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CRYOPRESERVATION OF GRAPE EMBRYOGENIC CELL SUSPENSIONS : 1-INFLUENCE OF PRETREATMENT, FREEZING AND THAWING CONDITIONS

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Summary : During this work, conditions allowing for the cryopreservation of an embryogenic grape cell suspension were defined. Survival rates higher than 60% could be obtained with cells at 30% PCV after 1-hour pretreatment at 0°C with 0.25 M maltose and 5% DMSO, freezing at 0.5°C.min^{-1} to -40°C followed by immersion in liquid nitrogen. After rapid thawing, the cells were allowed to grow for 18 days on a semi-solid medium containing activated charcoal. The cells were then transferred to liquid medium. One and a half months after thawing, the growth of the cryopreserved cells was similar to that of the unfrozen control.

Key-words : cryopreservation, grape, *Vitis* sp., embryogenic cell suspension, cryoprotective substances, maltose.

INTRODUCTION

Today, *in vitro* culture techniques are routinely used for the propagation of numerous plant species. With serial subcultures, a progressive decrease in the morphogenetic or biosynthetic capacities of the cell lines is often observed. Risks of somaclonal variation, which can lead to the loss of trueness to type when compared with the starting material, generally increase with the duration of *in vitro* culture. Moreover, the material remains exposed to contamination. Finally, culture maintenance is costly both in terms of time and labour. Only cryopreservation in liquid nitrogen (LN, -196°C) can presently ensure the conservation of cell strains for theoretically unlimited time durations, without alterations and sheltered from contamination. Resistance to freezing in LN has been obtained for more than 70 plant species, including about ten cell suspensions of tree species (1). For example, plantlets have been regenerated from cryopreserved embryogenic cell suspensions of *Picea glauca* (2) and *Citrus sinensis* (3).

The propagation of grape by means of somatic embryogenesis has been dealt with in several papers (4, 5, 6, 7). Embryogenic cell suspension culture in liquid medium was carried out by Lebrun and Branchard (8). This technique is used in France for the regeneration and genetic transformation of important vines (Chardonnay, Pinot noir, Gamay, Cabernet Sauvignon) as well as some rootstocks (41 B, *rupestris* du Lot, 110 R) (9). It is particularly interesting to be able to conserve these cell suspensions, which are often difficult to obtain.

In this study, we present the first results concerning the setting up of a cryopreservation process for an embryogenic cell suspension of grape.

MATERIALS AND METHODS

Plant material

The trials were carried out on the rootstock 41 B, a hybrid of *Vitis vinifera* var. Chasselas and *Vitis berlandiri*. The cell suspension used for these experiments was supplied by the Moët & Chandon viticulture laboratory. It was obtained from calluses originating from anthers, and cultivated according to the method described by Deloire (9).

Cryopreservation method

Pregrowth/Cryoprotection

The cell aggregates, taken 2 to 3 days after their last transfer, were placed in a 100 ml Erlenmeyer flask and the standard medium was replaced with medium

containing sorbitol, mannitol or glycerol at various concentrations, in order to obtain the desired Packed Cell Volume (PCV). A range of 10 to 70% was used to test the effect of PCV. A PCV of 30% was used in experiments to test other variables. In some cases, the cells were pregrown for 24 hours under the previously described culture conditions. The flask was then placed in an ice bath for 1 hour, and, in some cases, dimethylsulfoxide (DMSO) was added every 6 minutes during the cryoprotective treatment.

At the end of cryoprotective treatment, 1 ml aliquots of the cell suspension were distributed in 1.8ml sterile polypropylene screw cap cryotubes, precooled to 0°C.

Freezing

Two types of freezing were tested. Rapid freezing experiments were carried out by directly plunging the samples into LN (120°C.min^{-1}). Slow freezing experiments were carried out using a programmable freezing apparatus (Minicool LC 40, from L'Air Liquide). A range of -15 to -80°C was used to test the effect of the prefreezing temperature. A prefreezing temperature of -40°C was used in experiments to test other variables. A range of 0.1 to 5°C.min^{-1} was used in order to test the effect of the freezing rate. A freezing rate of 0.5°C.min^{-1} was used in experiments to test other variables. Crystallisation in the cryoprotective medium was induced manually at a temperature intermediate between the crystallisation and the nucleation temperatures of the medium, by briefly pinching the ampoules with forceps previously cooled in LN. Once the prefreezing temperature was reached, the samples were thawed directly or immersed in LN, where they were kept for 24 hours.

Thawing

The cryotubes were placed in a water-bath thermostated at 40°C (30 to 60°C in the experiment concerning the effect of the thawing rate) or at room temperature in laminar air flow, until melting of ice was complete.

Post-treatment

Each sample was poured on a Petri dish containing 20 ml of standard semi-solid medium, supplemented with 1 g.l^{-1} activated charcoal and 2 mg.l^{-1} naphthoxyacetic acid, covered with 2 filter papers. After 1 hour, the filter papers with the cells were transferred to another Petri dish. A new transfer was carried out after 24 hours. It was assumed that all cryoprotective substances had then been removed (2). The cells were transferred to fresh semi-solid medium after 7 and 14 days and then to liquid medium after 18 days. They were filtered after 1 week and then cultured according to the standard protocol.

Viability assessment

Two separate measurements were carried out in order to estimate cell survival. Immediately after thawing, viability was measured by staining with fluorescein diacetate (FDA, 10). The survival rate of a sample was assessed by calculating the mean percentage of living cells measured on 20 cell aggregates chosen randomly on a plate observed with a microscope. All the survival rates presented in the results are expressed as a percentage of the control value.

The fresh weight increase of the cells was also measured after 18 days of culture on a semi-solid culture medium using the technique developed by Horsch and Jones (11). The regrowth of cryoprotected or frozen suspensions is expressed as a percentage of the control value.

Analysis of the results

In the experiment concerning the effect of the glycerol, sorbitol and maltose concentrations, each set of conditions was represented by one sample. In all other experiments, each set of conditions was represented by 3 samples. The data concerning the survival rate and growth recovery represent the mean value for the 3 samples.

RESULTS

Preliminary experiments indicated that survival was impossible without cryoprotective treatment (data not shown). The results concerning the survival of the cells after freezing, as a function of the various cryoprotective substances employed, are presented in Table 1. Without pregrowth, there was no or a very low survival if no DMSO was added during the cryoprotective treatment. With the addition of DMSO during the cryoprotective treatment, a slight improvement was observed with glycerol and sorbitol. With maltose, viability rates increased dramatically, reaching the maximal value of 63.8% for 0.25 M. Growth recovery was possible with 0.25 and 0.5 M maltose.

A 24-hour pregrowth gave slightly improved results with sorbitol and glycerol, if the pretreatment was performed in the presence of DMSO. Growth recovery thus became possible after pregrowth with 0.75 M sorbitol. In contrast, a statistically significant decrease in the survival rates was observed with maltose ; growth recovery was no longer possible.

Four DMSO concentrations (5, 10, 15 and 20%) were used during cryoprotective treatment with 0.25 M maltose (Table 2). The increase in DMSO concentration did not modify the viability of the control cell suspensions which remained around 90% under all conditions. Cell viability decreased after freezing. With 5% DMSO, survival was

significantly higher than that obtained under the other conditions. Regrowth was possible with 5 and 10% DMSO.

The variation of cell viability as a function of the prefreezing temperature is illustrated in Fig. 1. With prefrozen controls, lowering the prefreezing temperature to -40°C induced no decrease in viability. No viability was observed for temperatures lower than -40°C. With cell suspensions frozen in LN, survival was maximal at -40°C. In this latter case, there was no difference with the prefrozen controls. From -15 to -35°C, the immersion in LN was responsible for the mortality: the viability rates differed significantly between prefrozen controls and samples submitted to freezing in LN. However, from -40 to -80°C, the freezing in LN was no longer selective: the viability rates obtained after immersing the cells in LN were not appreciably different from those of the prefrozen controls.

After prefreezing to below -40°C, regrowth of the prefrozen controls was no longer possible (Table 3). Only a prefreezing temperature of -40°C allowed for regrowth of the suspensions frozen in LN.

As concerns the cooling rate (Table 4), no survival was obtained after fast freezing. Survival was maximal after freezing at 0.5 or 1°C.min^{-1}. Regrowth was obtained for these two cooling rates only, but was lower with 1°C.min^{-1}. With a freezing rate of 0.1°C.min^{-1}, prefreezing was the limiting factor of the viability. With cooling rates of 2.5 and 5°C.min^{-1}, both prefreezing and immersion in LN were limiting.

The viability rates were not appreciably different after slow thawing, in ambient air, or rapid thawing, in a water-bath thermostated at 30 or 40°C (Table 5). When the temperature of the water-bath was raised to 60°C, a significant decrease in viability was noted. Regrowth was observed only after thawing at 40°C.

Although increasing the PCV to 70% led to an increased initial viability, this was not reflected in regrowth (Table 6) and the cells browned rapidly on transfer to semi-solid medium. Regrowth was optimal for a 30% PCV.

After an 18-day culture on semi-solid medium, cells originating from material frozen under the optimal conditions were resuspended in liquid medium. During the first three subcultures, cell growth was lower than that of the control. A weekly doubling of the PCV, i.e. a growth similar to that of the controls, could be observed afterwards.

DISCUSSION/CONCLUSIONS

The cryopreservation process set up for this grape embryogenic cell suspension regularly gave viability rates around 60%, and soon yielded after thawing a cell suspension showing the same growth rate as that of the original strain. Numerous

remarks can be made concerning the conditions required for the successive steps of the proposed protocol.

For preculture and pretreatment, glycerol, sorbitol and maltose, with addition of DMSO in some cases, were used during the pretreatment. Glycerol and sorbitol are commonly employed as cryoprotectants. Glycerol is generally used at high molarities (1 to 2 M), in mixtures with other compounds like DMSO (12, 13) or sucrose (14). When employed alone, it gives poor results : in a comparative experiment with DMSO and proline carried out with *Puccinellia distans* cell suspensions, Heszky *et al.* (12) showed that it was the least efficient cryoprotectant. Sorbitol appears to be an efficient cryoprotectant, even when employed alone, at around 1 M (15, 16). It also gives good results with DMSO (2, 17). Maltose is sometimes employed as carbon source for the *in vitro* culture of plants (8, 18), but its use as cryoprotectant has never been mentioned (19). With grape cell suspensions, glycerol and sorbitol showed only a very low cryoprotective effect, unlike maltose. In this study, the optimal maltose concentration was 0.25 M. However the effect of lower concentrations was not investigated. It was interesting to note that, as in the case of oil palm somatic embryos (20), a compound present in the standard culture medium gave the best cryoprotection.

The addition of DMSO at a concentration of 5% during the pretreatment increased the survival of the cells under all the conditions tested, as is generally observed (21). This substance enters the cells easily and facilitates the penetration of other compounds, notably sugars (22). Maltose, which is a source of carbon for the cells and is thus metabolized, may have entered the cells more rapidly. This could explain the toxicity which led to a decrease of viability after 24-hour pretreatment. With glycerol and sorbitol, which are not or only slightly metabolized by plant cells, the pregrowth effect took longer to appear, since the extension of pretreatment to 24 hours led to an improvement of survival. A further increase in the length of pretreatment may amplify this trend.

The two main parameters of freezing are the cooling rate and the prefreezing temperature. The range of freezing rates ensuring cell survival varies with the species : indeed, 50% of the cells of a *Pucinellia distans* cell suspension survived a cooling rate of $1°C.min^{-1}$ but less than 15% a freezing rate of $0.5°C.min^{-1}$ (12). With a carrot cell suspension, the range of freezing rates allowing for cell survival is wider, from 0.5 to $8°C.min^{-1}$ (23). The grape cell suspensions studied in this work showed an intermediate behaviour, since good viability and regrowth could be obtained with cooling rates of 0.5 and $1°C.min^{-1}$. For cell suspensions of many plant species, the optimal prefreezing temperature is between -30 and -50°C. In the case of grape cell suspensions, this parameter had to be very precisely determined since even if good viability was obtained for temperatures between -30 and -60°C, only a prefreezing temperature of -40°C ensured growth recovery.

Thawing is still only rarely studied. In most cases, rapid thawing is carried out using a water-bath thermostated at 37-40°C, so as to avoid recrystallisation phenomena which occur between -80 and -30°C if the thawing rate is too slow and which can be lethal to the cells (24). However, Reuff *et al.* (16) obtained a two-fold increase in survival of a *Coleus blumei* cell suspension simply by increasing the water-bath temperature to 60°C. With grape cell suspensions, on the other hand, a sharp decrease in the survival rate was obtained using this thawing temperature. The cells of these two species may have different structures (e.g. size, water content) which could explain why the same fast thawing rate allows the cells of *Coleus blumei* to survive but leads to the death of grape cells.

Cell suspensions are usually frozen with a 30% PCV. However, Bajaj indicated that the quantity of cells was an important criterion for survival (25). Higher densities ensured higher survival. We made the same observation with grape cell suspensions, since with a 70% PCV the survival rate was significantly higher than under the other conditions. The browning of the cells observed during regrowth on semi-solid medium may have been caused by nutritional problems. It could also be due to autotoxicity by products of cell damage.

Finally, during all these experiments, it was noted that the FDA test was very precise for measuring viability but did not give information on the multiplication potential of the cells. Indeed, under some conditions, high viability rates (around 30%) were measured after thawing but it was impossible to obtain regrowth of the cell suspension. This observation confirms that made recently by Panis *et al.* (26) during the cryopreservation of embryogenic cell suspensions of *Musa*. These authors obtained 40% viability under some conditions, measured with FDA, but no further regrowth. The use of FDA gives important indications concerning viability, but regrowth of the material must always be checked. Diettrich *et al.* (14) mentioned that in the case of a *Digitalis lanata* cell suspension, a minimum of 30% living cells was necessary in order to obtain recovery growth.

In conclusion, the technique proposed in this paper is efficient, since one and a half months after thawing the growth rate of cryopreserved cells was equivalent to that of unfrozen control. Additional experiments are presently under way in order to check that freezing in LN does not modify the conversion rate of the embryos into plantlets and their development *in vitro* and *in vivo*. This process will have to be extended to other clones. It seems that in the near future cryopreservation will allow the long-term storage of grape embryogenic cell suspensions.

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Table 1: Viability and regrowth (% of the control) of a cell suspension frozen in LN as a function of the concentration of glycerol, sorbitol or maltose, in the cryoprotective medium, with or without 5% DMSO, and with or without a 24 hour pregrowth.

				Cryoprotectant concentration (M)			
				0.25	0.5	0.75	1
Glycerol	no pregrowth	no DMSO	Viability	0.0	0.1	0.0	0.1
			Regrowth	0	0	0	0
		DMSO	Viability	1.0	1.1	0.3	0.2
			Regrowth	0	0	0	0
	pregrowth	no DMSO	Viability	0.2	0.3	0.8	2.2
			Regrowth	0	0	0	0
		DMSO	Viability	4.3	2.3	2.6	4.3
			Regrowth	0	0	0	0
Sorbitol	no pregrowth	no DMSO	Viability	0.0	0.1	0.2	0.1
			Regrowth	0	0	0	0
		DMSO	Viability	3.1	2.4	0.4	0.4
			Regrowth	0	0	0	0
	pregrowth	no DMSO	Viability	0.1	0.6	0.9	0.5
			Regrowth	0	0	0	0
		DMSO	Viability	10.1	5.4	13.7	7.5
			Regrowth	0	0	25	0
Maltose	no pregrowth	no DMSO	Viability	0.2	0.3	0.7	1.1
			Regrowth	0	0	0	0
		DMSO	Viability	63.8	26.1	15.6	23.9
			Regrowth	75	50	0	0
	pregrowth	no DMSO	Viability	0.4	1.6	4.5	4.6
			Regrowth	0	0	0	0
		DMSO	Viability	39.0	10.6	9.3	8.1
			Regrowth	0	0	0	0

Table 2: Viability and regrowth (% of the control) of a cell suspension cryoprotected or frozen in LN, as a function of the DMSO concentration in the cryoprotection medium.

		DMSO concentration (%)				
		0	5	10	15	20
Cryoprotected	Viability	93.0	91.2	92.2	89.6	90.6
	Regrowth	100	90	85	90	90
LN	Viability	0.6	54.1	21.5	1.2	0.8
	Regrowth	0	75	50	0	0

Table 3: Regrowth (% of the control) of a cell suspension cryoprotected or frozen in LN, as a function of the prefreezing temperature.

	Prefreezing temperature (°C)						
	-20	-30	-35	-40	-45	-60	-80
Prefreezing	95	92	90	90	0	0	0
LN	0	0	0	87	0	0	0

Table 4: Viability and regrowth (% of the control) of a cell suspension prefrozen or frozen in LN, as a function of the cooling rate during the prefreezing.

		Freezing rate (°C.min^{-1})					
		0.1	0.5	1	2.5	5	120 (rapid)
Prefreezing	Viability	13.8	96.2	60.5	32.5	25.9	/
	Regrowth	0	82	80	0	0	0
LN	Viability	12.5	78.6	61.9	25.7	0.0	0.0
	Regrowth	0	75	25	0	0	0

Table 5: Viability and regrowth (% of the control) of a cell suspension frozen in LN, as a function of the thawing temperature.

	Thawing temperature (°C)			
	Ambient	30	40	60
Viability	24.7	28.7	34.9	9.4
Regrowth	0	0	63	0

Table 6: Viability and regrowth (% of the control) of a cell suspension frozen in LN, as a function of the PCV in the cryotubes.

	PCV (%)				
	10	20	30	50	70
Viability	49.4	55.4	51.8	50.6	75.0
Regrowth	50	63	75	50	50

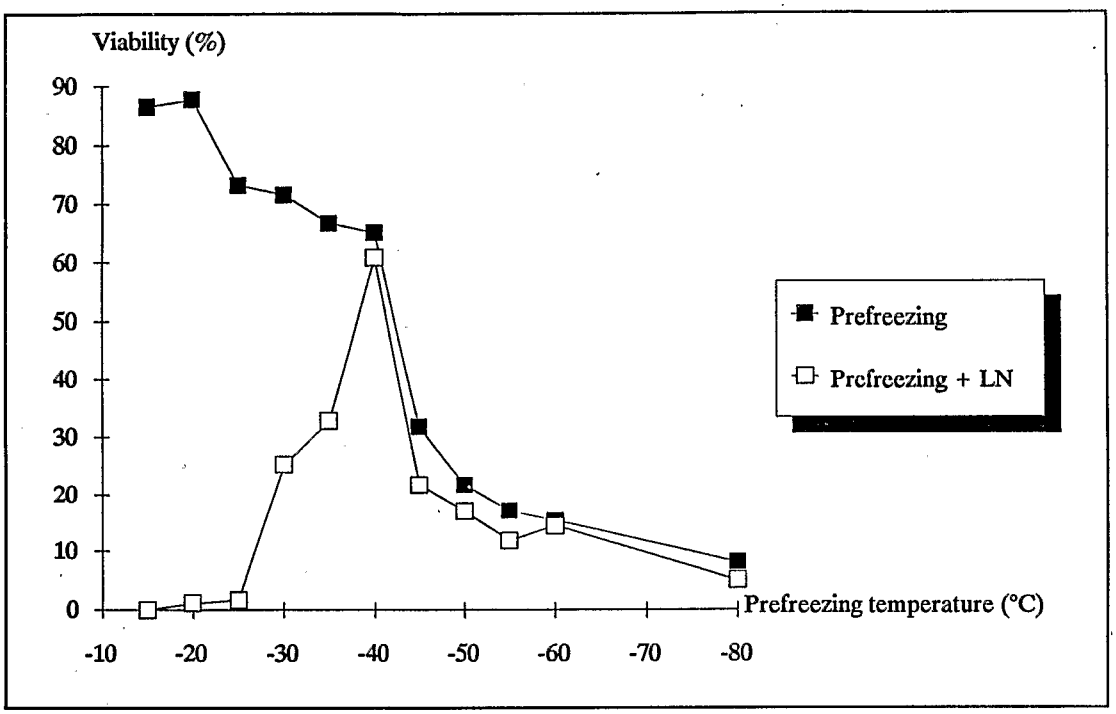

Fig. 1 : Viability, in % of the control, of a cell suspension prefrozen or frozen in LN, as a function of the prefreezing temperature.