite. Transfers were performed every 4-5 weeks. Calli were placed in the dark at 27 ± 1°C. For the cryopreservation experiments, calli were used 3-4 weeks after the last transfer.

Cell suspensions were cultivated in the same liquid medium on rotary shakers (100 rpm), with transfers every 18 days. They were placed in a growth chamber thermostated at 27 ± 1°C, under a light intensity of 30.5 µmol/m^2/s with a 12L/12D photoperiod. For the cryopreservation experiments, cells were sampled at the beginning of their proliferation phase, i.e. 8-10 days after the last transfer.

cell suspensions

cryopreservation

Cell suspensions were frozen using the standard process described by AGUILAR *et al.* (1993) or the simplified method of ENGELMANN *et al.* (1994).

Cells underwent a 1 h pretreatment at 0°C in a cryoprotective medium containing sucrose (0.15 to 0.9 M) and dimethylsulfoxide (DMSO, 0 to 25%). They were then transferred into 2 ml sterile cryotubes each containing 0.3 ml cells and 0.7 ml cryoprotective medium.

By the standard process, samples were frozen in a programmable freezer (Minicool, L'Air Liquide). The freezing rates tested varied from 0.2 to 5°C/min, with prefreezing temperatures (end of controlled freezing) of – 15 to – 80°C. After prefreezing, cryotubes containing the cells were rapidly immersed in liquid nitrogen, where they were stored for a minimum of 1 h (up to 10 months during the storage experiment). After rapid thawing by plunging the cryotubes in a 40°C water-bath until the ice had completly melted, the cryotube contents were plated on filter paper placed in a petri dish containing solid culture medium. The filter paper with the cells was transferred onto new medium after 1 h and 24 h to eliminate the cryoprotectants. Cells were then subcultured into liquid medium immediately after thawing or after 3-14 days culture periods on solid medium.

For the simplified freezing experiments, we used a thermocooler 1°C/min (Nalge Company, USA) consisting of a polycarbonate box containing 250 ml isopropylic alcohol. After pretreatment, cryotubes containing the cells were placed on the tube rack and the box was placed in a – 80°C deep freezer. The decrease in temperature was monitored with a thermocouple placed in a control sample. When the temperature reached – 40°C (after around 65 min), the cryotubes were either immersed in liquid nitrogen or thawed immediately.

estimate of viability and recovery

Cell viability was estimated immediately after thawing by staining with fluorescein diacetate (FDA, WIDHOLM, 1977). Survival rates were expressed in percentage of the untreated control cell suspension.

In some trials, growth recovery on solid medium was estimated on an intensity scale (– to 3+). In other experiments, proliferation recovery of cells in liquid medium was assessed by measuring the increase in settled cell volume (SCV) for 21 to 30 days.

plant regeneration

In vitro plantlets were regenerated from control and cryopreserved cell suspensions. After one month on solid medium, cells were transferred onto a new embryogenesis induction medium (HIDAKA and OMURA, 1989) supplemented with 0.15 M galactose and 2 g/l gelrite. The proembryos formed were subcultured on the same medium supplemented with 0.1 M sorbitol, 0.1 M galactose, 1 mM gibberellic acid and 7 g/l agar. After 15 days, the young plantlets obtained were subcultured on a half strength MT medium supplemented with 20 g/l sucrose and 7 g/l agar.

embryogenic calli

cryopreservation

Embryogenic calli were frozen using both methods that were developed with cell suspensions. Pretreatment was performed with a cryoprotective mixture containing 0.15 M sucrose and 0 to 15% DMSO. The efficiency of both methods was compared with Shamouti orange and Chios mandarin calli. Willow leaf mandarin and Mexican lime calli were cryopreserved using the standard process, and those of Hamlin orange and Cleopatra mandarin using the simplified process only. After rapid thawing (40°C water-bath), calli were placed on a filter paper, subcultured onto new medium after 1 h and 24 h, and then cultured on standard medium to assess their growth recovery.

growth recovery

Callus growth recovery was estimated after one month of culture according to a fresh matter increase scale (– to 5+).

results

Survival and proliferation recovery of cryopreserved cells varied according to the sucrose and DMSO concentrations during pretreatment (Table 1). Without DMSO, survival increased with increased sucrose concentrations. DMSO proved to be toxic at more than 15% concentrations. Growth recovery was noted after 12 days when pretreatment was carried out with 0.15 M sucrose and 5 or 10% DMSO, and after only 18 days with 0.3 or 0.6 M sucrose. No recovery was obtained with 0.9 M sucrose. Optimal pretreatment conditions were thus obtained with 0.15 M sucrose and 5-10% DMSO.

Survival rates increased progressively with prefreezing temperatures of – 15 to – 40°C, the latter being the optimal temperature (57% survival) (Table 2). Growth recovery on solid medium was obtained with all prefrozen controls. In cryopreserved suspensions, growth recovery could be obtained with prefreezing temperatures of – 25 to – 80°C and was optimal from – 35 to – 45°C.

Survival was obtained at freezing rates of 0.2 to 1°C/min, and was maximal at 0.5°C/min (Table 3). Growth recovery on solid medium was very high with prefrozen controls. After freezing in liquid nitrogen, growth recovery was obtained at freezing rates of 0.2 to 1°C/min, and was maximal at 0.5°C/min.

The effect of culture duration for cells on solid medium on the proliferation recovery in liquid medium was observed with control (Figure 1) and cryopreserved (Figure 2) cell suspensions. After 30 days in liquid medium, the SCV levels were similar in control cells regardless of the

Table 1
Effect of sucrose and DMSO concentration on the survival rate and proliferation recovery of cryopreserved willow leaf mandarin cell suspension.*

Sucrose (M)		DMSO 0	5	10	15	20	25
0.15	Survival (%)	0	55	38	26	3	1
	Recovery	–	3+	3+	+	–	–
0.3	Survival (%)	16	39	37	16	0	0
	Recovery	–	+	2 +	– /+	–	–
0.6	Survival (%)	26	31	21	12	2	4
	Recovery	– /+	2+	2+	– /+	–	–
0.9	Survival (%)	37	19	16	10	0	0
	Recovery	–	–	–	–	–	–

* Growth recovery on solid medium was estimated on an intensity scale (– to 3+).

Table 2
Effect of prefreezing temperature on the survival rate and proliferation recovery of prefrozen control (–LN) and cryopreserved (+LN) willow leaf mandarin cell suspension.*

		Prefreezing temperature (°C) – 15	– 20	– 25	– 30	– 35	– 40	– 45	– 50	– 55	– 80
– LN	Survival (%)	65	79	69	81	44	72	58	36	32	23
	Recovery	3+	3+	2+	3+	3+	3+	3+	2+	2+	2+
+ LN	Survival (%)	0	0	8	19	43	57	48	38	29	20
	Recovery	–	–	+	2+	3+	3+	3+	2+	2+	2+

* Growth recovery on solid medium was estimated on an intensity scale (– to 3+).

culture duration on solid medium. With cryopreserved cells, the SCV increase was the highest with 3 to 7 days recovery on solid medium, and was optimal at 5 days. In optimal conditions, proliferation recovery in liquid medium was slightly higher in cryopreserved cells than in control cells.

Differences in the recovery of adventive embryogenesis were noted between control and cryopreserved cells. Indeed, somatic embryo production was more rapid and intense with cryopreserved cells than with control cells. Globular and heart stage embryos were obtained within 15 days with frozen cells and after only 22 days with control cells. Embryos germinated after 15 days and developed into young plantlets, similar to those developed in the control cultures, after an additional month in culture (Photo 1).

We also found that the storage duration in liquid nitrogen had no influence on the survival rate of cells since no difference was observed after 1 h or 10 months at – 196°C (Table 4). The intensity of cell proliferation recovery in liquid medium was the same regardless of the storage period.

We performed an experiment to compare the efficiencies of the standard freezing process and the simplified freezing process. Although the survival rate of cell suspensions prefrozen to – 40°C was appreciably higher with the standard freezing process, the results were comparable for both techniques after freezing in liquid nitrogen (Table 5).

Growth recovery in liquid medium of cells frozen by the simplified process was monitored for 21 days and compared to pretreated and prefrozen control cells (Figure 3). Cells were separated into two groups showing similar growth: control and pretreated cells had higher growth than prefrozen and cryopreserved cells.

Both techniques developed for cell suspensions were then investigated with embryogenic calli of several *Citrus* varieties. In the first step, we tested the standard freezing technique with willow leaf mandarin and Mexican lime calli. With both varieties, increasing the DMSO concentration in the cryoprotective

Table 3
Effect of freezing rate on the survival rate and proliferation recovery of prefrozen control (– LN) and cryopreserved (+LN) willow leaf mandarin cell suspensions.*

		Freezing rate (°C/min)				
		0,2	0,5	1,0	2,0	5,0
– LN	Survival (%)	44	72	31	30	64
	Recovery	3+	3+	3+	3+	3+
+ LN	Survival (%)	41	57	16	0	1
	Recovery	2+	3+	+	–	–

* Growth recovery on solid medium was estimated on an intensity scale (– to 3+).

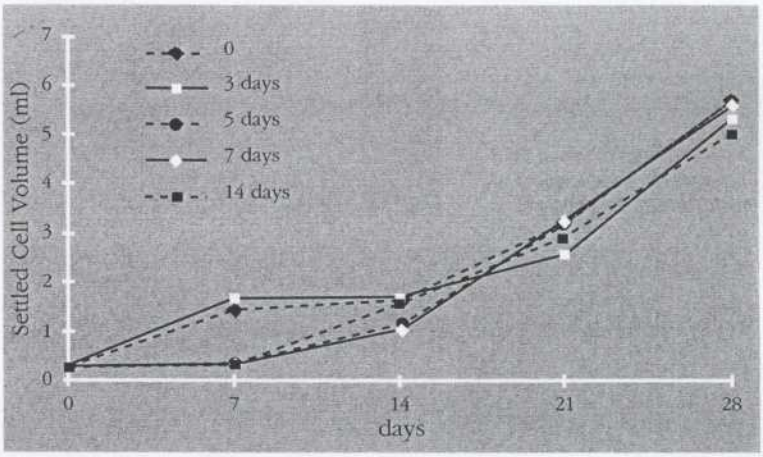

Figure 1
Effect of culture duration on solid medium on growth recovery in liquid medium of control willow leaf mandarin cell suspensions.

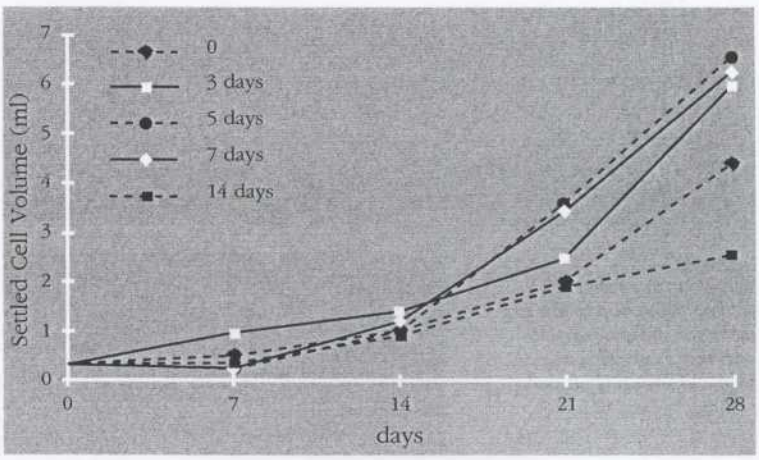

Figure 2
Effect of culture duration on solid medium on growth recovery in liquid medium of cryopreserved willow leaf mandarin cell suspensions.

Photo 1
In vitro *plantlet obtained from a cryopreserved willow leaf mandarin cell suspension.*

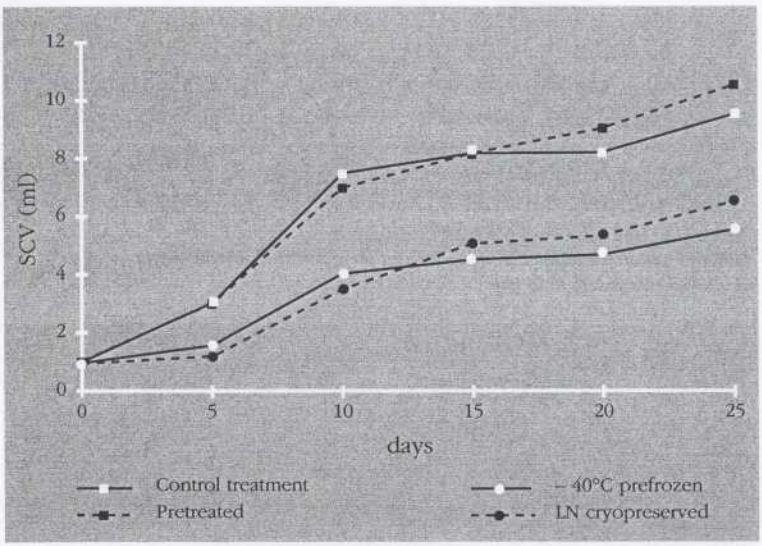

Figure 3
Variations in settled cell volume (SCV) of a willow leaf mandarin cell suspension cryopreserved using a simplified freezing process.

Table 4
Effect of storage duration in liquid nitrogen on the survival rate of a willow leaf mandarin cell suspension.

	Storage duration	
	1 hour	10 months
Survival rate	70	78

Table 5
Effect of the freezing process on the survival of a prefrozen (– 40°C) and cryopreserved (– 196°C) willow leaf mandarin cell suspension.*

	Freezing process	
	CFP	SFP
– 40°C	72	57
– 196°C	55	52

*CFP: standard process; SFP: simplified process.

medium increased the survival rates (Table 6). There was equal growth recovery for pretreated, prefrozen and cryopreserved calli with 10 and 15% DMSO, whereas with at lower DMSO concentrations, growth recovery of control calli was much higher than that of prefrozen and cryopreserved calli. Mexican lime calli were more sensitive to the various treatments than those of willow leaf mandarin. Indeed, their growth recovery was generally lower in all conditions.

We then studied the effect of the freezing process on growth recovery of Shamouti orange and Chios mandarin calli after various pretreatments. With both varieties, increasing the DMSO concentration had no effect on growth recovery of pretreated calli (Table 7). The growth recovery of prefrozen and cryopreserved calli was very low without DMSO in the cryoprotective medium. It was higher in the presence of DMSO during pretreatment with both freezing methods. Shamouti orange calli were more sensitive to pretreatment, prefreezing and freezing than those of Chios mandarin, since their growth recovery was lower in all conditions. With the simplified freezing process, growth recovery was generally more intense when cryoprotective medium contained 10 or 15% DMSO.

Finally, the simplified freezing process was applied to Hamlin orange and Cleopatra mandarin calli (Table 8). Here again, increasing the DMSO concentration improved growth recovery after freezing in liquid nitrogen. Growth recovery appeared to be better with Hamlin orange than with Cleopatra mandarin.

discussion conclusion

In the present study, we developed and applied two freezing techniques to willow leaf mandarin cell suspensions and embryogenic calli from six *Citrus* varieties. Comparable results were obtained with both methods, i.e. the standard protocol using a programmable freezer or the simplified freezing technique. We also found that the survival rate and proliferation recovery of cell suspensions were not modified after 10 months of storage at – 196°C. Finally, we were able to regenerate plantlets *in vitro* from cryopreserved cell suspensions.

Pretreatment of cell suspensions with 0.15 M sucrose and 5% DMSO followed by freezing at 0.5°C/min to – 40°C resulted in constant survival rates of around 50-60% of that of the control after freezing in liquid nitrogen. For embryogenic calli, there were marked differences in sensitivity to cryopreservation between varieties. However, satisfactory results were obtained with all varieties after pretreatment with 0.15 M sucrose and 10 or 15% DMSO. These conditions could thus be recommended for cryopreservation of other *Citrus* varieties.

From a practical viewpoint, the possibility of cryopreserving cell suspensions and embryogenic calli without a programmable freezer considerably simplifies the protocol. Cryopreservation of *Citrus* cell suspensions and calli can thus be easily carried out even in laboratories without this equipment.

In conclusion, this control of cryopreservation for *Citrus* calli and embryogenic cell suspensions will allow routine use of the technique. This should make it safer and easier to manage collections of embryogenic calli to be used for varietal creation via somatic fusion and genetic transformation. Moreover, for the same programmes, duplicates of transformed or fused cell lines could now be stored during the whole regeneration and evaluation period, permitting rapid initiation of multiplication in the best genotypes through somatic embryogenesis. Concerning genetic resources, cryopreservation of apices or axillary buds sampled on virus-free mother plants could also effectively hunder accidental recontamination of plant material and avoid problems of juvenility associated with somatic embryogenesis.

Table 6
Effect of DMSO concentration during pretreatment on the recovery of pretreated (Pt), prefrozen (– 40°C) and cryopreserved (LN) willow leaf mandarin and Mexican lime embryogenic calli using a standard freezing process.*

	DMSO (%)											
	0			5			10			15		
	Pt	– 40°C	LN	Pt	– 40°C	LN	Pt	– 40°C	LN	Pt	– 40°C	LN
Mandarin	5+	+	+	5+	2+	+	5+	5+	4+	5+	5+	5+
Lime	3+	2+	2+	4+	2+	4+	4+	4+	4+	4+	4+	4+

* Growth recovery was estimated after one month of culture according to a fresh matter increase scale (– to 5+).

Table 7
Effect of DMSO concentration during pretreatment on the recovery of pretreated (Pt), prefrozen (– 40°C) and cryopreserved (LN) Shamouti orange and chios mandarin embryogenic calli using a conventional (CFP) or a simplified freezing process (SFP). The sucrose concentration in the cryoprotective medium was 0,15 M.*

	Shamouti orange					Chios mandarin				
	CFP			SFP		CFP			SFP	
DMSO (%)	Pt	– 40°C	LN	– 40°C	LN	Pt	– 40°C	LN	– 40°C	LN
0	4+	2+	+	+	+	5+	+/–	+/–	+/–	+/–
5	4+	+	2+	2+	+	5+	4+	4+	3+	3+
10	4+	2+	+	2+	2+	5+	3+	3+	5+	5+
15	4+	3+	2+	2+	2+	5+	5+	5+	5+	5+

* Growth recovery was estimated after one month of culture according to a fresh matter increase scale (– to 5+).

Table 8
Effect of DMSO concentration during pretreatment on the recovery of pretreated (Pt), prefrozen (– 40°C) and cryopreserved (LN) of Hamlin orange and Cleopatra mandarin embryogenic calli (Cleo) using a simplified freezing process.*

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

* Growth recovery was estimated after one month of culture according to a fresh matter increase scale (– to 5+).

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