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CRYOPRESERVATION OF CELL SUSPENSIONS OF *Citrus deliciosa* Tan. AND HISTOLOGICAL STUDY

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SUMMARY: During this work, optimal conditions for the cryopreservation of cell suspensions of *Citrus deliciosa* Tan. were defined. After a one-hour cryoprotective treatment with 0.15M sucrose and 5% DMSO, cells were prefrozen to -40°C at 0.5°C.min^{-1} and immersed in liquid nitrogen. After rapid thawing, survival was comprized between 50 and 60% of unfrozen controls. Proliferation in liquid medium was optimal after a 5-day recovery period on solid medium. Production of somatic embryos was higher with cryopreserved cultures than with unfrozen control cultures. Histological examination showed that in the cell aggregates, proembryogenic cells which displayed meristematic characters were little harmed after freezing whereas more differentiated cells were severely damaged. Proembryogenic cells could start dividing within two days after thawing.

KEY-WORDS: Cryopreservation; *Citrus deliciosa* Tan.; cell suspension; histology.

INTRODUCTION

Cryopreservation (liquid nitrogen, -196°C) is particularly interesting for the storage of plants with recalcitrant seeds and for those which are vegetatively propagated (1). These techniques have been applied to around 70 different plant species, 40 of which are from tropical origin and 20 woody species (2, 3). Even though seeds of *Citrus* are not typically recalcitrant, their viability is very short. Therefore, the availability of safe long-term conservation techniques is of paramount importance for this species (4, 5). A limited amount of work only has been performed with *Citrus*, using various types of materials. Bajaj (6) could successfully freeze ovules of *Citrus sp.* Somatic embryos of *Citrus sinensis* (Washington navel) survived after cryopreservation, however with a low recovery rate, and

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gave rise to whole plants which were transferred *in vivo* (4). More recently, japanese researchers developed cryopreservation techniques for nucellar cells of *Citrus sinensis* var. *brasiliensis* using a classical cryopreservation process (7) or a vitrification procedure (8, 9; 10).

The aim of the present study was to set up a cryopreservation protocol for a cell suspension of *Citrus deliciosa* Tan. and to observe the changes occurring during the freeze-thaw cycle at the cellular level by using photonic microscopy.

MATERIALS AND METHODS

Plant Material

The cell suspensions used in this study were obtained from calluses derived from nucellar embryos of *Citrus deliciosa* Tan. produced at the INRA/IRFA (Institut National pour la Recherche Agronomique/Institut de Recherche pour les Fruits et Agrumes Tropicaux) Research Center of San Giuliano (Corsica) according to the method of Ollitrault *et al.* (11).

Methods

In vitro culture

The cell suspensions were cultured on rotary shakers (96 rpm) in a liquid Murashige and Tucker (12) basal medium (MT) supplemented with 0.5 $g.l^{-1}$ malt extract and 0.15M sucrose. Transfers were performed every two weeks. They were placed in a culture room at 25±1°C, under a light intensity of 30.5 $\mu mol.m^{-2}.s^{-1}$, with a photoperiod of 12 hours light/12 hours dark. For cryopreservation experiments, cells were harvested at the beginning of their exponential growth phase (8-10 days after the last transfer).

Cryopreservation

For pretreatment, various sucrose concentrations were used (0.15 to 0.9M), in combination with 0 to 25% dimethylsulfoxide (DMSO). Cells were pretreated for 1 h in an ice-bath. DMSO was added progressively in ten aliquots every 3 min over the first 30 min of the pretreatment. Aliquots of 1 ml with a 30% Packed Cell Volume (PCV) were then distributed in 2 ml cryotubes. In experiments performed to test other variables, cells were pretreated with 0.15M sucrose and 5% DMSO.

Cells were frozen either rapidly by direct immersion of the cryotubes in liquid nitrogen or by using slow, controlled freezing (Programmable freezer Minicool, by L'Air Liquide). The prefreezing temperature varied between -15 and -80°C, and the freezing rate between 0.2 and 5°$C.min^{-1}$. When the prefreezing temperature was reached, cryotubes were immersed rapidly

in liquid nitrogen where they were kept for a minimum of 1 h. Crystallization of the cryoprotective medium was induced manually at a temperature intermediate between the crystallization and the nucleation temperature of the cryoprotective medium, by pinching briefly the cryotubes with forceps precooled in liquid nitrogen. In experiments performed to test other variables, samples were prefrozen to -40°C at 0.5°C.min^{-1}.

In the experiment concerning the effect of thawing temperature, samples were rewarmed either slowly at room temperature, or in a water-bath thermostated at 40° or 80°C, until melting of ice was complete. In experiments performed to test other variables, a thawing temperature of 40°C was employed.

For regrowth, the contents of the cryotubes was poured on filter papers placed in Petri dishes filled with 20 ml of solid standard medium. Filter papers with the cells were transferred onto fresh medium after 1 h and again after 24 hrs. It was then assumed that all cryoprotective substances were eliminated. In the experiment performed to test the effect of duration of growth on solid medium on proliferation recovery of cells in liquid medium, various culture periods on solid medium were experimented: 0, 1, 24 hrs; 2, 5, 7, 14, 21 days.

Viability and recovery assessment

Viability of cells was estimated immediately after thawing using staining with fluorescein diacetate (FDA, 13). Survival rate of a sample was assessed by calculating the mean percentage of living cells measured on 20 cell aggregates chosen randomly on a plate observed with a microscope, according to the method of Dussert *et al.* (14). Survival rates were expressed as a percentage of the control value.

During experiments aiming at determining the optimal pregrowth, freezing and thawing conditions, growth recovery of cells was estimated visually every 4 days during one month using a scale reflecting its intensity (-, -/+, +, ++, +++). Once optimal conditions were set up, growth recovery on solid medium was measured by weighting the filter paper and the cells every 4 days during one month. It was expressed in % of the initial fresh weight of the samples. In the experiment concerning the effect of culture duration on solid medium on proliferation of cells after transfer in liquid medium, regrowth was followed by measuring the Packed Cell Volume (PCV) increase of the cell suspensions during 30 days.

Regeneration of plants

Regeneration of plants was carried out with control and cryopreserved cells pretreated with 0.15M sucrose and 5% DMSO, prefrozen to -40°C at 0.5°C.min^{-1} before immersion in liquid nitrogen. After one month recovery on solid standard medium, they were transferred

onto a new medium for induction of embryogenesis (15). This medium consisted of a basal MT medium supplemented with 0.15M galactose and 2 $g.l^{-1}$ gelrite. The young proembryos formed were then transferred onto the same basal medium supplemented with 0.1M sorbitol, 0.1M galactose, 1 µM gibberellic acid (GA_3) and 7 $g.l^{-1}$ agar. After 15 days, the young plantlets obtained were transferred in individual test tubes containing half strength MT basal medium with 20 $g.l^{-1}$ sucrose and 7 $g.l^{-1}$ agar.

Expression of the results

One sample per condition only was used to determine the optimal freezing conditions (pretreatment, freezing parameters, thawing). In all other experiments, three samples per condition were used. The results represent the mean value of the three samples used per condition. In each of the freezing experiments, various controls were made. They consisted of untreated cells, pretreated cells, prefrozen cells and cryopreserved cells.

Histological study

Histological examination was performed on samples after the following steps of the cryopreservation process: control cells during the exponential growth phase; cells pretreated with 0.15M sucrose and 5% DMSO; cells prefrozen to -40°C at 0.5°$C.min^{-1}$; cells after freezing in liquid nitrogen; cells after various culture periods on solid recovery medium (1, 24 hrs; 2, 5, 7, 14 and 21 days).

Samples were fixed for 48 hrs in a phosphate buffer (pH 7.2) containing 2% paraformaldehyde, 1% acroleine, 2% glutaraldehyde and 1% caffeine. They were then dehydrated by successive transfers in alcohol baths with progressively increased alcohol concentrations. Inclusion was carried out in Kulser 7100 resin (Labonord). 3 µm thick sections were cut using an automatic microtome (Historange 2218, LKB). Samples were treated with periodic acid-Schiff reaction and naphthol blue black. This double staining technique allows specific characterization of polysaccharide compounds which stain red and of the soluble and non-soluble proteins which stain blue black (16).

RESULTS

Survival and proliferation recovery of cryopreserved cells varied depending on the concentration of sucrose and DMSO employed during pregrowth treatment (Table 1). Without DMSO, survival increased in line with increasing sucrose concentrations. DMSO was toxic to the cells above 15%. Growth recovery on solid medium was visible after 12 days when pretreatment was performed with 0.15M sucrose and 5 or 10% DMSO and after 18 days only with 0.3 or 0.6M sucrose. No regrowth was obtained with 0.9M sucrose. The optimal pretreatment conditions were 0.15M sucrose and 5 or 10% DMSO.

Table 1: Effect of sucrose and DMSO concentration on the survival rate and intensity of proliferation recovery of cryopreserved cell suspensions of *Citrus deliciosa* Tan.

		DMSO (%)					
Sucrose (M)		0	5	10	15	20	25
0.15	Survival (%)	0	55	38	26	3	1
	Recovery	-	+++	+++	+	-	-
0.3	Survival (%)	16	39	37	16	0	0
	Recovery	-	+	++	-/+	-	-
0.6	Survival (%)	26	31	21	12	2	4
	Recovery	-/+	++	++	-/+	-	-
0.9	Survival (%)	37	19	16	10	0	0
	Recovery	-	-	-	-	-	-

No survival was noted after rapid freezing. Survival progressively increased for prefreezing temperatures comprized between -15 and -40°C which represented the optimal value (57% survival) (Table 2). It decreased for lower prefreezing temperatures to reach 20% for -80°C. Growth recovery could be obtained with all prefrozen controls. With cryopreserved samples, it was possible for prefreezing temperatures between -25 and -80°C and was maximal between -35 and -45°C.

Table 2: Effect of prefreezing temperature on the survival rate and the intensity of proliferation recovery of prefrozen control (-LN) and cryopreserved (+LN) cell suspensions of *Citrus deliciosa* Tan.

		Prefreezing temperature (°C)									
		-15	-20	-25	-30	-35	-40	-45	-50	-55	-80
-LN	Survival (%)	65	79	69	81	44	72	58	36	32	23
	Recovery	+++	+++	++	+++	+++	+++	+++	++	++	++
+LN	Survival (%)	0	0	8	19	43	57	48	38	29	20
	Recovery	-	-	+	++	+++	+++	+++	++	++	++

Survival was noted for cooling rates comprized between 0.2 and 1°C.min^{-1}, with an optimum at 0.5°C.min^{-1} (Table 3). Growth recovery was very intense with all prefrozen controls. After cryopreservation, regrowth was possible for freezing rates of 0.2, 0.5 and 1.0°C.min^{-1} but was maximal with 0.5°C.min^{-1}.

Table 3: Effect of freezing rate on the survival rate and the intensity of proliferation recovery of prefrozen control (-LN) and cryooreserved (+LN) cell suspensions of *Citrus deliciosa* Tan.

		Freezing rate (°C.min^{-1})				
		0.2	0.5	1.0	2.0	5.0
-LN	Survival (%)	44	72	31	30	64
	Recovery	+++	+++	+++	+++	+++
+LN	Survival (%)	41	57	16	0	1
	Recovery	++	+++	+	-	-

Survival was satisfactory whatever the thawing conditions (Table 4), with an optimum for 40°C. However, no growth recovery was observed after slow thawing, whereas it was equivalent for thawing temperatures of 40 and 80°C.

Table 4: Effect of the thawing procedure on the survival rate and intensity of proliferation recovery of cryopreserved cell suspensions of *Citrus deliciosa* Tan.

	Thawing procedure		
	Air	40°C	80°C
Survival (%)	32	60	42
Recovery	+	+++	+++

Fresh weight increase on solid medium of untreated, pretreated, prefrozen controls and cryopreserved cells was measured. Growth recovery started in all samples after 4 days of culture (Fig. 1). However, regrowth of prefrozen and cryopreserved cells was lower than that of untreated and pretreated controls.

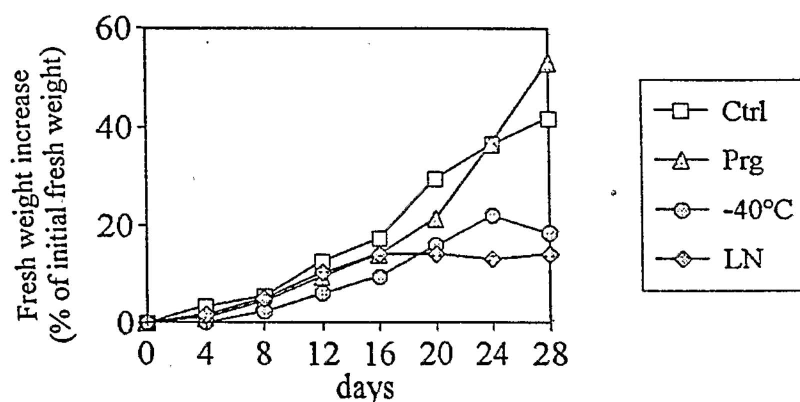

Figure 1: Growth recovery on solid medium of control (ctrl), pregrown (prg), prefrozen (-40°C) and cryopreserved cell suspensions.

The effect of culture duration on solid medium on the multiplication of cells in liquid medium was observed with control (Fig. 2A) and cryopreserved (Fig. 2B) cells. After 30 days of culture in liquid medium, PCV values of control cells were comparable for culture periods comprized between 2 and 21 days. With cryopreserved cells, it was highest after 2 to 7 days of culture on solid medium and optimal for 5 days. In optimal conditions, PCV values were slightly higher with cryopreserved cells than with unfrozen controls.

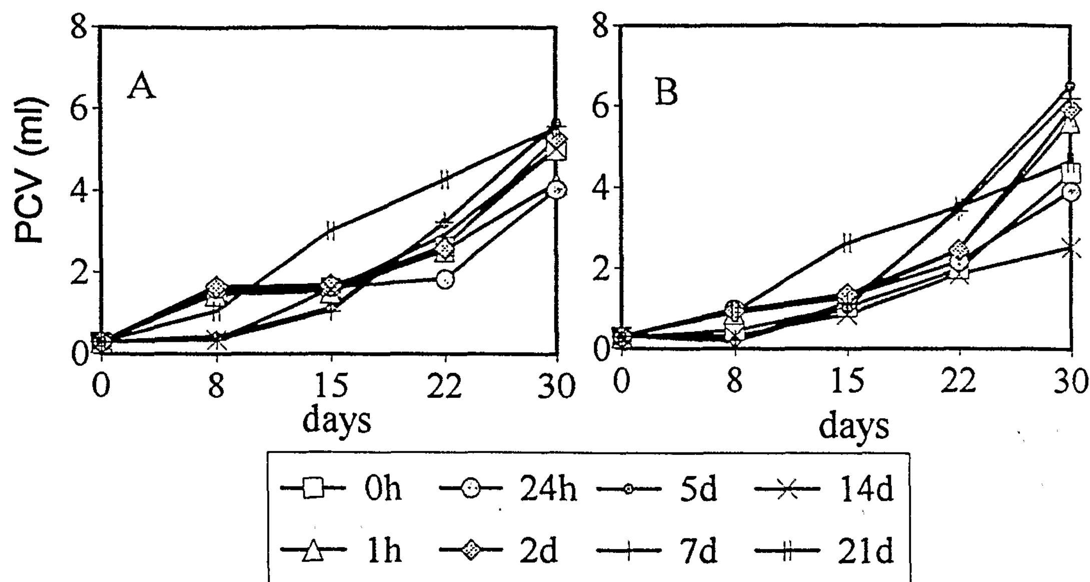

Figure 2: Effect of culture duration on solid medium on growth recovery of control (A) and cryopreserved (B) cell suspensions.

Recovery of the embryogenic potential was different between control and frozen cells. Production of somatic embryos was more rapid and intense with cryopreserved cells than with controls. Globular and heart-shaped embryos were obtained within 15 days with cryopreserved cultures and after 22 days only with controls. Embryos germinated after 15 days and developed into small plantlets similar to controls after one additional month in culture (Plate I, Fig. a).

The cell suspension studied was formed of cell aggregates comprizing two clearly defined types of cells (Plate I, Fig. b). A first type (type 1, Plate I, Fig. c) consisted of small, proembryogenic cells, localized at the periphery of the aggregates. They were poorly vacuolated, with a dense cytoplasm, one or two well defined spherical nucleoli and grains of starch. The center of the aggregates was formed of large, differentiated cells (type 2, Plate I, Fig. d) with large vacuoles and numerous grains of starch. The quantity of starch increased in these cells after pretreatment (compare Plate II, Fig. a with Plate I, Fig. b). In

proembryogenic cells (type 1), freezing induced either deformation of the nucleus which lost its spherical shape or disappearance of the nuclear membrane; some nuclei acquired a very strong staining due to the contraction of chromatin (Plate II, Fig. b, N). In some cells (Plate II, Fig. b, arrows) retraction of the cytoplasm was observed. In type 2 cells, damages consisted mostly of rupture and deformation of the cell wall (Plate II, Fig. c, large arrows) associated with retraction of the cytoplasm (Plate II, Fig. c, small arrows).

Recovery of cryopreserved cells on solid medium was observed after different periods of culture. After 5 days on solid medium, cellular zones necrosed after cryopreservation degenerated whereas proembryogenic aggregates proliferated actively (Plate II, Fig. d). Culture periods longer than 7 days on solid medium were detrimental to growth recovery of proembryogenic cells since cells started to accumulate starch and to differentiate.

DISCUSSION/CONCLUSION

The present study allowed to determine the optimal procedure for the cryopreservation of *Citrus deliciosa* Tan. cell suspensions. After pretreatment with 0.15M sucrose and 5% DMSO followed by prefreezing to -40°C at 0.5°C.min^{-1} and immersion in liquid nitrogen, survival rates reached 50-60% of unfrozen controls. Growth recovery in liquid medium was maximal after a prerequisite 5-day culture on solid medium. Plantlets similar to unfrozen controls were obtained from cryopreserved cultures.

This work confirmed that, as already observed in previous studies with *Citrus* (6, 7) a cryoprotective solution comprizing sucrose and DMSO was the most efficient. Mixtures of cryoprotectants generally have a higher cryoprotective effect than a single substance employed at the same osmolarity (17).

Survival after freezing in liquid nitrogen depends on the optimal dehydration of the cell suspension which is achieved by optimizing the freezing parameters, i.e. prefreezing temperature and cooling rate (18). The range of prefreezing temperatures ensuring acceptable survival rates vary among species: -23 to -40°C with sugarcane calluses (19), -30 to -60°C with grape cell suspensions (14). It was even broader with *Citrus deliciosa* since it was comprized between -30 and -80°C. The choice of an adequate freezing rate was also determinant to obtain high survival rates. If survival was satisfactory after prefreezing to -40°C whatever the cooling rate employed, it was no more the case after immersion in liquid nitrogen. Indeed, survival dropped dramatically for cooling rates higher than 1°C.min^{-1}. This was also observed notably with *Citrus* by Kobayashi *et al.* (7) for freezing rates higher than 0.5°C.min^{-1}. However, as already noted with grape cell suspensions (14), growth recovery

was possible within a much narrower range of prefreezing temperatures, from -35 to -45°C and for freezing rates of 0.2 to 0.5°C.min^{-1} only. These freezing parameters may allow optimal dehydration of the cells.

If slow thawing allowed to obtain survival, growth recovery was impossible to achieve. Cells of *Citrus deliciosa* were not sensitive to rapid rewarming, since growth recovery could be obtained whatever the temperature of the water-bath. On the contrary, in the case of grape embryogenic cell suspensions, recovery was obtained only after thawing at 40°C (14).

The differences noted in the intensity of recovery on solid medium reflected the various extent of damage caused to the cells in the different conditions. This was particularly obvious as concerned the optimal culture period on solid medium before transfer to liquid medium. Indeed, after 5 days on solid medium, PCV increase in liquid medium was even slightly higher with cryopreserved cells than with control cells. The same observation was made as regards the number of embryos produced. This has been observed notably in the case of *Picea abies* embryogenic calluses (20). This was certainly due to the cell selection process caused by cryopreservation. Indeed, histological examination showed that only cells showing meristematic characters survived after cryopreservation whereas all differentiated cells were killed or severely damaged during the freeze-thaw cycle. In optimal recovery conditions, the nuclear membrane and the nucleoli of proembryogenic cells retrieved their normal shape within two days after thawing and cells started to multiply rapidly. On the contrary, when cells were kept for too long on solid medium, PCV increase was much lower than that of the control. In this case, histological examination revealed that proembryogenic cells progressively lost their meristematic characteristics and started to differentiate, leading to a lower multiplication rate after transfer to liquid medium.

In vitro plantlets produced from cryopreserved material did not display morphological differences compared to controls. This implies that cryopreservation did not induce modifications in the cultures. However, biochemical and molecular studies should be performed in order to confirm the genetic stability of plants produced from cyropreserved material.

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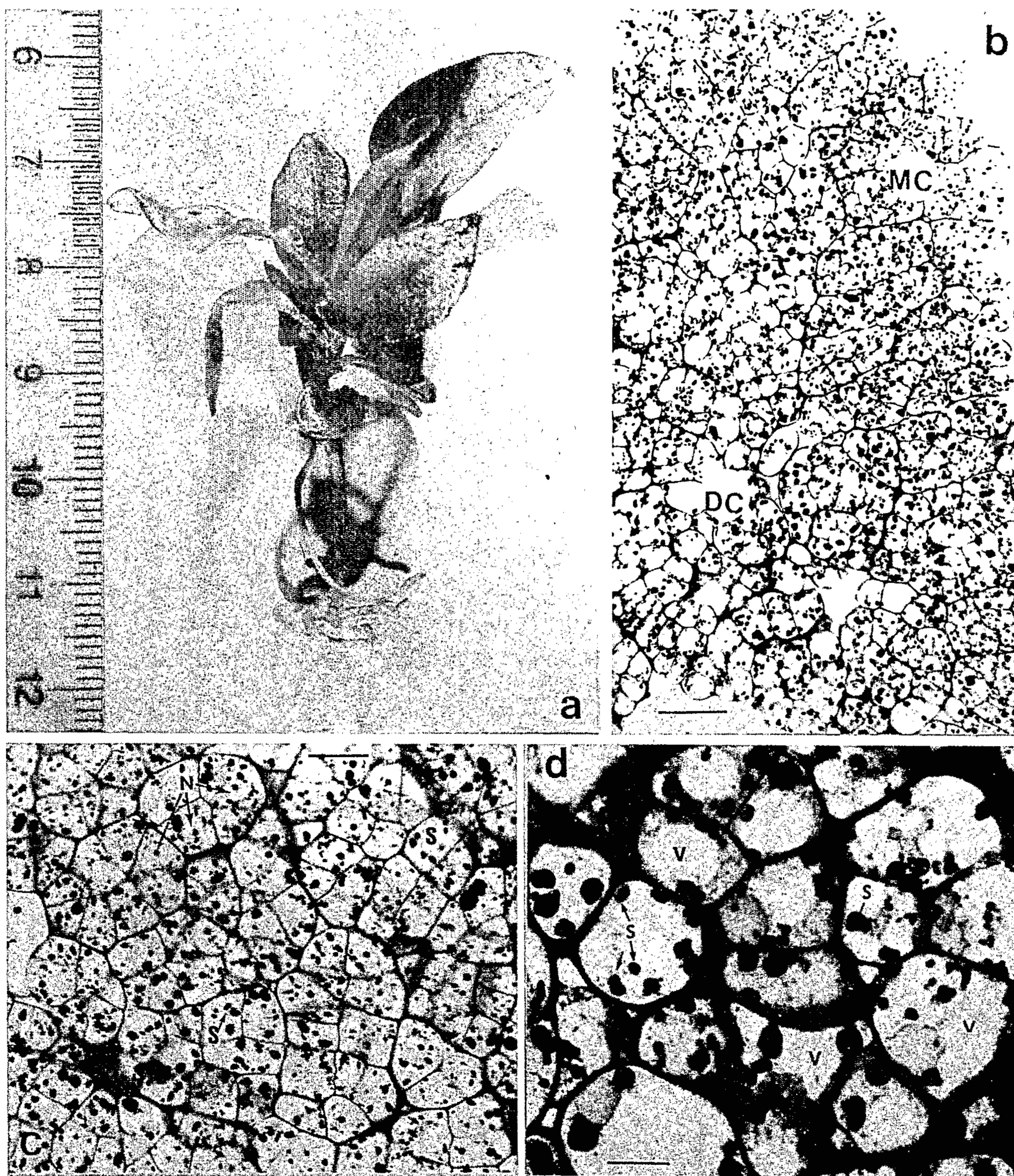

Plate I

Fig. a: Young *in vitro* plantlet regenerated from a cryopreserved embryogenic suspension.

Fig.b: Histological structure of a cell aggregate constituting the embryogenic cell suspension. MC: proembryogenic cells at the periphery; DC: differentiated cells in the center. Starch appears dark inside cells. Bar: 40 µm.

Fig. c: Proembryogenic cells. N: nuclei; s: starch. Bar: 20 µm.

Fig. d: Differentiated cells. s: starch; v: vacuoles. Bar: 10 µm.

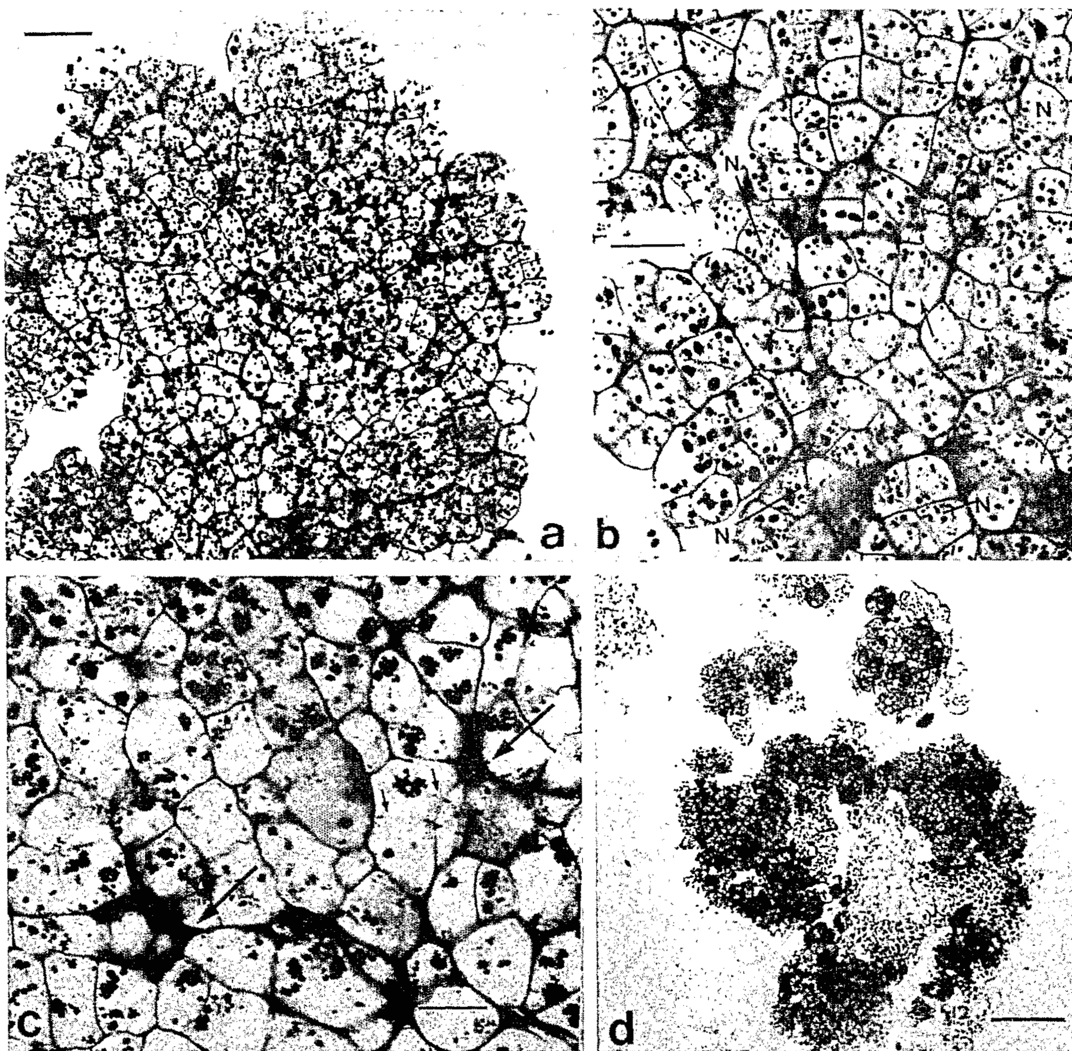

Plate II

Fig. a: Accumulation of starch in cells of the aggregates after pretreatment. Bar: 40 µm.

Fig. b and c: Structural alterations after freezing in liquid nitrogen. Bar: 20 µm.

b: in type 1 cells (meristematic cells): nuclear membrane became less apparent and some nuclei acquired a very dark staining. N: nuclei.

c: in type 2 cells (differentiated cells): rupture and deformation of cell walls (large arrows) and contraction of cytoplasm (small arrows).

Fig. d: Aspect of aggregates during recovery on solid medium after freezing in liquid nitrogen. Proliferation recovery occurred in meristematic zones (dark) only. Bar: 125 µm.