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CRYOPRESERVATION OF ENCAPSULATED APICES OF SUGARCANE: EFFECT OF FREEZING PROCEDURE AND HISTOLOGY

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SUMMARY: The effect of sucrose concentration during the pregrowth treatment and of the freezing procedure on the survival of encapsulated apices of 6 sugarcane varieties was investigated. The optimal sucrose concentration was 0.75M. Survival was noted with both rapid freezing by direct immersion in liquid nitrogen and slow freezing using a programmable freezer but it was generally higher after rapid cooling. Important genotypic variation was observed for both parameters studied. Histological examination revealed that cells were slightly harmed during pregrowth and freezing. Apices could withstand freezing as a whole. Direct regrowth of cryopreserved apices occurred within 3 days after thawing.

KEY-WORDS: sugarcane, cryopreservation, apex, encapsulation/dehydration, freezing procedure, histology.

INTRODUCTION

Apices oftenly represent the material of choice for germplasm conservation of species with recalcitrant seeds and of those which reproduce asexually. Until recently, apices were cryopreserved using classical procedures including pretreatment with cryoprotectants in liquid medium and controlled slow freezing (1). However, survival rates were generally low, growth recovery was slow, often including a transitory callusing phase and results were not always reproducible. A new cryopreservation method termed encapsulation/dehydration was developed for apices. It appears to offer high survival rates, rapid growth recovery and reproducible results. It was applied firstly to species from temperate climates (2, 3, 4, 5) and experimented more recently with plants from tropical origin (6, 7, 8, 9).

In the encapsulation/dehydration process, freezing is generally rapid but slow controlled cooling proved beneficial in some cases (2, 4). With sugarcane apices, this technique has so far been tested with a total of 8 varieties but using rapid freezing only (7, 8).

In this paper, the effect of freezing procedure on the survival rate of 6 commercial sugarcane varieties was investigated and histological study of apices was performed during the whole cryopreservation process.

MATERIALS AND METHODS

Plant material

Plants of 6 commercial sugarcane varieties were used: 3 coming from the *in vitro* collection of CIRAD-CA, Montpellier (B69566, My5514, IAC54480), 3 from that of CNIC, La Habana (C8751, C26670, B34104).

In vitro **culture conditions of mother-plants**

Plants originating from CIRAD were cultivated on semi-solid medium devoid of growth regulators and containing 50 $g.l^{-1}$ sucrose (7). At CNIC, mother-plants were maintained on semi-solid medium supplemented with 0.1 $mg.l^{-1}$ 6-benzylaminopurine (BAP), 1 $mg.l^{-1}$ kinetin (KIN), 0.5 $mg.l^{-1}$ indole acetic acid (AIA) and 20 $g.l^{-1}$ sucrose (10). Plants were placed at 25±1°C, with a photoperiod of 16 hrs light/8 hrs dark with a photon dose of 36 $\mu mol.m^{-2}s^{-1}$.

Cryopreservation protocol

The cryopreservation protocol employed consisted of a combination of the conditions used by Paulet *et al.* (7) and Gonzalez Arnao *et al.* (8). After dissection, apices were placed overnight on CNIC's standard medium (8), encapsulated in the same medium supplemented with 3% alginate and pregrown for 24 hrs in liquid medium with various sucrose concentrations. Beads were dehydrated for 6 hours in the air current of the laminar flow cabinet down to 20% water content (fresh weight basis), then placed in 2 ml polypropylene sterile cryotubes and submitted to one of the three following freezing procedures: rapid freezing by direct immersion in liquid nitrogen, slow freezing either at $0.5°C.min^{-1}$ from 20°C to -40°C (Minicool, L'Air Liquide, France) or at $0.5°C.min^{-1}$ from 20°C to -40°C and $1°C.min^{-1}$ from -40°C to -80°C (Bio-Cool, FTS Systems, USA), followed by immersion in liquid nitrogen. After one hour of storage at -196°C, beads were thawed by placing the cryotubes for 10-15 min at room temperature. Recovery took place on semi-solid medium containing 0.5 $mg.l^{-1}$ BAP, 0.1 $mg.l^{-1}$ KIN, 2.5 $g.l^{-1}$ activated charcoal and 20 $g.l^{-1}$ sucrose (8). After one week in the dark, apices were transferred to standard conditions of illumination. Survival was noted after 3 weeks by counting the number of apices which had developed into shoots. Four to 30 apices were used per condition. Pregrown and desiccated apices were used as controls.

Histology

Samples were fixed for 48 hours at room temperature in a phosphate buffer (pH 7.2) containing 10% paraformaldehyde, 1% glutaraldehyde and 1% caffeine. They were then dehydrated by successive transfers in alcohol baths with progressively increasing alcohol concentrations. Inclusion was carried out in Kulser 7100 resin. Three µm thick sections were cut using an automatic microtome (Historange 2218, LKB). Samples were treated with periodic acid-Schiff reaction and naphtol blue black. This double staining technique allows specific characterization of polysaccharide compounds which stain red and the soluble and non-soluble proteins which stain blue black (11). Five apices were prepared and examined after each of the following steps: dissection, overnight culture on standard medium, 24-hr pregrowth with 0.75M sucrose, 6-hr desiccation, rapid freezing and thawing, 1, 3 and 7 days of recovery.

RESULTS

Survival of pregrown non desiccated control apices was close to 100% in all conditions (data not shown). With two varieties (B34104 and B69566) control apices pregrown with the lowest sucrose concentration (0.3 M) did not withstand the 6-hour desiccation period. In general, survival after freezing in liquid nitrogen increased in line with increasing sucrose concentrations and was optimal for 0.75M (Table 1). Higher concentrations led to a drastic decrease in survival (data not shown).There was no apparent effect of the culture medium of the mother-plants on survival of apices after the various treatments. Direct immersion in liquid nitrogen (RF) generally gave better results than controlled freezing (SF1 and SF2). However, a high genotypic effect was noted regarding the resistance to pregrowth and freezing with the different varieties used.

Figure 1A corresponds to a longitudinal section of an untreated control apex. It consisted of the apical dome, several foliar primordia and of a basal part. Cells of the apical

region displayed characteristics of meristematic cells: they had a high nucleocytoplasmic ratio, a dense cytoplasm, small vacuoles, a large nucleus with one or two apparent nucleoli. Basal cells contained a high protein concentration and numerous lipoproteic bodies (Fig. 1B).

Table 1: Effect of sucrose concentration during pregrowth and of freezing procedure on the survival rate (%) of control (Ctrl) and cryopreserved (RF, SF1, SF2) apices of 6 varieties of sugarcane.
RF: rapid freezing; SF1: controlled freezing, 0.5°C.min^{-1} to -40°C; SF2: controlled freezing, 0.5°C.min^{-1} to -40°C+ 1°C.min^{-1} from -40°C to -80°C. -: not tested.
1: mother-plants cultivated on CNIC medium; 2: mother-plants cultivated on CIRAD medium.

Sucrose (M)		C26670$_1$	C8751$_1$	B34104$_1$	My5514$_2$	IAC5448$_2$	B69566$_2$
0.3	Ctrl	63	71	0	66	87	0
	RF	0	0	0	0	10	0
	SF1	0	0	0	12	10	0
0.5	Ctrl	68	80	83	67	44	33
	RF	90	0	57	25	25	0
	SF1	93	0	17	25	22	0
	SF2	76	0	11	-	-	-
0.75	Ctrl	100	90	50	83	50	29
	RF	90	70	67	75	38	38
	SF1	90	11	17	50	13	50
	SF2	29	22	14	-	-	-

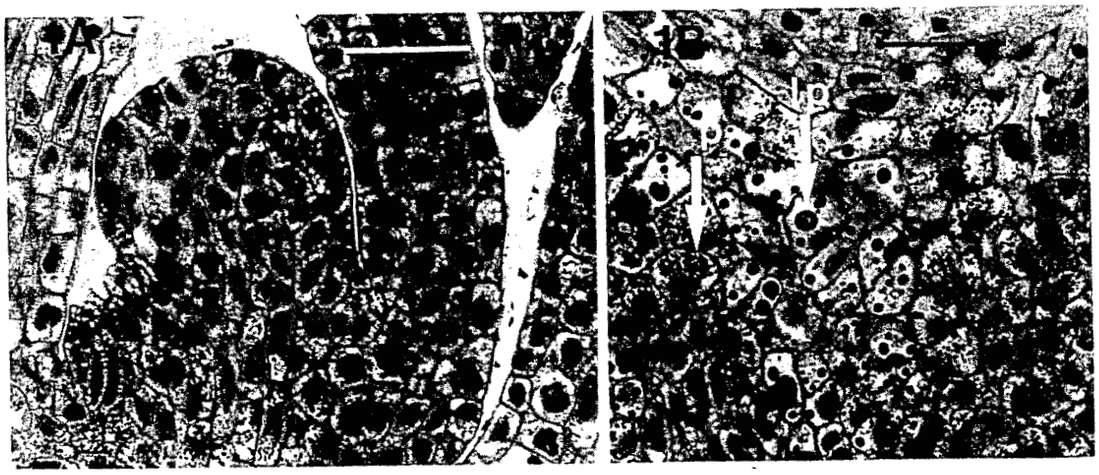

Fig. 1A: Control apex immediately after dissection. Bar: 50 µm.
Fig. 1B: Basal cells of the same apex containing numerous lipoproteic bodies (lp) and proteins (p). Bar: 50 µm.

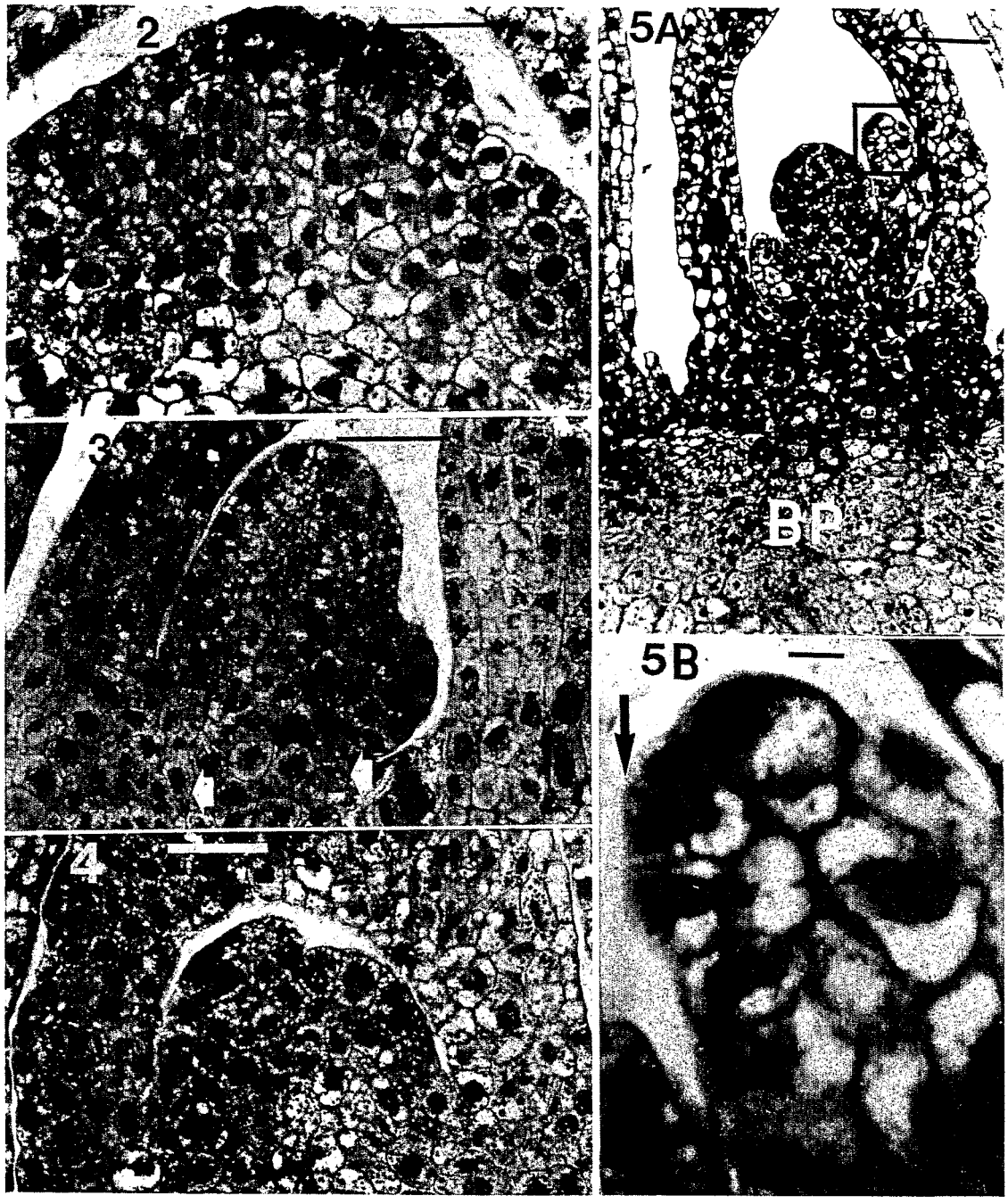

Fig. 2: Apex after overnight culture on standard medium. Note the accumulation of starch grains in cells of the meristematic region. Bar: 50 µm.
Fig. 3: Apex after pregrowth and desiccation. Note retracted and intensely stained nuclei and cytoplasm retracted from the cell wall in some cells (arrows). Bar: 50 µm.
Fig. 4: Apex 24 hrs after the freeze-thaw cycle. Bar: 50 µm.
Fig. 5A: Apex 3 days after the freeze-thaw cycle. Note damaged and destroyed cells in the basal part (BP) and in the larger primordia. Bar: 100 µm.
Fig. 5B: Magnification of boxed area on Fig. 5A: detail of the same apex showing recovery of cell divisions (arrow) in a young foliar primordium. Bar: 10 µm.

After overnight culture on standard medium, accumulation of starch was observed, mostly in cells of the meristematic region (Fig. 2). Vacuolization seemed to increase in cells of the underlying zone. An important decrease in the quantity of proteins was noted. After the 24-hr pregrowth in medium with 0.75M sucrose, synthesis of starch increased in intensity. After desiccation, the structure of cells was drastically modified (Fig. 3): nuclei were retracted and displayed a particular aspect, being intensely and uniformely stained. Nucleoli were no longer visible. The cytoplasm was oftenly retracted, detached from the cell wall. Cells of samples observed immediately after the freeze-thaw cycle displayed a similar structure. However, recovery of apices was very rapid, as shown by examining samples 24 hrs after thawing (Fig. 4). At this stage, almost all cells of the apical region had recovered their initial appearance, as noted in comparison with Fig. 1. The cytoplasm had reexpended and was densely stained and nuclei, some of which were lobed, showing several well defined nucleoli. Three days after thawing (Fig. 5A), most cells of the whole apical zone were alive whereas those of the basal part were severely damaged or destroyed. Some cells of the foliar primordia were also destroyed. Cell divisions could be observed at the extremity of one young foliar primordium (Fig. 5B).

DISCUSSION/CONCLUSION

During this work, we showed that encapsulated apices of sugarcane could withstand freezing in liquid nitrogen using various freezing procedures after a suitable pregrowth treatment with sucrose. Growth recovery of apices after thawing was very rapid and direct, due to the fact that most cells of the apical region had been only slightly harmed, as revealed by histocytological examination.

Cryoprotection with sucrose at high concentrations was employed in some cases during classical freezing processes (12, 13, 14) and is used during all encapsulation/dehydration procedures (15). In optimal pregrowth conditions, the decrease in survival rate between desiccated and cryopreserved sugarcane apices was low, thus indicating that desiccation was the key step in reducing viability and not cryopreservation. A large increase in starch content was observed during pregrowth of sugarcane apices, as noted in the case of date palm (16). This allows us to assume that intracellular concentration of free sugars was drastically increased, as measured in *Asparagus* apices (17) and oil palm somatic embryos (18). In contrast to the osmotic dehydration phenomenon occurring during a classical freezing process where only freezable water is extracted during pretreatment and prefreezing (19), the extensive dehydration performed during desiccation in air allows to remove both freezable and non freezable water (20). This was confirmed by microcalorimetric studies which showed that a glass transition only was recorded in dehydrated samples (15, 22, 23). Sucrose may protect the structural integrity of cells by stabilizing proteins and phospholipid bilayers (21).

Histological studies performed on apices of various species frozen using a classical procedure revealed that a considerable number of cells are damaged and only groups of cells, oftenly localized in primordial leaf tissues and the meristematic dome area, remained alive (16, 23, 24, 25, 26). Intense callusing (16, 27, 28, 29) and sometimes production of multiple shoots from a single meristem (23) were oftenly reported. On the contrary, the present study showed that almost all cells of the meristematic area and of young leaf primordia survived after freezing in liquid nitrogen, thus allowing rapid and direct growth recovery of the whole structure.

Contrarily to what was observed with potato and grape (2, 4) controlled freezing did not improve the survival rate of sugarcane apices. The present study also confirmed the high genotypic variability of response to cryopreservation which has already been observed in the first report on cryopreservation of sugarcane apices (7). However, from a practical point of view, it is essential to note that even though the present experiments were performed on a limited number of apices, satisfactory survival rates could be obtained with 6 new sugarcane varieties using the same freezing procedure.

In conclusion, encapsulation/dehydration, which has already been successfully applied to several plant species from different origins appears as a very promising process for the cryopreservation of sugarcane apices and should be developed for a larger range of plant species.

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REFERENCES

1 L.A. Withers, in "Biotechnology of Perennial Fruit Crops", Biotechnology in Agriculture n°8, F.A. Hammerschlag and R.E. Litz eds., CAB Intl., Wallingford, UK, 1992, pp. 169-200.
2 J. Fabre and J. Dereuddre, Cryo-Letters 11, 413-426 (1990).
3 J. Dereuddre, C. Scottez, Y. Arnaud and M. Duron, C.R. Acad. Sci. Paris 310, Sér. III, 265-272 (1990).
4 P. Plessis, C. Leddet and J. Dereuddre, C.R. Acad. Sci. Paris 313, Sér. III, 373-380 (1991).
5 C. Scottez, E. Chevreau, N. Godard, Y. Arnaud, M. Duron and J. Dereuddre, Cryobiology 29, 671-700 (1992).
6 E.E. Benson, N. Chabrillange and F. Engelmann, in Proc. Soc. for Low Temp. Biol. Autumn Meeting, Stirling, UK, 8-9/09/92, 1992 (in press).
7 F. Paulet, F. Engelmann and J.C. Glaszmann, Plant Cell Rep. in press (1993).
8 M.T. Gonzalez Arnao, F. Engelmann and C. Urra, Biotecnologia Aplicada (submitted).
9 S. Mari, F. Engelmann, N. Chabrillange and A. Charrier, in Proc. 15th Intl. Coll. on Coffee, Montpellier, France, 6-11/06/93, in press (1993).
10 A. Korneva, (personal communication).
11 D.B. Fisher, Histochemie 16, 92-96 (1968).
12 J. Fabre and J. Dereuddre, C.R. Acad. Sci. Paris 304, Sér. III, 485-487 (1987).
13 H. Bouman and G.J. de Klerk, Vakblad voor Bloemsterij 12, 52-53 (1990).
14 S. Bagniol and F. Engelmann, Cryo-Letters, 12, 279-286 (1991).
15 J. Dereuddre, in "Reproductive Biology and Plant Breeding", Y. Dattée, C. Dumas and A. Gallais eds., Springer Verlag, Berlin, 1992, pp. 291-300.
16 S. Bagniol, F. Engelmann and N. Michaux-Ferrière, Cryo-Letters 13, 405-412 (1992).
17 A. Uragami, PhD Thesis, Kyushu Univ., Japan, 121 p. (1991).
18 D. Dumet, (personal communication).
19 P. Mazur, Am. J. Physiol. 247, 125-142 (1984).
20 J.H. Crowe, J.F. Carpenter, L.M. Crowe and T.J. Anchordoguy, Cryobiology 27, 219-231 (1990).
21 J. Dereuddre, N. Hassen, S. Blandin and M. Kaminski, Cryo-Letters 12, 135-148 (1991).
22 D. Dumet, F. Engelmann, N. Chabrillange, Y. Duval and J. Dereuddre, Cryo-Letters 14 (in press).
23 R.H. Haskins and K.K. Kartha, Can. J. Bot. 58, 833-840 (1980).
24 B.W.W. Grout and G.G. Henshaw, Ann. Bot. 46, 243-248 (1990).
25 K. Taniguchi, R. Tanaka, N. Ashitani and H. Miyagawa, Jpn. J. Genet. 63, 267-272 (1988).
26 S. Fukai and M. Oe, J. Jpn. Soc. Hort. Sci. 59, 383-387 (1990).
27 K.K. Kartha, N.L. Leung and L.A. Mroginski, Zeitschrift Planzenphysiol. 107, 133-140 (1982).
28 L.E. Towill, Cryobiology 20, 567-573 (1983).
29 S. Fukai, Scientia Hort. 45, 167-174 (1990).