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CRYOPRESERVATION OF ENCAPSULATED SUGARCANE APICES: EFFECT OF STORAGE TEMPERATURE AND STORAGE DURATION

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SUMMARY: Apices sampled on *in vitro* plantlets of sugarcane were cryopreserved using the encapsulation-dehydration technique, comprising pregrowth for 24 h in liquid medium with 0.75M sucrose, desiccation to 20-25% moisture content (fresh weight basis) and rapid immersion in liquid nitrogen. Apices of one variety were stored for up to one year at -196°C, -70°C or -25°C, with or without previous transitory immersion in liquid nitrogen. Whereas the recovery percentage of apices stored at -196°C remained high and constant throughout the experiment, recovery of apices conserved at -70°C and -25°C decreased dramatically within 0.5 day, and was nil after 10 or 120 days in storage at -25°C and -70°C, respectively. The recovery percentage of apices of four additional varieties remained stable throughout a 1 year storage period at -196°C. No differences were noted for 6 agronomic traits between plants regenerated from control and cryopreserved apices of two sugarcane varieties.

KEYWORDS: sugarcane; apex; cryopreservation; storage temperature; storage duration; genetic stability.

INTRODUCTION

Cryopreservation protocols have been established for cell suspensions, calluses, apices, zygotic and somatic embryos of numerous species (2, 7). Samples are usually stored immersed in liquid nitrogen at -196°C, or in the vapours of liquid nitrogen at approximately -150°C. However, several authors have experimented higher storage temperatures (-12 to -150°C) using domestic and laboratory deep freezers (5, 14, 18, 20). This could represent an interesting alternative for laboratories where liquid nitrogen supply is difficult or limited.

There is an increasing amount of evidence which shows that cryopreservation does not affect trueness to type of regenerated material (7). However, only a limited number of reports are available on the field evaluation of agronomic traits of whole plants regenerated from cryopreservation (14, 16, 19).

In the case of sugarcane, cryopreservation of apices has been achieved using the encapsulation-dehydration technique (7, 11). The application of a protocol developed on fifteen different varieties allowed high and reproducible recovery rates to be achieved, irrespective of the geographic origin and growth habit *in vitro* of the plant material (8, 16). As regards the assessment of genetic stability of plants regenerated from cryopreserved samples, it was demonstrated with two varieties that the electrophoretic profiles of two isoenzymatic systems were not modified by cryopreservation (9, 17).

In the present work, we observed the effect of storage temperature and storage period on the recovery of apices of several sugarcane varieties cryopreserved using the encapsulation-dehydration technique. Six agronomic traits were also evaluated on field-grown plants regenerated from control and cryopreserved apices of two different sugarcane varieties.

MATERIAL AND METHODS

Plant material

In vitro plantlets of sugarcane varieties C266-70, C87-51, B4362, B34104 and Ja 60-5 maintained at CNIC, La Habana were used for these experiments.

Methods

In vitro *culture*

Mother-plants were cultivated on semi-solid MS medium supplemented with 0.1 mg/L 6-benzylaminopurine (BAP), 1 mg/L kinetin (KIN), 0.5 mg/L indole acetic acid (AIA) and 20 g/L sucrose (11). Plantlets were cultivated at 26±1°C, under a photoperiod of 16 h light/8 h dark with a photon dose of 36 $\mu mol.m^{-2}s^{-1}$. Subcultures were performed every 4 weeks.

Cryopreservation protocol

For cryopreservation experiments, apices of 0.5-1.0 mm were sampled on *in vitro* plantlets and subjected to the encapsulation-dehydration protocol established by González-Arnao *et al.* (11, 12). After an overnight recovery period on standard medium following dissection, apices were encapsulated in calcium alginate (3%) beads with a diameter of approximately 4 mm, pregrown for 24 h in liquid medium with 0.75M sucrose, desiccated to 20-25% moisture content (MC, fresh weight basis) and immersed directly in liquid nitrogen. For rewarming, the encapsulated apices were placed in Petri dishes under the air current of the laminar flow cabinet for 2-3 min. Recovery of encapsulated apices took place on semi-solid medium containing 0.5 mg/L BAP, 0.1 mg/L KIN, 2.5 g/L activated charcoal and 20 g/L sucrose. After one week in the dark, apices were transferred to standard conditions of illumination.

Recovery assessment

Recovery was evaluated after 5 weeks by counting the number of apices that turned green and broke the synthetic beads, developing into new plantlets. Three replicates of 50 apices were used per experimental condition. The results presented correspond to the average recovery of these 3 replicates. They were analyzed statistically using the Newman and Keuls test ($p < 0.05$).

Storage experiments

Apices of variety C266-70 were stored at -196°C, -70°C or -25°C, with or without previous transitory immersion (1h) in liquid nitrogen, and their recovery measured after various storage periods (0.5d, 10d, 120d, 180d and 365d). The recovery of apices of four additional varieties (C87-51, B4362, B34104, Ja60-5) stored at -196°C was measured after the same storage periods.

Evaluation of agronomic traits

In vitro plantlets of varieties C87-51 and B34104, regenerated from control and cryopreserved apices were multiplied *in vitro* for 3 months (3 subcultures), rooted, then transferred to the greenhouse and planted in the field. Six agronomic traits were evaluated on control and cryopreserved plants: number, weight, height, diameter of stems, number of active leaves per stem and Brix (i.e. % total soluble solids). The observations were performed on three groups of 10 plants randomly chosen among the 800 plants of each variety planted in the field. The results correspond to the average of the measurements performed on the three groups of plants.

RESULTS

Recovery of apices stored in liquid nitrogen remained constant (around 80%) throughout the experiment (Table 1). By contrast, viability of samples at -70°C and -25°C decreased drastically after 0.5 d in storage and was nil after 10 d (-25°C) and 120 d (-70°C). The transitory immersion in liquid nitrogen of apices before their transfer to higher temperatures had no effect on their recovery.

Table 1: Effect of storage temperature and duration on the recovery (%) of apices of sugarcane var. C266-70, without (-LN) or with (+LN) previous transitory immersion in liquid nitrogen. Figures followed by different letters are significantly different ($p < 0.05$).

Storage Period (d)	Storage temperature				
	-25°C		-70°C		-196°C
	-LN	+LN	-LN	+LN	
0.5	25±4 c	12±2 d	18±3 c	32±2 b	86±4 a
10	0 e	0 e	0 e	26±4 c	84±4 a
120	0 e	0 e	0 e	0 e	80±6 a
180	0 e	0 e	0 e	0 e	80±7 a
365	0 e	0 e	0 e	0 e	81±6 a

The sugarcane varieties employed had very different recovery percentages, ranging from 14% (Ja60-5) to 86% (C266-70) (Table 2). However, no significant difference in their recovery was observed throughout the storage experiment. Regrowth of cryopreserved apices occurred directly, without callus formation.

Table 2. Effect of storage duration in liquid nitrogen on the recovery (%) of cryopreserved apices of different sugarcane varieties. -: not tested.

	Storage duration (days)				
Variety	0.5	10	120	180	365
C. 266-70	86±4	84±4	80±6	80±7	81±6
C. 87-51	70±4	68±3	67±6	66±5	67±7
B 4362	76±5	74±6	68±7	68±7	67±7
B 34104	67±4	-	67±5	65±4	66±6
Ja 60-5	14±5	-	-	20±5	21±3

No significant differences between plants regenerated from control and cryopreserved apices were noted in the six agronomic traits observed (Table 3). The morphological development of plants under field conditions took place according that normally observed with each sugarcane variety.

Table 3: Evaluation of some agronomic traits on *in vivo* plants of sugarcane varieties B34104 and C87-51 regenerated from control (-LN) and cryopreserved (+LN) apices.

	Variety B34104		Variety C87-51	
Agronomic trait	-LN	+LN	-LN	+LN
Number of stems/plant	12.8±2.0	10.5±3.6	15.6±2.6	14.0±3.3
Stem diameter (mm)	32.3±3.4	29.7±2.8	28.4±4.4	37.3±6.1
Stem height (mm)	277.0±10.2	256.0±11.3	266.0±4.2	267.0±5.3
Number of active leaves/stem	10.8±2.0	10.6±2.6	9.6±1.8	9.7±2.3
Stem weight (Kg)	1.6±0.3	1.5±0.2	1.6±0.3	1.5±0.4
°Brix	20.6±1.4	20.5±1.8	20.8±2.0	19.5±1.5

DISCUSSION/CONCLUSION

In this study, apices sampled on *in vitro* plantlets of different varieties could be cryopreserved using the encapsulation-dehydration technique and stored for one year at the temperature of liquid nitrogen without modification in their recovery percentage. By contrast, apices placed at -70°C or -25°C lost viability very rapidly. It was also shown that cryopreservation did not affect six agronomic traits, which were evaluated on field-grown plants regenerated from control and cryopreserved apices. The present work therefore confirmed that storage in liquid nitrogen is a reliable method for germplasm conservation, both in terms of recovery percentage and stability of the plant material.

As regards the effect of storage duration in liquid nitrogen on the regeneration of plant material, all studies performed to date did not reveal any difference as observed notably with apices of potato and cassava (48 months in storage; 1), pear (36 months; 20), mulberry (36 months; 15), carnation (36 months; 21), embryogenic suspensions of white spruce (48 months; 16) and somatic embryos of oil palm (52 months; 6).

With regards to storage of samples at different negative temperatures, results vary depending on the temperature range and the plant material. In all experiments where samples were stored at temperatures close to 0°C, comprized between -12 and -25°C, survival dropped rapidly to zero, regardless of whether or not the explants had been temporarily immersed in liquid nitrogen before storage (5, 18, 20). Similar observations were made with encapsulated sugarcane apices. There are several explanations for this result: even though vitrification of internal solutes has been observed during freezing of these materials, including sugarcane (10) devitrification and recrystallization processes, which are detrimental to cellular integrity, take place at these temperatures (13). Damages to the samples caused by free radical attacks also occur (3). At lower storage temperatures, in the range of -70 to -80°C, different results have been obtained: no modification was noted in the recovery percentage of oil palm somatic embryos and pear apices after storage for 6 months at -80°C and one year at -75°C, respectively (5, 20), whereas a rapid decrease in survival was noted with citrus embryogenic callus cultures (18), apple shoot tips (14), as well as sugarcane apices, as shown in the present study. These contrasting results might be linked to the presence of higher levels of residual free water in the latter systems, which would recrystallize rapidly at these temperatures and result in the death of the explants. At lower temperatures comprised between -135 and -196°C, no differences were noted in the regrowth capacity of all materials mentioned above whatever the storage duration tested.

The results obtained concerning the stability of the six agronomic characters observed on field-grown plants of two sugarcane varieties confirm similar information already available with several other plans such as banana, (4) white spruce (16), potato (19) and oil palm (8).

In conclusion, the additional information provided by this study concerning the stability of regrowth capacities of sugarcane over extended storage periods at the temperature of liquid nitrogen, as well as the trueness to type of plants regenerated from cryopreserved apices calls for the rapid routine application of cryopreservation for the long-term conservation of sugarcane germplasm.

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