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HISTO-CYTOLOGICAL STUDY OF APICES OF COFFEE (*COFFEA RACEMOSA* AND *C. SESSILIFLORA*) *IN VITRO* PLANTLETS DURING THEIR CRYOPRESERVATION USING THE ENCAPSULATION-DEHYDRATION TECHNIQUE.

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Abstract: Apices of *in vitro* plantlets of *Coffea racemosa* and *C. sessiliflora* withstood freezing in liquid nitrogen using the encapsulation-dehydration technique. An intense intracellular starch accumulation was noted after overnight culture of apices on standard medium. Apices of *C. sessiliflora* required a 3 to 10-d pregrowth in liquid medium containing 0.75 M sucrose, whereas those of *C. racemosa* required a progressive increase of the sucrose concentration from 0.5 to 1 M. After the dehydration phase, cells of some apices were severely damaged, showing osmiophilic granules on the surface of the plasmalemma. In other apices, cells were less severely damaged, showing exocytosis vesicles at the periphery of the plasma membrane. After freezing, survival rates of 27 and 37% were obtained for *C. racemosa* and *C. sessiliflora*, respectively. Two growth recovery patterns were observed: direct development of foliar primordia and callusing.

Key words: coffee, *C. racemosa*, *C. sessiliflora*, apex, cryopreservation, encapsulation-dehydration, histology.

Introduction

As regards conservation possibilities, seeds of coffee, which represents around 80 species of the sub-genus *Coffea*, are classified intermediate (1). Even though storage duration of coffee seeds could be extended to more than one year, long-term conservation of coffee genetic resources in seed form is still impossible. Cryopreservation is the only current method allowing the long-term conservation of genetic resources of recalcitrant and intermediate seed species.

Encapsulation-dehydration is a new cryopreservation technique which has been successfully applied to apices of several temperate and tropical plant species (2, 3). This technique comprizes the following successive steps: encapsulation of apices in calcium alginate beads, pregrowth of encapsulated apices in liquid medium containing high sucrose concentrations, partial dehydration followed by the freeze-thaw cycle. The modifications induced by these treatments at the cellular level have been studied with various species (4, 5, 6, 7). Apical cells generally accumulate large starch quantities during pregrowth treatment. After freezing, the state of apices varies depending on the species: with sugarcane, most meristematic cells were alive (4) whereas with other species (5, 6, 7) the coexistence of dead and living cells was observed. In most cases, growth recovery of apices is direct, with the rapid development of new foliar primordia. However, transitory callusing could be observed with cassava apices (8).

In this paper, the application of the encapsulation-dehydration technique to apices of two coffee species, *C. racemosa* and *C. sessiliflora*, is reported and the effects of pregrowth, desiccation and freezing on the structure of apices at the cellular and ultrastructural level are presented.

Materials and Methods

Plant material

Plantlets of *C. racemosa* and *C. sessiliflora* were obtained from zygotic embryos extracted from seeds and germinated *in vitro* (9). They were cultivated on a modified MS medium (10) containing 40 $g.l^{-1}$ sucrose, 0.3 $mg.l^{-1}$ 6-benzylaminopurine (BAP) and 2 $g.l^{-1}$ phytagel, at 27±1°C, under a light intensity of 50 $\mu E.m^{-2}.s^{-1}$ (PAR) with a 12 h light/ 12 h dark period. Microcuttings comprizing two nodes were placed on standard medium supplemented with 0.5 $mg.l^{-1}$ BAP and 0.2 $mg.l^{-1}$ naphtalene acetic acid (NAA). After three weeks, apices consisting of the meristematic dome, the subapical zone and one or two leaf primordia, were sampled on microcuttings.

Methods

Cryopreservation: After dissection, apices were left overnight on the standard microcutting culture medium to recover from dissection stress, then encapsulated in 3% alginate beads. For pregrowth, encapsulated apices were cultured for 1 to 10 d in liquid medium with various sucrose concentrations (0.1 to 1.25 M) on a rotary shaker (91 rpm). In some cases, apices were transferred at 24-h intervals in media with progressively increased sucrose concentration with the following sequence: 0.5, 0.75, 1 M. Beads were then dehydrated for 0 to 6 h in the air current of a laminar flow cabinet. Encapsulated apices were transferred in sterile 2 ml cryotubes and immersed rapidly in liquid nitrogen where they were kept for at least 25 min. Beads were rewarmed for 2-3 min in the air current of the laminar flow cabinet and placed onto the standard culture medium (0.3 $mg.l^{-1}$ BAP) for recovery.

Recovery of apices after the various treatments was estimated 3 weeks after freezing by counting the number of apices showing any sign of regrowth, i.e. development of new foliar primordia and / or callusing. Seven to 17 apices were used per condition.

Histology: For both techniques utilized (photonic and electronic microscopy) 5 apices of *C. racemosa* were sampled at the end of each of the following steps of the cryopreservation protocol: dissection, overnight culture on standard medium, pregrowth treatment (3 d with 0.5 and 0.75 M sucrose), dehydration (4.5 and 6 h), freeze-thaw cycle, visible regrowth of the apex (i.e. when growth of new foliar primordia could be observed).

- **Photonic microscopy**: Samples were fixed for 1 to 3 d at room temperature in a 0.1 M phosphate buffer (pH 7) enriched with sucrose at the concentration used during pregrowth treatment, and containing 1% glutaraldehyde, 2% paraformaldehyde and 1% caffein. They were then dehydrated by successive transfers in alcohol baths with progressively increasing alcohol grades (30 to 100°). Apices were then treated with chloroform for 1 d and with butanol for 3 d, in order to allow a better penetration of the inclusion resin. For impregnation apices were placed at 4 °C for 3 d in Kulzer Technovit 7100 resin. Inclusion was performed with the same resin supplemented with Kulzer Technovit 7100 hardener. Three and a half µm thick sections were cut using an automatic microtome (Historange 2218, LKB). Sections were double-stained using the periodic acid/Schiff reaction (APS) and naphthol blue black. This double staining allows specific characterization of polysaccharide compounds which stain red and the soluble and non-soluble proteins which stain blue black (11).

- **Electronic microscopy**: Apices were fixed by immersion for 2 h in a 0.1 M cacodylate buffer (pH 7.2) containing 2% glutaraldehyde and 1% caffein. They were then rinsed three times for 30 min each with cacodylate buffer, post-fixed for 1 h at 4°C with 2% osmic acid and rinsed three times with distilled water. Samples were then dehydrated by successive tranfers in alcohol baths with progressively increasing alcohol grades (10 to 100°) and included using the technique described by Spurr (12). Ultra-thin sections (65 nm) were cut using a Leica Reichert Ultracut S ultramicrotome, and placed on copper grids. They were then contrasted with 2% uranyle acetate for 30 min in the dark, then for 10 min in the dark with lead citrate (1.33 g lead citrate + 1.76 g sodium citrate in 50 ml distilled water).

Results

Definition of parameters of the cryopreservation procedure

- Pregrowth treatment

With both species, survival of apices was very high for sucrose concentrations comprized between 0.1 and 0.75 M whatever the duration of the pregrowth treatment (Table 1). Survival decreased rapidly when pregrowth treatment was performed with 1 M sucrose.

Table 1: Effect of sucrose concentration and of pregrowth treatment duration (days) on the survival rate (%) of apices of *C. racemosa* and *C. sessiliflora*.

	C. racemosa					*C. sessiliflora*				
	1 d	3 d	5 d	7 d	10 d	1 d	3 d	5 d	7 d	10 d
0.1 M	100	93	100	80	80	100	100	100	93	100
0.3 M	100	87	93	53	80	100	100	100	100	100
0.5 M	100	93	67	80	87	100	100	100	93	100
0.75M	100	73	60	40	87	100	100	80	73	100
1 M	77	47	47	40	27	40	93	53	47	40

- Resistance to desiccation and freezing

Control apices withstood dehydration up to 3 h whatever the pregrowth treatment. For longer desiccation periods, a large drop in survival was noted with both species (Tables 2 and 3). In the case of *C. racemosa*, survival of control apices decreased more rapidly when apices were placed directly in medium with 1 M sucrose, in comparison with a progressive increase in sucrose concentration (Table 2).

Table 2: Effect of pregrowth conditions (progressive increase in sucrose concentration from 0.5 to 1 M, or direct pregrowth in 1 M sucrose) and of desiccation on the survival rate (%) of control (-LN) and cryopreserved (+LN) apices of *C. racemosa*.

		Progressive pregrowth		Direct pregrowth	
		- LN	+ LN	- LN	+ LN
Desiccation	0	100	0	60	0
(h)	3	43	0	29	0
	4.5	29	25	14	0
	6	29	27	0	0

After freezing no survival was generally obtained for desiccation periods shorter than 3 h (Tables 2 and 3). The maximal survival rates obtained were similar with both species. In the case of *C. racemosa* (Table 2), survival was noted only if pregrowth consisted of a daily increase in sucrose concentration from 0.5 to 1 M and was optimal for 6 h desiccation.

Table 3: Effect of pregrowth duration in liquid medium with 0.75 M sucrose and of desiccation period on the survival rate (%) of control (-LN) and cryopreserved (+LN) apices of *C. sessiliflora.*

	Pregrowth duration (days)							
	3		5		7		10	
Desiccation (h)	- LN	+ LN	- LN	+ LN	- LN	+ LN	- LN	+ LN
0	100	0	100	0	100	0	100	0
3	43	0	57	0	86	0	100	13
4.5	43	0	57	38	57	0	100	13
6	57	13	57	0	57	12	71	25

Apices of *C. sessiliflora* withstood freezing after all pregrowth treatment durations comprized between 3 and 10 days (Table 3). However, extending the pregrowth duration delayed the recovery of frozen apices and increased callusing. In the case of *C. sessiliflora* the highest survival rates were obtained after a 5-d pregrowth and a 4.5 h-desiccation period.

Histo-cytological study of apices during the cryopreservation protocol

- Control apices

Explants were formed of the apical meristem, the sub-apical zone and two developing foliar primordia. The meristem comprized a limited number of cells arranged in 4 to 5 layers (Pl. I, Fig. a). These cells had a high nucleoplasmic ratio, a circular nucleus often situated in central position, a little dense chromatin, some clusters of heterochromatin and one or two nucleoli (Pl. I, Fig. b). Only few organites were observed in their dense cytoplasm. Cells of the peripheric layer were more vacuolated and were covered with a thin cuticle. Under these typically meristematic cell layers, the underneath zone was formed of differentiating cells which were more voluminous and more vacuolated (Pl. I, Fig. a)

- Apices after overnight culture on standard medium

Starch accumulation was noted during this phase of the cryopreservation protocol. This accumulation was intense in cells adjacent to procambial vessels and in the medullar zone (not illustrated). It was also visible in cells of the meristematic dome and was detected only with electronic microscopic (Pl. I, Fig. c)

- Apices after pregrowth treatment

During the pregrowth treatment, the nucleoplasmic ratio of the most meristematic cells tended to decrease and nuclei became less spherical (Pl. I, Fig. d) in comparison with the previous stage (Pl. I, Fig. c). However, limited modifications were noted during this phase.

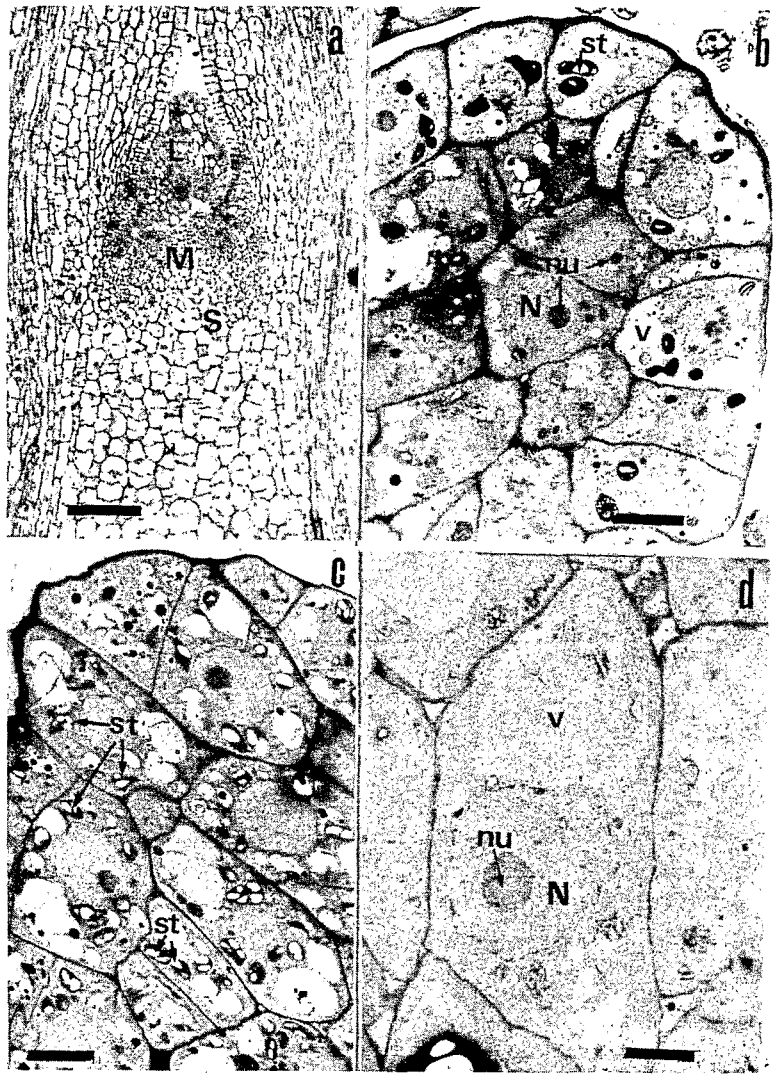

Plate I

Figs. a and b. Control apices

a. Histological aspect of a longitudinal section. L, leaf primordium; M, meristem; S, subapical zone. (bar: 25 µm)

b. Ultrastructural aspect of the apical cells. N, nucleus; nu, nucleolus; st, starch; v, vacuole. (bar: 2 µm)

Fig. c. Accumulation of starch (st), in the apical cells after an overnight culture on standard medium. (bar: 2 µm)

Fig. d. Ultrastructural aspect of a meristematic cell after pregrowth treatment. N, nucleus; nu, nucleolus; v, vacuole. (bar: 2 µm)

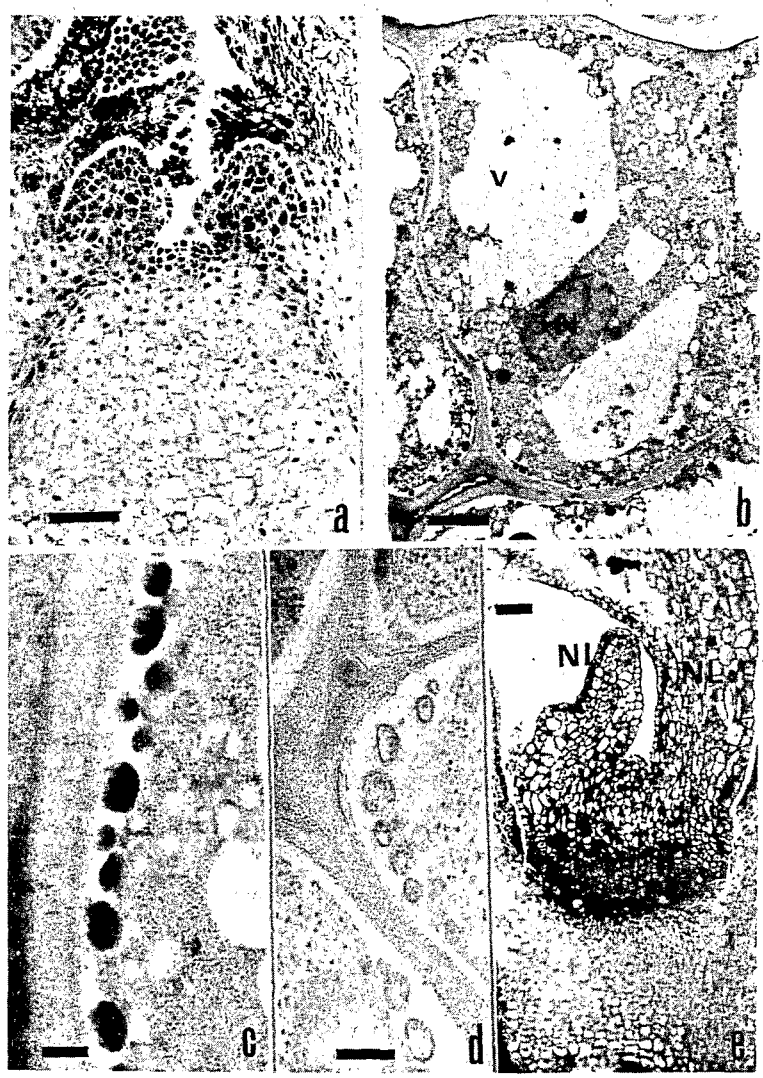

Plate II

Figs. a to d. Cytological aspect of apices after desiccation

a. Longitudinal section of an apex. Note the very strong staining of the nuclei. (bar: 25 µm)

Figs. b to d. Ultrastructural alterations after dehydration of a meristematic cell

b. N, nucleus; v, vacuole. (bar: 2 µm)

c. Osmiophilic granules at the periphery of the plasmalemma. (bar: 200 nm)

d. Clear cytoplasmic vesicles between the plasmalemma and the cell wall. (bar: 200 nm)

Fig. e. Histological aspect of the apical zone after freezing. NL, new leaf primordia. (bar: 25 µm)

- Apices after desiccation

On the opposite, after desiccation, the majority of meristematic cells were plasmolysed; retracted cytoplasm appeared more dense and nuclei were often pycnotic (Pl. II, Fig. a). Three cases were observed: i) cells were severely damaged with retracted cytoplasm, no more visible organites, disrupted membranes, clustered chromatin and nucleoli no more visible; ii) cells showed the same alterations with the appearance of osmiophilic granules at the periphery of the plasmalemma (Pl. II, Fig. b and c); iii) cells were plasmolysed but appeared less harmed; nuclei had an aspect similar to that of control cells and clear cytoplasmic vesicles were visible between the plasmalemma and the cell wall (Pl. II, Fig. d). The large cells of the medullar zone were rarely unharmed: their nuclei were little visible and their cytoplasm was destructured.

- Apices after freezing

Pre-existing foliar primordia withstood severe damage during the freeze-thaw cycle which led to their necrosis shortly after thawing. Half of apices which survived after freezing developed directly, with the production of new leaf primordia (Pl. II, Fig. e), while only non-chlorophyllian calluses appeared on the other half. Regrowth was observable macroscopically 3 to 4 weeks after thawing. The cytological examination of a living apex showed that most meristematic cells had recovered from freezing and the apex was still capable of forming new foliar primordia (Pl. II, Fig. e).

Discussion /Conclusion

This study demonstrated that apices of *C. racemosa* and *C. sessilifora* could withstand freezing in liquid nitrogen using the encapsulation / dehydration technique. Direct pregrowth treatment with high sucrose concentration was used with *C. sessiliflora* whereas progressive increase in sucrose concentration was necessary with *C. racemosa*. Optimal desiccation periods were comprized between 3 and 6 h. With *C. racemosa*, the little difference in survival between dehydrated and frozen apices showed that resistance to the cryopreservation protocol was achieved when resistance to desiccation had been obtained. At the histo-cytological level, if the accumulation of starch during culture on standard medium was the first modification observed, the main changes were noted during the desiccation step. Cells of some apices were severely damaged during dehydration with osmiophilic granules at the periphery of the plasmalemma whereas cells of others, which formed exocytosis membrane vesicles were less damaged. Growth recovery of frozen apices took place either directly with the production foliar primordia, or through callusing, with a frequency varying according to the pregrowth treatment.

Culture of apices on standard medium after dissection resulted in the reactivation of metabolism through starch accumulation in all cells. This was also observed with apices of date palm (13) and sugarcane (4). This culture period may also allow to reduce the physiological heterogeneity of apices by synchronizing their metabolism (14). This step appeared indispensable to obtain survival of apices of numerous

species after cryopreservation using both classical (13, 15) and encapsulation-dehydration protocols (4, 6, 7, 14).

The two species studied differed in their sensitivity to sucrose since a progressive pregrowth treatment was necessary for *C. racemosa* whereas *C. sessiliflora* withstood a direct pregrowth treatment. Little modifications were obtained during this step at the histological level. Starch accumulation was stopped with coffee apices whereas it went on during pregrowth treatment in apices of potato and sugarcane (4, 5).

Pregrowth treatments with high sucrose concentrations were necessary to obtain resistance to dehydration during which major alterations occurred, which concerned mainly membranes and the nucleus. The observation that cells of different apices were differently harmed could correspond to different levels of resistance to the cryopreservation protocol. Thus, the presence of osmiophilic granules at the periphery of the plasmalemma in cells of some apices might correspond to an irreversible loss of membrane material caused by too intense dehydration. Such granules were observed in cells of apices of carnation and potato submitted to lethal plasmolysis during a cryopreservation protocol (5, 16). They were also noted in rye cell suspensions after osmotic stress leading to cellular lysis during rehydration (17). By contrast, dehydration caused little alterations in one of the coffee apices observed. The appearence of the nucleus which was similar to that of control apices and the absence of osmiophilic granules allowed to consider that this particular apex had withstood better the cryopreservation process. The production of exocytosis membrane vesicles might be an adaptative mechanism to reduction in cell volume induced by various treatments. Steponkus demonstrated the existence of exo- or endocytosis flows to adjust the cell volume during osmotic stress (18). Exocytosis vesicles could be reincorporated into the plasmalemma during rehydration, thus allowing survival (19). The formation of membrane vesicles was also observed in rice and *Panicum maximum* cell suspensions which withstood freezing (20, 21). Further studies are necessary to confirm these preliminary observations.

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