Cryopreservation of sugarcane embryogenic callus

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Introduction

Classical freezing protocols, which include slow controlled cooling (0.5–1°C/min) to around –40°C followed by immersion of samples in liquid nitrogen, are usually employed for cell suspensions and embryogenic calluses (Kartha and Engelmann 1994). Storing embryogenic cell suspensions or calluses in liquid nitrogen preserves their regeneration capacities and limits the risks of somaclonal variation which increase with culture duration.

In the case of sugarcane, cryopreservation protocols have been developed for various materials: apices of *in vitro* plantlets using the encapsulation-dehydration technique (González-Arnao *et al.* 1993a, 1993b; Paulet *et al.* 1993); cell suspensions (Finkle and Ulrich 1979) and embryogenic calluses using classical freezing protocols (Jian *et al.* 1987; Gnanapragasam and Vasil 1990; Eksomtramage *et al.* 1992).

Simplified freezing protocols in which the expensive programmable freezers required for classical protocols are replaced with simpler freezing devices including alcohol baths and/or domestic freezers have been established for various materials (Sakai *et al.* 1991; Nishizawa *et al.* 1992; Engelmann *et al.* 1994, 1997).

In this paper, we describe a simplified cryopreservation protocol and its application to embryogenic calluses of three commercial varieties of sugarcane. We also present data on the effect of extended cryopreserved storage duration on the survival and plantlet production of calluses of one sugarcane variety.

Materials and methods

In vitro culture

Embryogenic calluses were initiated by culturing 3 to 5-mm-long immature inflorescence segments on a modified MS medium as described by Martínez-Montero *et al.* (1998). Calluses were cultured in the dark at 25±2°C, with monthly subcultures. For plantlet regeneration, calluses were transferred to a medium devoid of 2,4–D at 27±2°C under a 16-h light/8-h dark photoperiod, with a 40 μmol m^{-2} s^{-1} photon dose.

Cryopreservation

For cryopreservation experiments, 15 to 25-d-old calluses, about 3–5 mm in diameter, were employed. About one-third of sterile 2-ml cryotubes were filled

with fragments of calluses which were pretreated in liquid medium with sucrose concentrations ranging between 0.3 and 0.75M for 1 h at 0°C. Dimethylsulfoxide (DMSO) was added progressively to the liquid medium over a period of 30 min until the desired final concentration (5, 10 or 15%, v/v) was reached.

Freezing was performed using a home-made ethanol bath, consisting of a polypropylene container filled with 700 ml of ethanol precooled at 0°C. Cryotubes were inserted in holes pierced in a thin polypropylene plate floating on top of the ethanol, which allowed immersion of the cryotubes in the coolant. The ethanol bath was then placed in a –40°C freezer, thus allowing an average cooling rate of 0.4–0.6°C/min between 0°C and –40°C. Crystallization was induced manually in the cryoprotective medium at a temperature intermediate between the nucleation and the crystallization temperature of the cryoprotective medium, by briefly putting the base of the cryotubes in contact with liquid nitrogen. Once the temperature of –40°C was reached, the cryotubes were kept for 2 h at this temperature, then immersed rapidly in liquid nitrogen. Samples were kept for a minimum of 2 h at –196°C. Rapid thawing was carried out by plunging the cryotubes in a +40°C water-bath. Calluses were then transferred directly (without washing) to recovery medium.

Three replicates, each consisting of six fragments of embryogenic calluses, were used for each experimental condition. The survival rate, evaluated 40–50 d after freezing, corresponded to the percentage of calluses which had increased in size during the recovery period. For the measurement of plantlet production, six calluses randomly chosen in each condition were transferred to standard medium without 2,4–D, and the number of plantlets produced was estimated after 80 d of culture. The survival rate and plantlet production of calluses of variety CP 5243 were evaluated after storage for 2 h, 4 and 14 months in liquid nitrogen.

Results

Survival of calluses after pretreatment was comparable between the three varieties. It was high with 5% and 10% DMSO, and decreased slightly with 15% DMSO. After freezing in liquid nitrogen, higher survival was achieved with variety CP 5243 than with the other two varieties. With varieties CP 5243 and C 91-301, high survival rates were obtained for a large range of pretreatment conditions. In contrast, for variety C 1051-73, high survival was achieved after pretreatment with 5% DMSO and 0.3M sucrose only.

Fully developed plants could be obtained from regenerating calluses of all three sugarcane varieties (Table 1). The number of plantlets produced from control calluses was higher than from cryopreserved ones and regeneration from calluses of variety CP 5243 was much higher than from the two other varieties. Regeneration of plantlets was obtained from a much broader range of experimental conditions than those which had ensured optimal survival.

Table 1. Effect of DMSO and sucrose concentration in the cryoprotective solution on the number of plantlets produced from control (-LN) and cryopreserved (+LN) embryogenic calluses of sugarcane varieties CP 5243, C 91-301 and C 1051-73 (Reprinted from Martinez Montero *et al.* 1998, with permission)

DMSO (%)	Sucrose (M)	Plantlets produced					
		CP 5243		C 91-301		C 1051-73	
		-LN	+LN	-LN	+LN	-LN	+LN
5	0.3	190	143	88	0	90	0
	0.5	196	165	69	33	90	35
	0.75	211	150	72	34	88	44
10	0.3	210	150	82	40	95	48
	0.5	213	106	73	42	99	51
	0.75	200	119	63	41	88	45
15	0.3	243	165	70	18	82	27
	0.5	209	171	65	18	81	29
	0.75	209	161	51	18	75	25

The effect of storage duration on the survival rate and plantlet regeneration of cryopreserved calluses of variety CP 5243 is presented in Table 2. Even though a slight decrease in survival between 2 h and 4 months in storage could be noted under some conditions, both the survival rate and the number of plantlets regenerated from cryopreserved calluses were not modified after 14 months under LN storage.

Table 2. Effect of extended storage duration on the survival rate and plantlet production of callus of variety CP 5243 (Reprinted from Martinez Montero *et al.* 1998, with permission)

DMSO (%)	Sucrose (M)	Survival (%)			Plantlets produced		
		2 h	4 mo	14 mo	2 h	4 mo	14 mo
10	0.3	81	75	77	150	113	95
	0.5	90	81	78	106	124	115
	0.75	94	73	77	119	99	100

Discussion/Conclusion

The simplified freezing protocol developed in this study was efficient since it achieved results comparable to those obtained with the classical protocols employed by other authors to cryopreserve sugarcane calluses (Jian *et al.* 1987; Gnanapragasam and Vasil 1990; Eksomtramage *et al.* 1992). Differences in sensitivity to the different cryoprotective mixtures were noted among clones, as previously reported by Eksomtramage *et al.* (1992).

Previous experiments (Jian *et al.* 1987; Eksomtramage *et al.* 1992) using a classical freezing procedure had indicated that sugarcane embryogenic calluses could be successfully frozen using a relatively wide range of cooling rates, between 0.1 and 1°C/min. This allowed us to obtain positive results after freezing the embryogenic calluses with a cooling rate ranging between 0.4 and 0.6°C/min.

In this work, the use of a simplified freezing protocol achieved good survival and numerous plantlets from regenerating calluses of all sugarcane varieties. The variety CP 5243 gave a much higher survival rate and production of plantlets than the other two varieties tested. However, it is important to mention that control calluses of these two varieties (C 91-301 and C 1051-73) had a much slower growth rate and released a large amount of phenolic compounds in the medium. This underlines the importance of the *in vitro* propagation procedure in the successful establishment of a cryopreservation protocol for any given material.

Extending storage duration in liquid nitrogen to 14 months did not induce any modification in the survival rate and plantlet production of cryopreserved calluses, which confirms the applicability of cryopreservation for the long-term conservation of material produced *in vitro*.

In conclusion, efficient and simple cryopreservation protocols have been developed for cell suspensions, embryogenic calluses and apices of sugarcane and successfully applied to a wide range of genotypes. Sugarcane could thus be one of the first tropical crops to which cryopreservation is applied routinely.

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