

CRYOPRESERVATION OF MANGO (*Mangifera indica* L.) EMBRYOGENIC CULTURES

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Abstract

Three techniques were compared for cryopreserving embryogenic masses (EMs) sampled from mango (*Mangifera indica* L.) cv. Zihua embryogenic cultures: (i) encapsulation/dehydration; (ii) pregrowth/dehydration; and (iii) vitrification. In all experiments, EMs were sampled from embryogenic cultures during their exponential growth phase and pretreated for 24 h on solid medium containing 0.5 M sucrose before freezing. No recovery was achieved after cryopreservation using the encapsulation/dehydration technique, whatever the moisture content (fresh weight basis) of EMs, which ranged from 78.3 % without dehydration to 40.8 % after 6 h dehydration. With the pregrowth/dehydration technique, limited recovery (8.3 %) was achieved after desiccation of EMs for 1 h, to 58.5 % MC. Using the vitrification technique, recovery ranged from 94.3 % after treatment of EMs with the PVS3 vitrification solution for 20 min (EM moisture content of 34.7 %) to 10.9 % after a 120 min treatment with the vitrification solution (EM moisture content of 26.0 %).

Keywords: *Mangifera indica*; embryogenic masses; vitrification; encapsulation/dehydration; pregrowth/dehydration.

INTRODUCTION

Mango is grown in tropical and subtropical regions worldwide and ranks 5th among tropical fruit crops, after *Citrus*, *Musa*, oranges and coconuts for its total annual production (11). The collecting and conservation of mango germplasm has a long history, especially in India, where it can be traced back to the 16th century. As a result, more than 1,000 varieties belonging to 41 mango species have been recorded and they are conserved in field genebanks or botanical gardens (2). However, due to increasing deforestation and urbanization, a large amount of mango germplasm with interesting characteristics has disappeared in India and

Southeast Asia, over recent years (2). It is therefore important to increase germplasm collecting activities and to improve conservation techniques for mango.

The technique most commonly employed for *ex situ* conservation of plant genetic resources is the storage of partly desiccated seeds at low temperature (8). However, such a technique is not available for mango. Indeed, as a typical recalcitrant seed species, mango seeds are highly sensitive to dehydration and low temperature and, in addition, they have an extremely long maturation period and a large size (3, 16). It is thus impossible to achieve long-term conservation of mango seeds in the conditions employed for orthodox seeds and only very short-term sub-imbibed storage (120 days) is possible even under optimal humidity and temperature conditions (15).

In vitro culture techniques can allow rapid multiplication of superior clones and long-term storage of germplasm by means of cryopreservation of tissue-cultured material. *In vitro* propagation protocols exist for well over one thousand plant species (13). Cryopreservation is now recognized as the only safe and cost-effective option for the long-term conservation of genetic resources of recalcitrant seed and vegetatively propagated species (7). Various cryopreservation techniques have been developed and protocols established for several hundred plant species (1, 10, 34). Cryopreservation of embryogenic cultures of a tree species was first reported in 1985 as a means of avoiding the loss of embryogenic potential over repeated subcultures and the potential risk of somaclonal variation caused by the long-term maintenance of actively growing embryogenic cultures, as well as for storing large numbers of genotypes until the results of progeny tests become available (4, 14).

For *in vitro* propagation of mango, early studies using leaf explants and shoot tips have shown that such materials produce high levels of phenolic exudates, are often severely infected with contaminants and display a poor growth response *in vitro* (29, 33). However, success has been achieved with somatic embryogenesis using nucellar tissues as primary explants, with the occurrence of a transitory callus phase. Somatic embryos could be induced and plants regenerated from embryogenic cultures of some polyembryonic (5, 18) and monoembryonic (17, 19) cultivars. More recently, direct somatic embryogenesis has been obtained from cotyledons of immature seeds of a monoembryonic mango cultivar (37). Even though progress has been made regarding the cryopreservation of embryogenic cultures of a number of species (30), no protocol is currently available for the cryopreservation of mango embryogenic cultures.

In the present paper, three cryopreservation techniques - encapsulation/dehydration, pregrowth/dehydration and vitrification - have been compared for the cryopreservation of embryogenic cultures of a mango monoembryonic cultivar and *in vitro* plantlets regenerated from cryopreserved cultures.

MATERIALS AND METHODS

Experiments were carried out using the mango (*Mangifera indica* L.) cultivar Zihua. The 3-5 cm long immature fruits were collected 35 to 40 days after flowering from 10-year old trees grown in zhongshan university gardens.

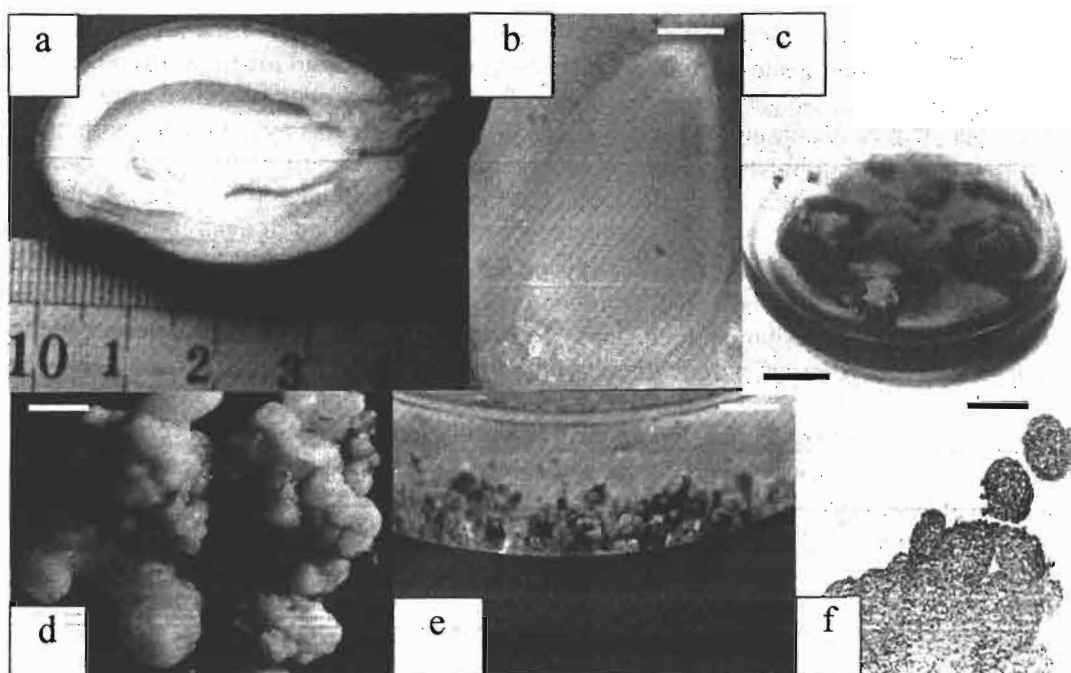


Figure 1: Induction and proliferation of embryogenic cultures and regeneration of EMs of mango cv. Zihua. a) transverse section of an immature fruit showing the embryo; b) immature embryo (Bar: 0.6 mm); c) embryogenic cultures produced from cotyledons of an immature embryo on induction medium (Bar: 1 cm); d) closer view of an EM (Bar: 0.2 mm); e) embryogenic cultures maintained in suspension medium for 20 days (Bar: 4 mm); f) histological section of an EM cultured in liquid medium (stained with haematoxylin) (Bar: 136 μ m).

Induction and proliferation of embryogenic cultures

Immature fruits were washed with tap water, then rinsed with 75 % ethanol and immersed in 75 % ethanol for 5 min. After three rinses in sterile distilled water, the fruits were dipped in absolute ethanol and flamed on a burner. The fruits were then bisected along the longitudinal axis (Fig. 1a) under sterile conditions and immature seeds were removed from each fruit. Each immature seed was also bisected along its longitudinal axis and the embryos (Fig. 1b) were dissected from each half of the immature seed. The cotyledons were cultured on solid modified B5 (MB) medium (containing B5 (12) macroelements except $(\text{NH}_4)_2\text{SO}_4$, MS (21) microelements and organic components), 500 mg/l glutamine, 10 % coconut water, 5.0 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D), 40 g/l sucrose, 6.5 g/l agar and 2 g/l activated charcoal. Cultures were maintained at $28 \pm 1^\circ\text{C}$ on 50 ml medium in 100 ml glass flasks, under white fluorescent light ($52 \mu\text{mol}/\text{m}^2/\text{s}$) with a 12 h light/12 h dark photoperiod.

Three weeks later, embryogenic cultures (Fig. 1c & d) induced from cotyledons were transferred on solid proliferation medium containing MB medium basic components (500 mg/l glutamine, 10 % coconut water, 2.0 mg/l 2,4-D, 1.0 mg/l kinetin, 40 g/l sucrose, 6.5 g/l agar and 2 g/l activated charcoal) or in liquid medium (Fig. 1e & f) containing the same components except agar and activated charcoal, and placed on a rotary shaker at 100 rpm. Five hundred mg of embryogenic cultures were inoculated in liquid medium and 150 mg on solid medium.

Growth characteristics of embryogenic cultures

Growth of embryogenic cultures on solid medium and in liquid medium was measured by following their fresh weight increase. Cultures from each flask were weighed after 5, 10, 20, 30, 40 and 50 days in culture. The growth ratio of the cultures was expressed as: final fresh weight /initial fresh weight.

Cryopreservation

Selection of experimental material: For cryopreservation, embryogenic masses (EMs) of 3 to 5 mm in diameter were selected from embryogenic suspension cultures that had been multiplied in liquid medium for 20 days. The cryopreservation techniques tested included encapsulation/dehydration, pregrowth/dehydration and vitrification.

Pretreatment of EMs: Before comparing the efficiency of the three cryopreservation techniques selected, an experiment was performed to test the effect of sucrose pretreatment on encapsulated and non-encapsulated EMs. Encapsulated and non-encapsulated EMs were either pretreated directly with a 0.5 M, 0.7 M, 1.0 M or 1.2 M sucrose solution for 24 h at $25 \pm 1^\circ\text{C}$, or transferred on media with daily increasing sucrose concentrations (0.5, 0.7, 1.0, 1.2M). In all further freezing experiments, encapsulated and non-encapsulated EMs were pretreated for 24 h at 25°C on solid medium with 0.5 M sucrose before cryopreservation.

Encapsulation/dehydration: The EMs were encapsulated in 4% calcium alginate beads containing 0.5 M sucrose (the diameter of beads was approximately 5 mm) and submitted to the sucrose pretreatment. The beads were then desiccated with silica gel (20 beads above 60 g silica gel in 250 ml flasks) to moisture contents between 78.3 and 40.8 % (fresh weight basis), placed in 2 ml cryotubes (20 beads per cryotube), and frozen by direct immersion in liquid nitrogen.

Pregrowth/dehydration: After the sucrose pretreatment, the EMs were dehydrated with silica gel (20 EM cultures above 60 g silica gel in 250 ml flasks) to moisture contents between 78.0 and 12.2% (fresh weight basis), placed in 2 ml cryotubes (20 EMs per cryotube), and frozen by direct immersion in liquid nitrogen.

Vitrification: After sucrose pretreatment, EMs were dehydrated with the PVS3 vitrification solution (50% sucrose (w/v) + 50% glycerol (w/v) in standard culture medium (24) (20 EMs in 20 ml PVS3 solution at room temperature) to moisture contents between 78.0 and 26.0% (fresh weight basis), placed in 2 ml cryotubes with 1.5 ml fresh PVS3 solution (20 EMs per cryotube), and frozen by direct immersion in liquid nitrogen.

Measurement of moisture content of EMs: After sucrose pretreatment and after different dehydration periods, encapsulated or non-encapsulated EMs were placed in an oven at 105°C for 8 h. Moisture content was expressed on a fresh weight basis. Three replicates of twenty samples were employed to measure the moisture content.

Rewarming, regrowth and recovery: After storage at -196°C for 24 h, samples were rewarmed for 2-3 min in a water-bath at 25°C and regrowth and recovery were estimated after placing the EMs on solid MB medium under standard culture conditions. Regrowth consisted of the appearance of the first new light yellow or green embryogenic structures on top of and/or surrounding the treated EMs. Recovery was defined as the percentage of treated EMs showing the development of cotyledonary embryos (direct regrowth) and/or producing new embryogenic cultures 40 days after thawing. Three replicates of twenty samples were

observed for both regrowth and recovery. Regrowth and recovery percentages are presented with their standard error.

Regeneration of plantlets: About 100 mg of embryogenic cultures regenerated from cryopreserved EMs were transferred on maturation medium containing MB basic medium, 500 mg/l glutamine, 10% coconut water, 40 g/l sucrose, 6.5 g/l agar and 2 g/l activated charcoal. After 40 days in culture, the cotyledonary embryos were transferred to conversion medium containing MS basic medium, 500 mg/l glutamine, 10 % coconut water, 0.5 ml/l kinetin, 40 g/l. sucrose and 6.5 g/l agar. Whole plantlets could be regenerated from cotyledonary embryos after 60 days of culture.

RESULTS

Growth characteristics of embryogenic cultures

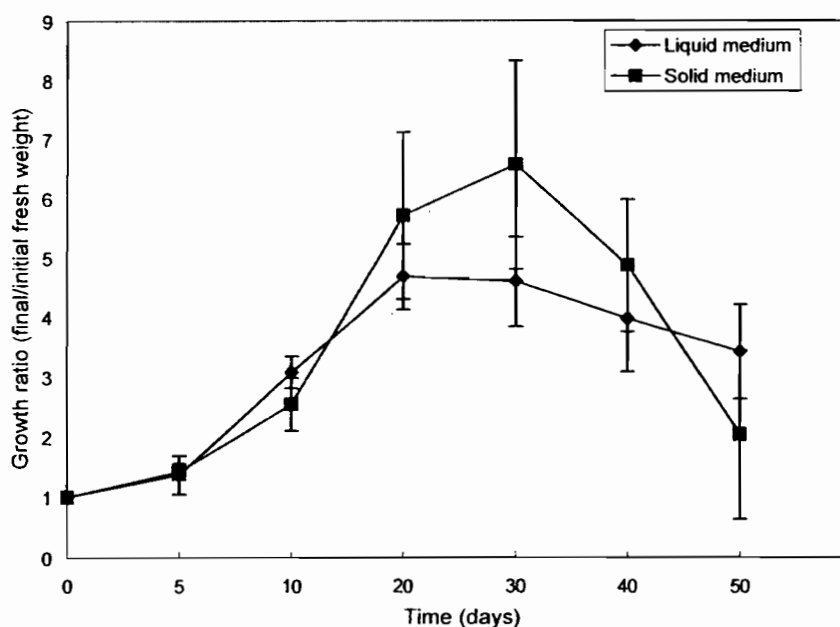


Figure 2: Growth ratio of embryogenic cultures in liquid or on solid medium over a 50 day period.

liquid medium. The fresh weight of cultures increased about 3.5-fold after 20 days in liquid medium and more than 5-fold after 30 days on solid medium (Fig. 2). Even though growth continued after 50 days, the growth rate of cultures decreased from 20 days onwards in liquid medium and from 30 days onwards on solid medium.

Cryopreservation

Pretreatment of encapsulated and non-encapsulated EMs: Recovery was 100 % after pretreatment with 0.5 and 0.7 M sucrose (Fig. 3). The highest sucrose concentration tested (1.2 M) reduced the viability of EMs, both encapsulated and non-encapsulated, even though encapsulation provided a higher level of protection. Recovery after a one day pretreatment decreased from 100 to 90% for encapsulated EMs and to 45 % for non-encapsulated EMs. Sequential pretreatments with progressively increasing sucrose concentrations were detrimental to recovery of EMs.

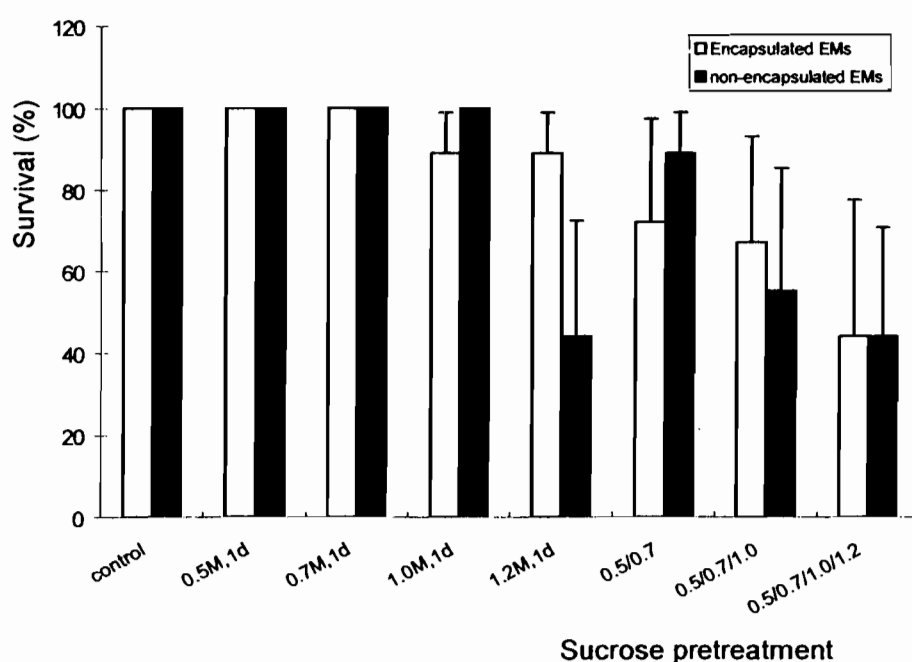


Figure 3: Effect of duration and sequence of pretreatment and of sucrose concentration in the pretreatment medium on the viability (%) of encapsulated and non-encapsulated EMs. After culture in liquid medium for 20 days, EMs were pretreated directly or encapsulated in 4% alginate beads containing 0.5 M sucrose before sucrose pretreatment. Control EMs were not submitted to the sucrose pretreatment.

The moisture content of EMs was $87.8 \pm 0.7\%$ after culture in liquid medium for 20 days and decreased to $78.0 \pm 0.7\%$ after pretreatment for 24 h on medium with 0.5 M sucrose.

Encapsulation-dehydration: Bead moisture content decreased from 78.3% after encapsulation to 40.8% after 6 h desiccation (Table 1). Recovery of desiccated controls remained 100% up to 3 h desiccation, and then decreased to 59.0% after 6 h desiccation. No recovery was achieved after cryopreservation, whatever the moisture content of encapsulated EMs.

Table 1: Effect of desiccation period (h) on the moisture content (% , fresh weight basis) and recovery (%) of EMs of mango cv. Zihua after desiccation (-LN) and desiccation and cryopreservation (+LN) using the encapsulation/dehydration technique.

Desiccation period (h)	Moisture content (%)	Recovery (%)	
		-LN	+LN
0	78.3 ± 0.3	100	0
1	72.1 ± 1.2	100	0
2	68.4 ± 1.6	100	0
3	60.9 ± 1.0	100	0
4	54.2 ± 2.0	94.0 ± 9.6	0
5	43.3 ± 4.6	77.0 ± 8.6	0
6	40.8 ± 6.6	59.0 ± 8.5	0

Pregrowth/dehydration: The moisture content of EMs decreased rapidly during the first 3 h of desiccation, from an initial 78.0 to 12.6 %, and then remained stable around 12 %, even when the desiccation period was extended up to 5 h (Table 2). Recovery of control EMs dropped from 100 % without desiccation to 55.7 % after 1 h desiccation and was nil after longer desiccation periods. After cryopreservation, limited recovery (8.3 %) was achieved only with EMs desiccated for 1 h.

Table 2: Effect of desiccation period (h) on the moisture content (% , fresh weight basis) and recovery (%) of EMs of mango cv. Zihua after desiccation (-LN) and desiccation and cryopreservation (+LN) using the pregrowth/dehydration technique.

Desiccation period (h)	Moisture content (%)	Survival (%)	
		-LN	+LN
0	78.0 ± 0.7	100	0
1	58.5 ± 0.9	55.7 ± 9.8	8.3 ± 9.8
2	22.4 ± 1.6	0	0
3	12.6 ± 1.3	0	-
4	12.6 ± 0.6	0	-
5	12.2 ± 2.6	0	-
6	-	0	-

Vitrification: In comparison with the other techniques tested, the decrease in moisture content of EMs was fastest with treatment with the PVS3 vitrification solution, as the moisture content of EMs went down from an initial 78.0% to 34.7% within 20 min, and reached 26.0% after 120 min (Table 3). Recovery of control EMs was very high for PVS3 treatment durations between 0 (100%) and 80 min (71.0%) but dropped rapidly to 44.0% (100 min) and 11.0% (120 min), due to the toxicity of the vitrification solution. After cryopreservation, no recovery of EMs was noted without treatment with the PVS3 solution. Recovery was very high (91.0-94.3%) after 20 and 40 min of treatment, then decreased progressively to 11.0% after 120 min.

Table 3: Effect of desiccation period (h) on the moisture content (% , fresh weight basis) and recovery (%) of EMs of mango cv. Zihua after desiccation (-LN) and desiccation and cryopreservation (+LN) using the vitrification technique.

Desiccation period (min)	Moisture content (%)	Recovery (%)	
		-LN	+LN
0	78.0 ± 0.7	100	0
20	34.7 ± 1.0	100	94.3 ± 9.8
40	30.5 ± 0.5	79.0 ± 8.5	91.0 ± 8.54
60	28.0 ± 0.6	78.0 ± 9.2	55.7 ± 9.8
80	27.9 ± 1.0	71.0 ± 7.5	45.3 ± 10.4
100	26.8 ± 0.8	44.0 ± 9.8	13.0 ± 6.9
120	26.0 ± 0.8	11.0 ± 10.1	10.9 ± 9.3

Regrowth of EMs and regeneration of plantlets: Control EMs turned dark brown one day after plating on solid culture medium. The first new light yellow or green embryogenic structures appeared on top of and/or surrounding the dark brown control EMs after an average of 5.7 days in culture (Table 4). After sucrose pretreatment, the EMs also became dark brown and regrowth, which followed the same pattern as that of control EMs, was noted after around 11.7 days in culture. Regrowth of EMs desiccated using the three techniques tested was delayed until 15.7-17.3 days, depending on the technique. After cryopreservation, regrowth was noted after 17.3 and 21.0 days for EMs cryopreserved using the vitrification and pregrowth/dehydration technique, respectively. No regrowth was noted from other treatment groups.

Table 4: Number of days necessary to observe the first visible signs of regrowth of EMs of mango cv Zihua without treatment (control), after pretreatment, after desiccation (-LN) and desiccation and freezing (+LN) using the three cryopreservation techniques tested. The dehydration periods of EMs employed were 5 h with encapsulation/dehydration, 1 h with pregrowth/dehydration and 20 min with vitrification. The first visible signs of regrowth noted were the appearance of new light yellow or green embryogenic structures on top of and/or surrounding the treated EMs. The results are presented with their standard deviation.

Treatment	Number of days necessary to observe first signs of regrowth	
	-LN	+LN
Control	5.7 ± 1.5	0.0
Pretreatment (0.5 M sucrose for 1 day)	11.7 ± 2.9	0.0
Encapsulation/dehydration	17.3 ± 2.5	0.0
Pregrowth/dehydration	17.0 ± 3.5	21.0 ± 5.7
Vitrification	15.7 ± 1.2	17.3 ± 2.3

After the first signs of regrowth were observed on treated EMs (Fig. 4a), some EMs started to proliferate (Fig. 4c) while others produced directly cotyledonary embryos (Fig. 4b). After 60 days in culture, the new embryogenic masses formed could be transferred on maturation medium and cotyledonary embryos were formed 40 days later (Fig. 4d). Following culture on conversion medium for 60 days, plantlets were regenerated from cotyledonary embryos (Fig. 4e & f). The plantlets produced are now being cultured *in vitro* and will be transferred *in vivo* for further studies when they reach the right developmental stage.

DISCUSSION

This study represents the first report of successful cryopreservation of mango embryogenic cultures. The EMs employed could be frozen using either the pregrowth/dehydration or the vitrification technique. The best results were obtained with a 24 h pretreatment on medium containing 0.5 M sucrose, desiccation with the PVS3 vitrification solution down to 30-35% moisture content, followed by rapid immersion in liquid nitrogen. The frozen EMs gave rise to new embryogenic cultures which proliferated normally after cryopreservation and produced *in vitro* plantlets through the development of somatic embryos.

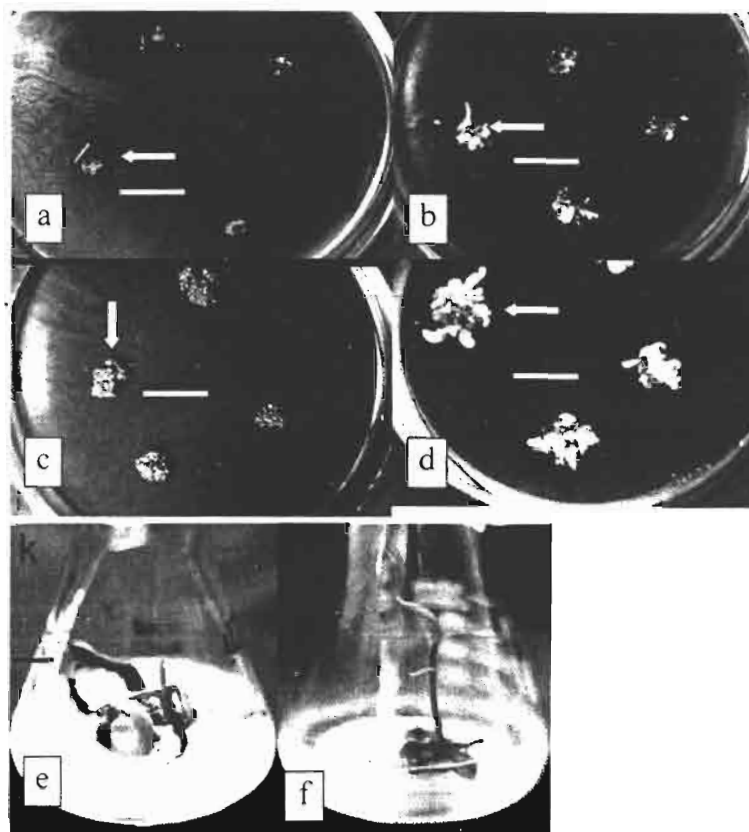


Figure 4: a) recovery of EMs (arrow) after freezing using vitrification; b) direct production of somatic embryos from cryopreserved EMs; c) proliferation of cryopreserved EMs into new embryogenic cultures (arrow); d) cotyledonary embryos (arrow) formed from new embryogenic cultures on maturation medium; e) shoots produced from frozen somatic embryos 60 days after transfer on conversion medium; f) *in vitro* plantlet regenerated from a cryopreserved EM. (Bars: 1 cm).

Somatic embryos of different species have been cryopreserved using both the classical slow cooling method based on freeze-induced dehydration and the new methods based on vitrification of internal solutes, including pregrowth/dehydration, vitrification, encapsulation/dehydration and encapsulation/vitrification (7, 9). An important parameter to take into consideration in the establishment of a cryopreservation protocol for any new species is the physiological state of the experimental material (35). In the case of suspension cultures, it has been shown that the highest cryotolerance is obtained when samples are collected during their exponential growth phase (31, 36). In this study, the growth rate of embryogenic cultures was highest after 20 days in liquid medium and 30 days on solid medium. Even though the growth rate of cultures on solid medium was higher than in liquid medium, germination of somatic embryos occurred on the former medium, whereas only proliferation was noted on the latter. Cultures multiplied in liquid medium for 20 days were thus selected as experimental material.

In order to withstand cryopreservation, tissues must undergo dehydration down to, or close to, their unfreezable water content, thereby suppressing or strongly reducing damage caused to intracellular structures by the crystallization of cellular water during freezing in liquid nitrogen (22, 23). Although, in general, tissues that have low initial water contents are

resistant to dehydration, this not the case for *in vitro* cultured materials, which are not inherently tolerant to dehydration, contain large amounts of water, and must thus be dehydrated artificially before freezing. Sucrose pretreatments have been employed to dehydrate samples in many cryopreservation protocols. It has been shown that sucrose acts both through its osmotic effect, through its accumulation within the cells and through the induction of metabolic changes (6, 32). Sucrose becomes toxic when relatively high concentrations, generally above 1.0 M, are employed during pretreatment and its toxicity increases in line with the duration of pretreatment. A progressive increase in sucrose concentration during pretreatment avoids the sensitivity to direct exposure to high sucrose levels which is encountered with some species such as eucalypt, grape and coffee (20, 27, 28). In this study, mango EMs were relatively resistant to sucrose, as only a pretreatment with 1.2 M for 24 h induced a reduction in recovery. They also appeared sensitive to the duration of pretreatment, as shown by the low recovery percentages obtained with all the progressive pretreatments tested.

In most cases, sucrose pretreatment alone does not dehydrate the samples to water contents sufficiently low for them to withstand freezing and thawing, and additional physical or osmotic dehydration is required. The experiments performed with mango EMs allowed comparison of three different desiccation procedures. Dehydration of encapsulated EMs was slowest. Encapsulated mango EMs appeared sensitive to dehydration since recovery of desiccation controls was only 59.0 % when they were dehydrated to 40.8 % MC. Recovery of EMs cryopreserved with 40.8 % MC was nil, most likely because this MC was still too high. Indeed, recovery of encapsulated samples is generally obtained for bead moisture contents between 20-30 % (fresh weight basis), a level at which most or all freezable water has been removed (7). Additional experiments should be performed to observe the recovery after freezing of encapsulated EMs dehydrated to lower MCs. However, in view of the high desiccation sensitivity of encapsulated EMs, high recovery percentages should not be expected after freezing EMs desiccated to low MCs. Physical desiccation of non-encapsulated EMs in the pregrowth-dehydration procedure was faster. Decrease in recovery of desiccation controls was also more rapid; for EMs at moisture contents around 55-58 %, recovery of encapsulated ones was 94.0 %, whereas it was only 55.7 % for non-encapsulated EMs. However, limited recovery (8.3 %) was achieved after cryopreservation of EMs with 58.5 % moisture content. It would be interesting to test the recovery of EMs frozen with moisture contents between this moisture content and 22.4 % (*i.e.* desiccation periods between 1 and 2 h) for which recovery was nil. Desiccation was extremely fast with the PVS3 vitrification solution; moisture content of EMs was reduced from 78.0 to 34.7 % within 20 min. At the same time, this rapid desiccation procedure ensured very high recovery of both desiccation controls and cryopreserved samples. Such a positive effect of high desiccation rates has been observed notably during the desiccation and freezing of recalcitrant seeds (25, 26). Elevated desiccation rates do not provide sufficient time for metabolic events that are detrimental to cell functional and structural integrity to take place (such as the production of free radicals, or changes in membrane structure), thereby ensuring high survival of rapidly desiccated samples.

Somatic embryogenesis plays a key role in mango propagation and germplasm conservation programmes, as it is the only method ensuring large scale multiplication of elite genotypes, and because EMs are at present the only materials that are amenable to cryopreservation. Cryopreserved storage of embryogenic cultures produced from an increasing number of mango accessions, to complement the traditional conservation of whole plants in field collections, should play a progressively more important role to ensure the safe and cost-effective long-term *ex situ* conservation of mango genetic resources.

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