Cryopreservation of sugarcane (*Saccharum* sp.) shoot tips using encapsulation-dehydration and droplet-vitrification

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1. Introduction

Over last years cryopreservation has been widely studied for its potential for long-term storage of plant germplasm. It allows overcoming the problems linked with field conservation (necessity of space and maintenance, risks of abiotic and biotic stresses) and with *in vitro* slow growth storage (high maintenance costs, risks of contaminations and somaclonal variation). Cryopreservation of sugarcane shoot tips, has already been successfully achieved using the encapsulation-dehydration technique (E-D, Gonzalez-Arnao *et al*. 1993; Paulet *et al*. 1993; Gonzalez-Arnao *et al*. 1999). New vitrification-based techniques have been developed recently. They could represent an opportunity to improve the efficiency of sugarcane cryopreservation. We thus compared E-D with the new droplet-vitrification technique (D-V, Panis *et al*. 2005).

2. Materials and methods

2.1. Plant material

Two sugarcane commercial clones were used in our experiments, H70-144, from Hawaii, and CP68-1026, from Canal Point (USA). Mother plants were transferred monthly on semi-solid Murashige and Skoog (1962) medium, enriched with 60 g L^{-1} sucrose and 7 g L^{-1} agar, at 27±1 °C, under a 12 h d^{-1}photoperiod, with a photon dose of 50 µmol m^{-2} s^{-1}.

2.2. Cryopreservation

For cryopreservation experiments we used explants of 0.5-1 mm in length consisting of the apical meristem, one or two leaf primordia and a basal part, which were dissected from *in vitro* plantlets 30-40 days after the last transfer. Dissected shoot tips were maintained overnight on standard medium in the dark to minimize the dissection stress.

During the E-D protocol, apices were encapsulated in 3 % calcium alginate beads (diameter of 4-5 mm) and cultured for 24 h in 0.75 M sucrose liquid medium. These pretreatment conditions had been established by Gonzalez-Arnao *et al*. (1993). The encapsulated shoot tips were dehydrated in containers (10 apices per container) filled with 80 g silica gel to moisture contents (MC, fresh weight basis) between 35 and 20 %, and then placed in 2 ml sterile polypropylene cryovials and plunged directly in liquid nitrogen (LN), where they were kept for a minimum of 15 min. Beads were rewarmed by transferring them directly on recovery medium in Petri dishes.

For D-V, after overnight recovery from dissection, explants were pretreated for 20 min in loading solution (2 M glycerol and 0.4 M sucrose) and osmotically dehydrated with PVS2 (30 % glycerol, 15% DMSO, 15% EG, 13.7% sucrose; Sakai *et al*. 1990), at 0 °C for 20 to 80

min or PVS3 (50 % glycerol and 50 % sucrose; Nishizawa *et al*. 1993) at room temperature for 20 to 100 min. Cooling was performed placing explants in 10 µL PVS droplets on aluminum foil strips and plugging them directly in LN. For rewarming, the aluminum foils were plunged rapidly in unloading solution containing 1.2 M sucrose for 20 min; explants were then transferred on recovery medium.
Recovery medium consisted of semi-solid MS medium with 30 g L^{-1} sucrose, 0.2 mg L^{-1} 6-benzylaminopurine (BAP), 0.1 mg L^{-1} kinetin, 7 g L^{-1} agar and 1 g L^{-1} Plant Preservative Mixture (PPM) to avoid the proliferation of endophytic bacteria. Shoot tips were kept in the dark for seven days after cryopreservation, then transferred under standard culture conditions.

2.3. Viability and plant growth
Results were collected by recording survival and recovery. Survival was evaluated after 10 days, by counting the explants showing green pigmentation and swelling. Recovery was measured after 40 days on regrowth medium, by counting the explants producing normal shoots through direct organogenesis. Results, presented in percent of plants surviving/recovering over the total number of plants treated per experimental condition, were analyzed using ANOVA following arcsin transformation, with Duncan's multiple range test (DMRT), using the SPSS 14.0 software.

3. Results

Explants of both clones tested were able to withstand cryopreservation. With E-D, apices of both clones displayed high survival and recovery, 60 % for H70-144 and 53 % for CP68-1026, after dehydration to around 20 % moisture content (Table 1). Regrowth of cryopreserved apices was rapid and direct (Fig. 1).
Following the D-V protocol, the highest recovery percentages were achieved for treatment durations of 20 and 40 min with PVS2 and PVS3, respectively (Table 1). The two clones reacted differently to the vitrification solutions employed. Following the treatment with PVS2, recovery was 37% for H70-144 and only 20% for CP68-1026. By contrast, following treatment with PVS3, recovery was similar for both clones, reaching 33% for H70-144 and 27% for CP68-1026. Regrowth of cryopreserved apices was also rapid and direct (Fig. 2).
When comparing E-D and D-V with the two clones tested (Table 1) it appeared that E-D ensured higher recovery after cryopreservation.

Table 1: Survival and recovery (%) of control and cryopreserved shoot tips of both clones treated by D-V (using PVS 2 and PVS 3) and E-D.

Clone/ treatment	Parameter	Protocol		
		PVS2	PVS3	ED
H70-144/ -LN	Survival	77 a	83 a	50 b
	Recovery	67 ab	73 a	47 b
H70-144/ +LN	Survival	43 a	53 a	60 a
	Recovery	37 b	33 b	60 a
CP68-1026/ -LN	Survival	50 b	87 a	83 a
	Recovery	50 b	87 a	83 a
CP68-1026/ +LN	Survival	23 b	40 ab	53 a
	Recovery	20 b	27 b	53 a

Values followed by the same letter in the same row are not significantly different at the 0.05 probability level.
In D-V samples were treated with VSs for 20 min for clone H70-144 and 40 min for clone CP68-1026; In E-D freezing was performed after dehydration to 20 % MC.

4. Discussion

Our results showed that sugarcane apices could be successfully cryopreserved using both E-D and D-V. They are in accordance with the results of Gonzalez-Arnao *et al*. (1993, 1999) and of Paulet *et al*. (1993) concerning the use of E-D. However, it is the first report of cryopreservation of sugarcane apices using D-V. E-D appeared more efficient compared to D-V, since shoots could be recovered from almost all explants which withstood cryopreservation. With D-V, the decrease noted between survival and recovery was due to the high toxicity of the PVSs employed (Kim *et al*. 2009). However, both techniques can be employed for cryopreservation of sugarcane, as they ensure rapid regrowth of phenotypically normal *in vitro* plantlets without callus formation from cryopreserved apices; this should be linked to the histological studies carried out by Gonzalez-Arnao *et al*. (1993), which showed that a high percentage of cells in the meristematic area of apices were still alive after cryopreservation. In conclusion, optimization of the successive steps of both protocols is still required to improve recovery of apices after cryopreservation, especially in the case of D-V, for which only preliminary investigations have been performed.

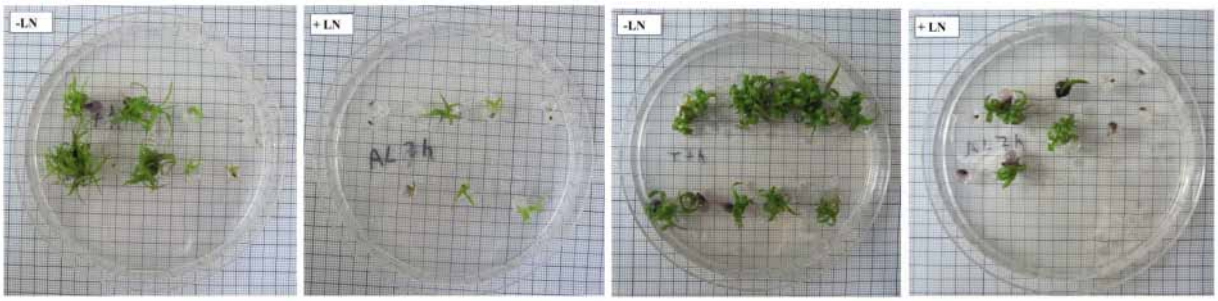

Figure 1: Survival and recovery of control (-LN) and cryopreserved (+LN) shoot tips of clone H70-144 (left) and CP68-1026 (right) treated by E-D.

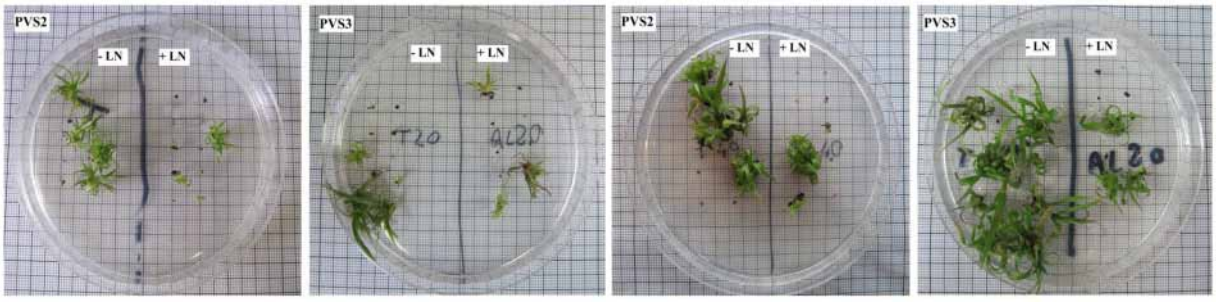

Figure 2: Recovery of control (-LN) and cryopreserved (+LN) shoot tips of clone H70-144 (left) and CP68-1026 treated by D-V using PVS 2 and PVS 3.

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