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Cryopreservation of apices of *in vitro* plantlets of sugarcane (*Saccharum* sp. hybrids) using encapsulation/dehydration

Florence Paulet [1], Florent Engelmann [2], and Jean-Christophe Glaszmann [1]

[1] CIRAD-CA, BP 5035, 34032 Montpellier cedex 01, France
[2] ORSTOM, Laboratoire de Ressources Génétiques et Amelioration des Plantes Tropicales, BP 5045, 34032 Montpellier cedex 01, France

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Summary: A cryopreservation process using encapsulation/dehydration was set up for apices sampled on *in vitro* plantlets of sugarcane. After dissection, apices were cultured for one day on standard medium and then encapsulated in medium with 3% alginate. Optimal conditions comprised preculture for 2 days in liquid medium with 250 $g.l^{-1}$ sucrose, desiccation for 6 hours under the laminar flow or for 10-11 hours with silicagel followed by rapid freezing and slow thawing. Survival after freezing in liquid nitrogen ranged between 38 and 91% for the 5 varieties experimented. Cryopreservation did not modify the electrophoretic profiles for aminoleucine peptidases and amylases with plants of the variety Co 6415.

Key-words: Sugarcane - *Saccharum* sp. hybrids - Apex -Cryopreservation - Encapsulation/dehydration - Germplasm conservation

Abbreviations: BAP: 6-benzylaminopurine - KIN: Kinetin - EDTA: ethylenediamine tetracetic acid - AMP: aminoleucine peptidases - AMY: amylases - RFLP: restriction fragment length polymorphism

Introduction

Sugarcane germplasm is conserved as *ex situ* collections of plants in several locations in India and in the USA. However, maintenance costs of large collections are very high and plants in natural conditions remain exposed to pests and pathogens as well as to natural disasters. Thus, 61 % of the clones of the US collections have been lost between 1957 and 1977 (Berding and Roach 1987). *In vitro* collections have been developed for a large number of plant species, which allow the reduction of these problems (Engelmann 1991a). In the case of sugarcane, the *in vitro* collection of CIRAD-CA Montpellier presently comprises more than 650 varieties which are conserved under slow growth conditions (Paulet *et al.* 1991). However, the maintenance of large *in vitro* collections is time consuming and the risks of contamination and of somaclonal variation increase with time (Withers 1987). Only cryopreservation (liquid nitrogen, -196°C) presently offers a long-term conservation option.

Cryopreservation has been applied presently to more than 70 plant species (Dereuddre and Engelmann 1987), among which around 40 species from tropical origin (Engelmann 1991a). With sugarcane, cryopreservation techniques have been developed with cell suspensions (Ulrich *et al.* 1984; Bajaj *et al.* 1987; Ling *et al.* 1987; Gnanapragasam and Vasil 1990) and embryogenic calluses (Bajaj *et al.* 1987; Ling *et al.* 1987; Eksomtramage *et al.* 1992a). One preliminary attempt (Bajaj *et al.* 1987) has been performed using meristems sampled on *in vivo* plants. In the latter case recovery of frozen material occurred in the form of limited callusing only and no plants could be regenerated.

Apices appear as ideal candidates for long-term germplasm conservation. Indeed, the constituent cells of shoot meristems are little differentiated and genetically stable, thus leading to maintenance of genetic stability in regenerated progenies in greater proportions when compared to other methods of *in vitro* plant regeneration such as those using embryogenic calluses or cell suspensions (Kartha 1985).

A new cryopreservation technique, encapsulation/dehydration, was developed recently for meristems (Dereuddre *et al.* 1990). It was successfully applied to several temperate plant species (Dereuddre 1992) and to one tropical species only, cassava (Benson *et al.* 1992). In this technique, apices are encapsulated in alginate beads, precultured in liquid medium with a high sucrose concentration, and partially desiccated before freezing in liquid nitrogen. Encapsulation allows apices to withstand drastic treatments (preculture with high sucrose, desiccation) which would be harmful to naked apices. Sugars play a very important role in the acquisition of resistance to desiccation (Crowe *et al.* 1988) and to freezing in liquid nitrogen (Dumet *et al.* personal communication).

Correspondence to: F. Engelmann

Preculture in the presence of high sucrose concentrations induces a dramatic increase of intracellular sugar concentration as observed by Uragami (1990) with *Asparagus* apices.

In this paper, we report the first successful attempt of the utilization of encapsulation/dehydration for the cryopreservation of apices of sugarcane.

Material and Methods

Plant material

Plant material consisted of apices sampled on *in vitro* plants of different sugarcane varieties from the collection of CIRAD-CA Montpellier. Two varieties originated from Coimbatore, India (Co 740 and Co 6415), one from Queensland, Australia (Q 90), one from Barbados (B 69566) and one from Canal Point, USA (CP 681026).

Explants consisted of the meristematic dome with 1 to 3 foliar primordia and a basal part. They measured 0.5±0.1 mm. Apices were sampled on shoots measuring 8-10 cm, 15 days after their last transfer.

Methods

- Maintenance of plants under slow growth

Plants of the *in vitro* collection were cultured on the basal medium defined by Chagvardieff (1980) comprizing Murashige and Skoog's (1962) macro- and microelements, Fuji's (1970) vitamins, 27.8 $mg.l^{-1}$ Fe EDTA, 20 $g.l^{-1}$ sucrose, solidified with 6 $g.l^{-1}$ agar (Touzard et Matignon). They were maintained at 18±1°C with a photoperiod of 12 hours light with a photon dose of 36 $\mu mol.m^{-2}s^{-1}$.

- Multiplication of plants before cryopreservation

Plants used for cryopreservation experiments were submitted to 5-6 monthly transfers on medium M 50 (basal medium + 50 $g.l^{-1}$ sucrose) before sampling of the apices. This medium proved to be optimal to stimulate growth and shoot multiplication (Paulet, unpublished). Plants were placed at 25±1°C under the same lighting conditions.

- Cryopreservation

After dissection, apices were placed for 24 hours on basal medium with 34 $g.l^{-1}$ sucrose. This sucrose concentration was determined as optimal for growth and recovery of apices (Paulet, unpublished). They were then encapsulated in the same medium containing 3% alginate. Preculture of apices in beads (4-5 mm diameter) consisted of a culture for 0-7 days in liquid medium supplemented with various concentrations of sucrose (34 to 427 $g.l^{-1}$). These sucrose concentrations are those commonly employed in encapsulation/dehydration processes (Dereuddre 1992). Erlenmeyer flasks containing the beads with preculture medium were placed on a rotary shaker (103 rpm). Beads were then desiccated either by placing them for 0 to 6 hours in the air current of a laminar flow cabinet or in 60 ml air-tight boxes containing 40 g of silicagel for 10 or 11 hours, according to the method of Dumet *et al.* (1993).

After desiccation, beads were placed in 2 ml polypropylene sterile cryovials which were immersed rapidly in liquid nitrogen where they were conserved for 1 hour. They were then thawed by placing the cryotubes for 10-15 min in the air current of the laminar flow at room temperature. Beads were then transferred to the recovery medium and cultivated as described previously.

The cryopreservation process was set up with the variety Co 6415 and then experimented with the four other varieties.

- Recovery of encapsulated meristems.

After cryopreservation, the encapsulated apices were cultured according to the following sequence:

- 1 week in the dark, on basal medium with 34 $g.l^{-1}$ sucrose, supplemented with 0.2 $mg.l^{-1}$ BAP and 0.1 $mg.l^{-1}$ KIN.
- 4-5 weeks under standard lighting conditions (photoperiod of 12 hours light, with a photon dose of 36 $\mu mol.m^{-2}s^{-1}$), on basal medium with 34 $g.l^{-1}$ sucrose, supplemented with 3.5 $g.l^{-1}$ activated charcoal.

Regenerated plantlets were then transferred to medium M 50 for regrowth, multiplication and rooting. Once they had reached a sufficient development (after 4-6 weeks), they were transferred to *in vivo* conditions and weaned according to the weaning process developed by CIRAD-CA (Paulet *et al.*1991).

- Recovery and viability assessment

Examination of the samples for survival was performed five weeks after thawing. Apices were considered alive after the various treatments when they had turned green and produced leaves. Controls consisted of apices which were submitted to preculture and desiccation treatments but not cryopreserved. Ten to 35 apices per condition were used in the different experiments.

- Stability test

Isoenzyme electrophoresis was performed on *in vivo* plants of the variety Co 6415 coming from the *in vitro* collection and from control and cryopreserved apices, using the technique developed by Feldmann (1984). Two isoenzymatic systems were studied: AMP and AMY.

Results

Survival of apices after a 24-hour preculture period varied depending on the sucrose concentration of the preculture medium and on the desiccation duration (Table 1). The highest sucrose concentrations (342 and 427 $g.l^{-1}$) were toxic whatever the dehydration duration. After preculture with 34 $g.l^{-1}$ sucrose, meristems did not withstand desiccation equal to or longer than 4 hours. Survival remained high up to 4 hours desiccation for intermediate sucrose concentrations (102 to 250 $g.l^{-1}$). After 6 hours dehydration, survival dropped for sucrose concentrations of 102 and 170 $g.l^{-1}$, but remained relatively high (8/15) for 250 $g.l^{-1}$ sugar. Preculture for 24 hours with 250 $g.l^{-1}$ sucrose thus allowed extended dehydration of apices whilst limiting their mortality.

Table 1: Number of apices surviving after a 24-hour preculture with different sucrose concentrations followed by various desiccation durations. 15 apices per condition were used.

Sucrose concentration (g/l)	Desiccation duration (hrs) 0	2	4	6
34	15	15	0	0
102	15	13	13	3
171	14	15	10	3
250	14	11	12	8
342	5	3	1	2
427	6	0	2	2

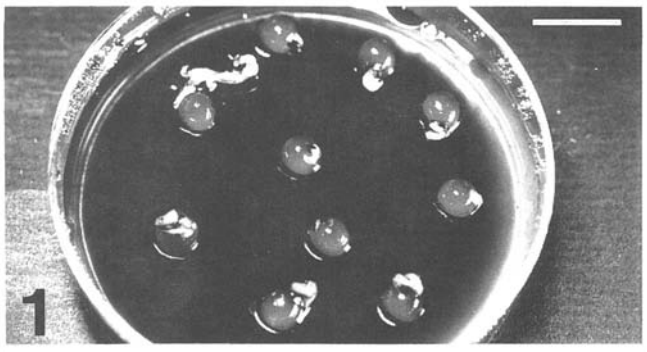

Figure 1: Growth recovery of cryopreserved apices ten days after thawing (Scale bar: 1 cm).

Table 2: Number of control (-LN) and cryopreserved (+LN) apices surviving after preculture in liquid medium with 250 $g.l^{-1}$ sucrose and various desiccation durations. 10 apices per condition were used.

	Preculture duration (days)									
Desiccation duration	0		1		2		3		7	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
4 hrs	0	0	9	7	8	8	8	4	7	5
6 hrs	0	0	9	9	9	9	9	6	7	6

The effect of extending the preculture duration in medium with 250 $g.l^{-1}$ sugar was studied (Table 2).

Preculture was necessary to obtain survival of control and cryopreserved apices whatever the desiccation duration. Survival of control and cryopreserved apices decreased slightly, in line with increasing preculture durations. For the shortest preculture treatments (1 and 2 days) cryopreservation had no effect on survival of the apices. For longer preculture durations (3 and 7 days), survival of cryopreserved apices decreased slightly in comparison with that of the controls. Growth recovery of control and cryopreserved apices was very rapid with only a short delay for cryopreserved material. Elongation of foliar primordia was observed within 1 week after thawing (Fig. 1). Rooted plantlets could be obtained after 4 weeks of culture (Fig. 2). They were then transferred onto medium M 50 for further growth (Fig. 3) and later to the greenhouse (Fig. 4).

Figure 2: Rooted plantlets originating from control (-LN) and cryopreserved (+LN) apices four weeks after thawing. (Scale bar: 1 cm).

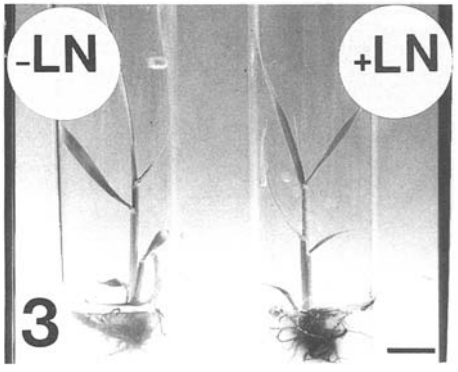

Figure 3: Plantlets originating from control (-LN) and cryopreserved (+LN) apices on medium M 50. (Scale bar: 1 cm).

Figure 4: Plantlets originating from cryopreserved apices after acclimatization. (Scale bar: 10 cm).

No difference was observed in the morphological development of plants coming from control and cryopreserved apices. After 6 hours of dehydration under the laminar flow, the water content of beads was around 0.5 g water/g dry weight. Similar water contents were obtained after 10-11 hours dehydration with silicagel. Survival of control and cryopreserved meristems was equivalent whatever the desiccation process (Table 3).

Table 3: Number of control (-LN) and cryopreserved (+LN) apices surviving after different desiccation processes. 10 apices per condition were used.

Desiccation process					
Laminar flow (6 hrs)		Silicagel (10 hrs)		Silicagel (11 hrs)	
-LN	+LN	-LN	+LN	-LN	+LN
9	6	6	6	6	6

When apices stored in liquid nitrogen are utilized, they have to be replaced as soon as possible using some of the plants regenerated from the thawed material. Therefore, we compared the results obtained with apices submitted to one freeze-thaw cycle and with apices sampled on plants regenerated from cryopreserved cultures and submitted to a second freeze-thaw cycle (Table 4). No differences were noted

in the survival of apices after one or two freeze-thaw cycles.

Table 4: Survival of control (-LN) and cryopreserved (+LN) apices after 1 and 2 freeze-thaw cycles (FTC).

1 FTC		2 FTC	
- LN	+ LN	- LN	+ LN
8/10	6/10	8/10	11/20

In order to evaluate the stability of plants of variety Co 6415 after cryopreservation comprizing a 2-day preculture with 250g.l^{-1} sucrose and 6 hrs of dehydration, we compared the electrophoretic profiles for AMP and AMY of 14 plants coming from 14 different cryopreserved apices with 6 plants coming from control meristems and 6 plants taken from the *in vitro* collection. No differences were observed between samples: the 3 bands characteristic of AMP and the two characteristic of AMY were present with the same intensity and no new band appeared.

The protocol set up with the variety Co 6415 comprising a 2-day preculture with 250g.l^{-1} sucrose and 6 hrs of dehydration was then experimented with 4 additional commercial varieties. Survival could be obtained in all cases (Table 5). It varied between 80 and 100 % for control meristems and between 38 % (Co 740) and 91% (B 69566) for cryopreserved apices.

Discussion/Conclusion

In the present study, it was demonstrated that cryopreservation of apices of *in vitro* plantlets of sugarcane could be achieved with high survival rates using encapsulation/dehydration.

Encapsulation induced a short delay only in the development of meristems, in comparison with non-encapsulated ones as observed with most species (Dereuddre 1992). Therefore, extraction of meristems from the beads was not necessary for regrowth after freezing as in the case of pear and grape apices (Dereuddre *et al.* 1990; Plessis *et al.* 1991).

Survival of apices after cryopreservation was achieved only after a preculture treatment. Sugarcane meristems appeared tolerant to high sucrose levels since they could be precultured directly in medium with 250 g.l^{-1} sugar and extension of preculture to 7 days induced limited mortality only. Therefore it was not necessary to progressively increase the sucrose concentration of the preculture medium as for grape meristems (Plessis *et al.* 1991).

The growth recovery conditions were very efficient since regrowth occurred very rapidly with only a slight delay between control and frozen meristems. Culture in the dark immediately after thawing may have improved recovery by limiting the detrimental oxidative effects of light (Benson and Noronha-Dutra 1988; Benson *et al.* 1989). Also, addition of activated charcoal to the medium when apices were transferred to lit conditions may have a positive effect by adsorbing toxic phenolic compounds which are produced in large quantities by sugarcane tissues. Growth recovery seemed to be direct without any transitory callus formation. However, this should be confirmed by an histological study of early phases of recovery.

No differences were noted in the survival rates of apices after one or two freeze-thaw cycles. Similar results were obtained with oil palm somatic embryos after 4 freeze-thaw cycles (Engelmann 1991b) and grape embryogenic cell suspensions after two freeze-thaw cycles (Dussert *et al.* 1992). On the contrary, resistance to freezing in liquid nitrogen progressively increased in line with the number of freeze-thaw cycles in the case of *Lavandula* and rice cell suspensions (Watanabe *et al.* 1985; 1990). Similarly, cold tolerance of plants regenerated from maize embryogenic calluses increased after successive freezings (Kendall *et al.* 1990). Therefore, the effect of an increased number of freeze-thaw cycles on the recovery of sugarcane apices should be experimented.

Genetic stability of cryopreserved material was tested by AMP and AMY electrophoresis. This was, however, a very preliminary study, since it concerned only 14 plants and two isozymes; it was conducted for comparison with a parallel study on plants regenerated from cryopreserved calluses with the technique developed by Eksomtramage *et al.* (1992b) which revealed variation for several isozymes including AMP.

Table 5: Survival of control (-LN) and cryopreserved (+LN) apices of different commercial varieties (* = total of 3 experiments).

B 69566		CP 681026		Q 90		Co 740		Co 6415 *	
-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
10/10	32/35 (91%)	10/10	18/28 (64%)	10/10	14/17 (82%)	4/5	7/18 (38%)	104/130 (80%)	83/130 (64%)

This latter study has progressed since then and nuclear as well as mitochondrial RFLP markers were found to be affected. In all cases, the modifications concerned the loss of genetic information (Eksomtramage 1993). However, these modifications could not be attributed to cryopreservation but they may be more likely due to the *in vitro* culture process. The question of how best to investigate genetic stability is unsolved. Testing the genetic stability with field experiments after *in vitro* culture is very labour intensive with sugarcane for specific reasons such as its vegetative multiplication which allows a long remnancy of contingent temporary physiological disturbances (Peros *et al.* 1989); the high coefficients of variation usually observed in field trials (Skinner *et al.* 1987), or; the necessity to study the plant crop as well as several ratoon crops. On the other hand, genetic changes induced by *in vitro* manipulation seem to be largely detected by molecular markers. Therefore, the genetic stability of materials regenerated from cryopreserved apices should be firstly investigated with molecular markers covering the whole genome. The development of molecular maps for sugarcane genome (Glaszmann *et al.* in press) will facilitate this process. Finally, the cryopreservation process developed with the variety Co 6415 could be applied to 4 additional varieties with satisfactory results. It was recently experimented successfully with 3 other varieties in a second laboratory in La Habana, Cuba (Gonzalez-Arnao *et al.* unpublished). The varieties used in the present study were chosen for the diversity of their geographic origin as well as for their different behaviour under *in vitro* conditions. Good results obtained when freezing the variety CP 681026 which is difficult to propagate *in vitro* allow to think that the present cryopreservation process will be applicable to any variety of sugarcane which has been introduced *in vitro*.

In conclusion, the safe long-term storage of sugarcane germplasm may be foreseable in a not too distant future using cryopreservation of encapsulated apices.

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