Cryo-Letters 16, 289-298(1995).
Published by Cryo-Letters, 7 Wootton Way, Cambridge CB3 9LX, U.K.

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

S. Mari[1], F. Engelmann[1,2*], N. Chabrillange[1], C. Huet[1] and N. Michaux-Ferrière[3]

1: ORSTOM, LRGAPT, BP 5045, 34032 Montpellier Cédex 1, France
2: IPGRI, Via delle Sette Chiese 142, 00145 Rome, Italy (present address)
3: CIRAD, BIOTROP, BP 5035 Montpellier Cédex 1, France

*: to whom correspondence should be addressed.

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

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)

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

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

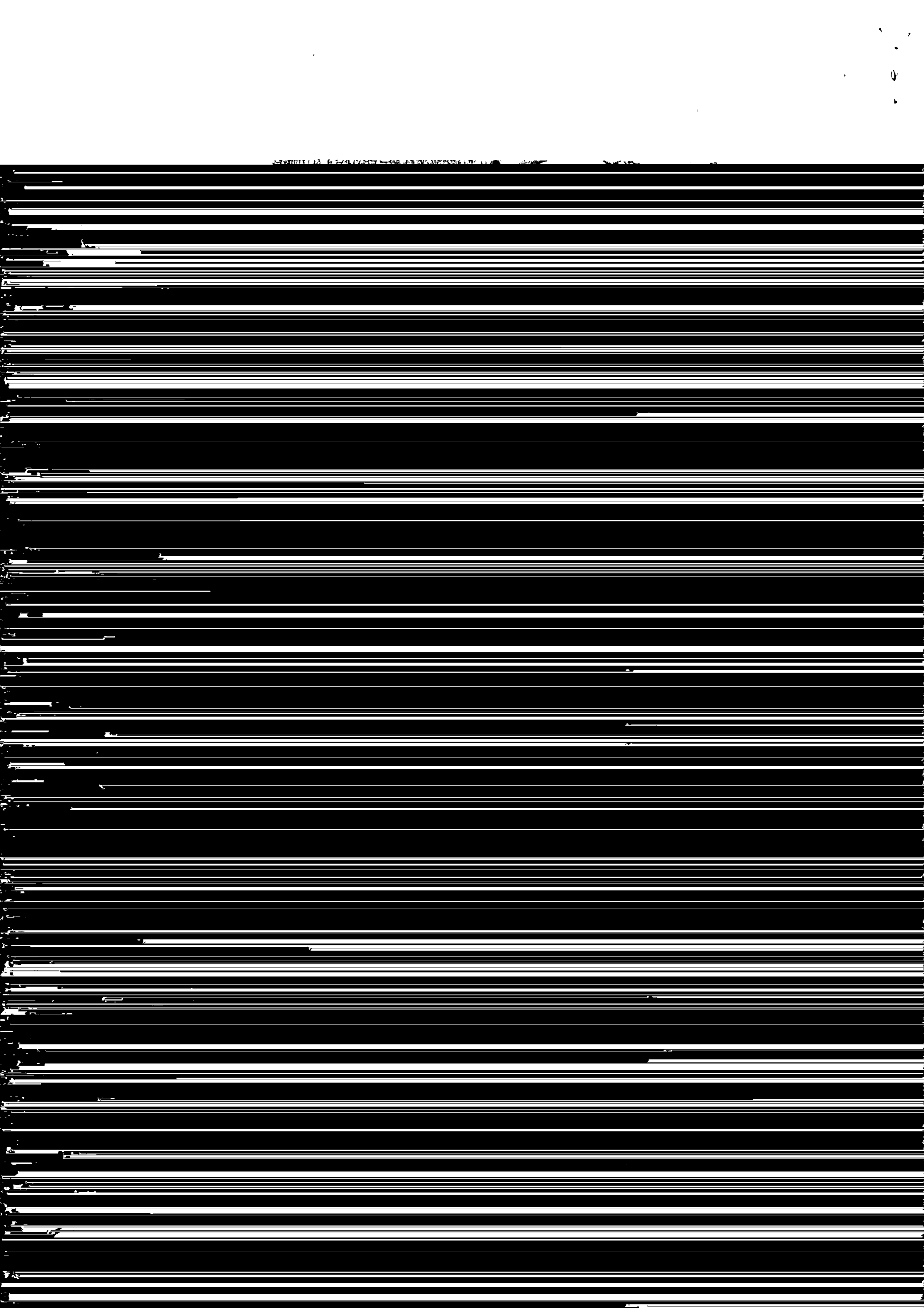
Control apices withstood dehydration up to 3 h whatever the pregrowth treatment. For longer desiccation periods, a large drop in survival was noted with both species (Tables 2 and 3). In the case of *C. racemosa*, survival of control apices decreased more rapidly when apices were placed directly in medium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

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)

	Pregrowth duration (days)							
	3		5		7		10	
Desiccation (h)	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
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 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

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.

Acknowledgements

The assistance of Alain Rival (ORSTOM/CIRAD, Montpellier) for the preparation of the illustrations is gratefully acknowledged.

References

(1) R. H. Ellis, T. D. Hong and E. H. Roberts, J. Exp. Bot., 41, 1167-1174 (1990).
(2) J. Dereuddre, in "Reproductive Biology and Plant Breeding", Y. Dattée, C. Dumas and A. Gallais (eds) Springer Verlag, Berlin, 1992, 291-300.
(3) F. Engelmann, E. E. Benson, N. Chabrillange, M. T. Gonzalez Arnao, S. Mari, N. Michaux-Ferrière, F. Paulet, J. C. Glaszmann and A. Charrier, in "Current Issues in Plant Molecular and Cellular Biology", M. Terzi, R. Cella and A. Falavigna (eds), Kluwer Academic Publishers, Dordrecht, 1993, 315-320.
(4) M. T. Gonzalez Arnao, F. Engelmann, C. Huet and C. Urra, Cryo-Letters, 14, 303-308 (1993).

(5) J. Fabre, Thèse d'Université, Université Paris VI, 1991, 123 p.
(6) C. Scottez, Thèse d'Université, Université Paris VI, 1993, 163 p.
(7) M. Tannoury, Thèse d'Université, Université Paris VI, 1993, 199 p.
(8) E. E. Benson, N. Chabrillange and F. Engelmann, Rapport de fin d'étude, ORSTOM, 1991, 49 p.
(9) A. Bertrand-Desbrunais, Thèse d'Université, Université Paris VI, 1991, 260 p.
(10) T. Murashige and F. Skoog, Physiol. Plant., 15, 473-497 (1962).
(11) D. B. Fisher, Histochemie, 16, 23-28 (1968).
(12) A. R. Spurr, J. Ultrastruc. Res., 26, 31-43 (1969).
(13) S. Bagniol, F. Engelmann and N. Michaux-Ferrière, Cryo-Letters, 13, 405-412 (1992).
(14) E. E. Benson, K. Harding and H. Smith, Cryo-Letters, 10, 323-344 (1992).
(15) P. Plessis, C. Leddet and J. Dereuddre, C. R. Acad. Sci., Paris, Sér. III, 373-378 (1991).
(16) C. Bassaglia, Diplome d'Etudes Approfondies, Université Paris VI, 1988, 81 p.
(17) J. Singh, Protoplasma, 98, 329-341 (1979).
(18) P. L. Steponkus, Ann. Rev. Plant Physiol., 35, 543-584 (1984).