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CRYOPRESERVATION OF SOMATIC EMBRYOS OF PARADISE TREE (*Melia azedarach* L.)

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Abstract

In paradise tree (*Melia azedarach* L.), immature zygotic embryos sampled from immature fruits are the starting material for the production of somatic embryos. These somatic embryos are employed for freezing experiments. Immature fruits could be stored at 25°C for up to 80 days without impairing the embryogenic potential of zygotic embryos, which represents a four-fold increase in immature fruit storage duration, compared with previous studies. Among the three cryopreservation techniques tested for freezing paradise tree somatic embryos, namely desiccation, encapsulation-dehydration and pregrowth-dehydration, only encapsulation-dehydration and pregrowth-dehydration led to successful results. The optimal protocol was the following: i) somatic embryos (encapsulated or not) pretreated in liquid Murashige & Skoog medium with daily increasing sucrose concentration (0.5 M / 0.75 M / 1.0 M); ii) dehydrated with silica gel to 21 - 26% moisture content (fresh weight basis), for encapsulation-dehydration, or to 19% moisture content, for pregrowth-dehydration; iii) frozen at 1°C/min from 20°C to -30°C with a programmable freezing apparatus; iv) rapid immersion in liquid nitrogen. The highest recovery achieved was 36% with encapsulation-dehydration and 30% with pregrowth-dehydration. Regrowth of frozen embryos was direct in most cases, as secondary embryogenesis originating from the root pole was observed on only around 10% of cryopreserved somatic embryos. Plants recovered from cryopreserved embryos presented the same phenotypic traits as non-frozen control plants.

Keywords: immature fruits, zygotic embryos, encapsulation-dehydration, pregrowth-dehydration, sucrose, desiccation, two-step freezing.

INTRODUCTION

Cryopreservation techniques have been developed for a broad range of materials, including cell suspensions, calluses, meristems, shoot tips, dormant buds, zygotic embryos, somatic embryos and seeds originating from temperate and tropical species (1,2,6,13,31). For

cryopreservation of somatic and zygotic embryos, the main techniques employed are desiccation (6,10,11), pregrowth-desiccation(11), encapsulation-dehydration (17), vitrification and encapsulation-vitrification (28).

In cryopreservation, the explants frozen are usually those which will directly produce whole plants after thawing, e.g. frozen shoot tips will develop into shoots, which will give rise to plantlets and whole plants. However, in some instances, a different strategy is employed: the explants frozen are the primary explants, from which whole plants will be produced only after a transitory organogenesis phase has taken place once they have been rewarmed. This is notably the case in the cryopreservation protocol established for coffee where competent leaf fragments (*i.e.* the primary explants), instead of embryogenic calluses are cryopreserved and stored for the long-term (16). A similar strategy is developed for cocoa cryopreservation (15). In this case, the primary explants which are frozen and stored for the long-term consist of floral parts.

The paradise tree (*Melia azedarach* L.) has raised a great interest in Argentina due to the rapidity of its growth (50 cm in diameter at the height of 1.30 m and a 6 m long trunk which can be cut after 10 years) and because its wood displays characteristics similar to cedar (*Cedrela odorata*), which makes of it an excellent forestry species (21). It shows a very good adaptability to different types of soils and climatic conditions, it is highly resistant to insects and therefore much valued for reforestation programmes (22). The wood is soft, easy to process manually or mechanically. It is used for making planks, furniture, coverings, frames, doors and windows (24). Selected trees are propagated through seeds, which leads to a large genetic variability between individuals. The solution to this problem is the application of vegetative propagation methods, which allow cloning selected trees. Conventional propagation methods have produced only limited results with paradise tree, which has led to the use of *in vitro* culture as an alternative means of propagation (5).

The first report on somatic embryogenesis in paradise tree was published by the staff of the Instituto de Botánica del Nordeste (32). In this protocol, somatic embryos were induced on zygotic embryos sampled from immature seeds, 8-9 weeks after pollination. After this period, embryo excision became impossible as the endocarp of the fruits became lignified and could not be broken with a lancet (Fig. 1A). Hence, in paradise tree, somatic embryogenesis can only be induced for the period of two weeks per year. In this paper, we explored the possibility of storing immature fruits at various temperatures ranging between +25°C and -196°C, in order to extend the period during which excised zygotic embryos can be used for induction of somatic embryogenesis.

There are only two reports on cryopreservation of paradise tree, the first one in which excised embryonic axes were frozen using the encapsulation-dehydration technique (3) and the second in which *in vitro* shoot tips were frozen using the same technique (29). We also established a cryopreservation protocol for paradise tree somatic embryos by comparing three cryopreservation methods, *i.e.* desiccation, encapsulation-dehydration and pregrowth-dehydration.

MATERIALS AND METHODS

Plant material

Immature fruits (8-9 weeks after pollination), of 11-13 mm in diameter, green-colored and egg-shaped (Fig. 1), selected based on their color and development were sampled from *Melia azedarach* L. var. *gigantea* adult trees located on the Campus of the Facultad de Ciencias Agrarias (UNNE), Corrientes, Argentina, from late November to early December in 2004 and 2005.

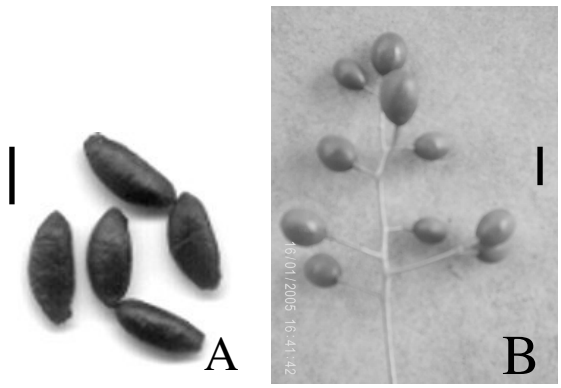

Figure 1: **A**, mature seeds of *Melia azedarach* L. (bar = 0.5 cm). **B**, infructescence with immature (8-9 weeks after pollination) fruits from which zygotic embryos are extracted for cryopreservation experiments (bar = 12 mm).

Storage of immature fruits

Immature fruits were stored at 25°C in an incubator, 4°C in a domestic refrigerator, -20°C in a domestic freezer and -196°C in a LN tank. Samples of fruits were removed from storage after 0, 20, 40 and 80 days.

In vitro *culture of zygotic and somatic embryos*

Fruits were surface sterilized by immersion in 70% ethanol for 5 min followed by 20 min in 3.6% (w/v) sodium hypochlorite solution with two drops of Tween®. They were then rinsed three times in sterile distilled water. Embryos (1.0-1.5 mm in length) were extracted aseptically from the seeds and placed on Murashige & Skoog [MS] (25) medium supplemented with 4.54 µM thidiazuron (TDZ) and 3% sucrose and 0.8% agar (Sigma A-1296). Embryos were cultured at 27 ± 2°C, under a 14 h light / 10 h dark photoperiod, with a light intensity of 116 µmol/m^2/s provided by cool white fluorescent lamps (32). After 4 weeks, the somatic embryos which had appeared on the surface of the zygotic embryos were transferred for germination on quarter-strength MS medium with 3% sucrose but without growth regulators under the same environmental conditions.

For each experimental condition, three replicates of 10 explants were used. Survival (%) of zygotic embryos was recorded after 7 days (living zygotic embryos were green, whereas dead zygotic embryos were white). Recovery was estimated after 6 weeks by measuring the embryogenic index of individual somatic embryos (EII) and of fused somatic embryos (EIF), calculated as follows:

EII = (% of explants with somatic embryos) x (number of individual somatic embryos/explant)/100;

EIF = (% of explants with somatic embryos) x (number of fused somatic embryos/explant)/100.

Cryopreservation of somatic embryos

Encapsulation-dehydration

The protocol employed in this study was adapted from that developed for freezing shoot tips of paradise tree (29). Mature somatic embryos (5 mm in length) were isolated from cultures and encapsulated individually. For encapsulation, somatic embryos were suspended in culture medium (MS medium with 4.54 µM TDZ) devoid of calcium and containing 3% sodium alginate. They were poured in a drop wise manner in a 0.1 M calcium chloride solution, thus forming beads of 4-5 mm diameter containing one embryo, which were left for 3-4 min in the calcium chloride solution. The beads were pretreated by transferring them every 24 h in liquid medium with progressively increasing sucrose concentration (0.5, 0.75 and 1.0 M). Ten beads were then dehydrated in 100 cm^3 air-tight containers with silica gel (30 g/container) for up to 4 h, thereby reaching 21-26% moisture content (MC, fresh weight basis). Some beads were dehydrated without sucrose pretreatment. Desiccated beads were

placed in sterile polypropylene 5 ml cryotubes (10 beads/tube), which were directly immersed in LN or submitted to a two-step freezing protocol: i) slow freezing at 1°C/min from 20°C to -30°C using a Controlled Rate Freezing System Model 9000 (Gordinier Electronics Inc., USA); followed by ii) direct immersion of the cryotubes in LN. Cryotubes were kept in LN for a minimum of 1 h and rewarmed rapidly by plunging them for 2 min in a water-bath thermostated at 30°C. Somatic embryos were then transferred to recovery medium (1/4 strength MS medium, 3% sucrose, no growth regulators) under standard culture conditions. The plants obtained after 2 months in culture were transferred to the greenhouse for further observation.

Pregrowth-dehydration

Non-encapsulated mature somatic embryos were submitted to the same pretreatment as for the encapsulation-dehydration technique, dehydrated with silica gel from 90.5% MC (0 h) to 13.5% MC (4 h), and then frozen rapidly (direct immersion of the cryotubes in LN) or slowly (1°C/min from 20°C to -30°C followed by direct immersion of the cryotubes in LN) and rewarded rapidly as described above. Growth recovery took place as described above.

Desiccation

Non-pretreated and non-encapsulated mature somatic embryos were desiccated with silica gel from 83.5% MC (0 h) to 9.3% MC (4 h) and then frozen rapidly (direct immersion of the cryotubes in LN) or slowly (1°C/min from 20°C to -30°C, followed by direct immersion of the cryotubes in LN). Rewarming and growth recovery took place as described previously.

Recovery (%) was measured after 3 weeks by counting the number of frozen somatic embryos which developed into plantlets and/or produced adventitious embryos, and gave rise to fully developed plantlets.

Statistical analyses

Experiments were repeated three times with 10 replicates per treatment. Results are presented as Tables and Figures with standard error (SE). Data were subjected to analysis of variance (ANOVA) and comparisons of means were performed using Tukey's Multiple Comparison test ($p<0.05$). In the experiments, controls correspond to embryos which have been submitted to all treatments but not cryopreserved.

RESULTS

When fruits were stored at sub-zero temperatures (-20°C and -196°C), no survival of zygotic embryos was observed after 20 days of storage (Fig. 2). Survival percentage of zygotic embryos remained high (equal to initial state) at 25°C and 4°C for 20 days in storage. However, the survival of zygotic embryos dropped to 0% after 40 days in storage at 4°C, whereas, in the case of fruits stored at 25°C survival decreased to around 60% after 40 days in storage and remained constant up to 80 days.

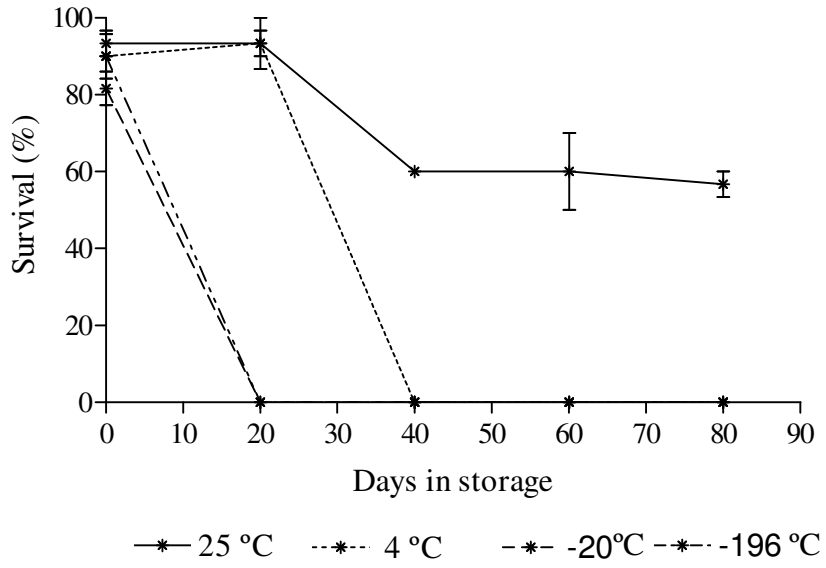

Figure 2. Effect of storage temperature and of storage duration of *M. azedarach* immature fruits on the survival percentage of excised zygotic embryos. Bars represent the SE of survival percentages.

Somatic embryos appeared most frequently on the hypocotyl of zygotic embryos, but they were also observed on the cotyledonary axis as well as on the surface of the radicle (Fig. 3A). Somatic embryos reached the globular stage after 2 weeks (Fig. 3B) and mature somatic embryos (Fig. 3C & D) were obtained after 4-6 weeks in culture.

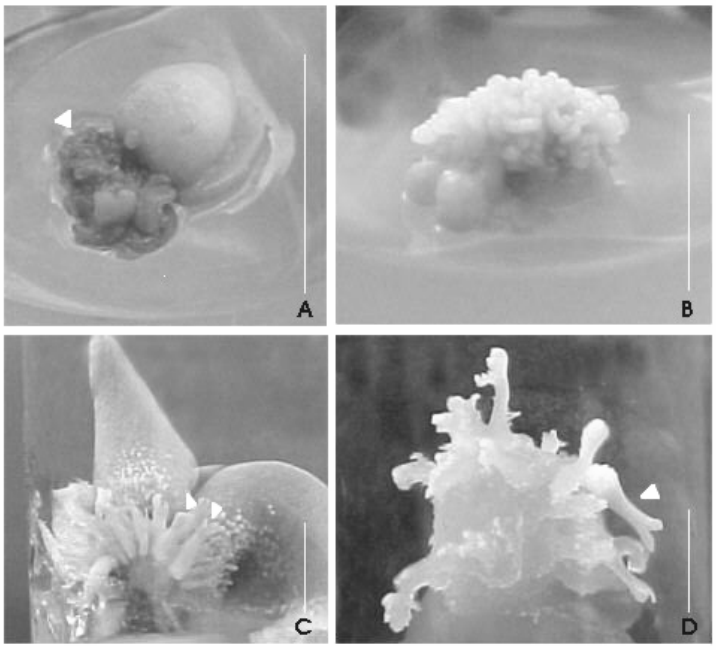

Figure 3: Somatic embryos of paradise tree at different stages of development.
A, formation of somatic embryos (arrow) on the hypocotyl and radicle of a zygotic embryo.
B, somatic embryos, mostly at the globular stage.
C and **D**, mature somatic embryos (arrows). Bars = 5 mm.

The production of individual embryos was higher than that of fused embryos (Fig. 4). The production of fused embryos decreased gradually over the period of storage and the EIF was nil after 40 days of storage at 25°C and at 4°C. As regards to individual embryos, the EII was stable after 0 and 20 days of storage at both temperatures tested. It then decreased

dramatically at 4°C to reach 0 after 40 days of storage and decreased only slightly at 25°C, reaching the value of 8 after 80 days in storage. The fact that the numerous fused embryos observed on explants at 20 days (Fig. 5A) disappeared on explants cultured at 25°C after 40 days was due to the continued growth of zygotic embryos over time at this temperature. Only isolated embryos were thus observed on explants after 40 days in storage (Fig. 5B). Isolated embryos germinated and developed into *in vitro* plantlets (Fig. 5C) which then grew into normal plants after transferring into pots (Fig. 5D).

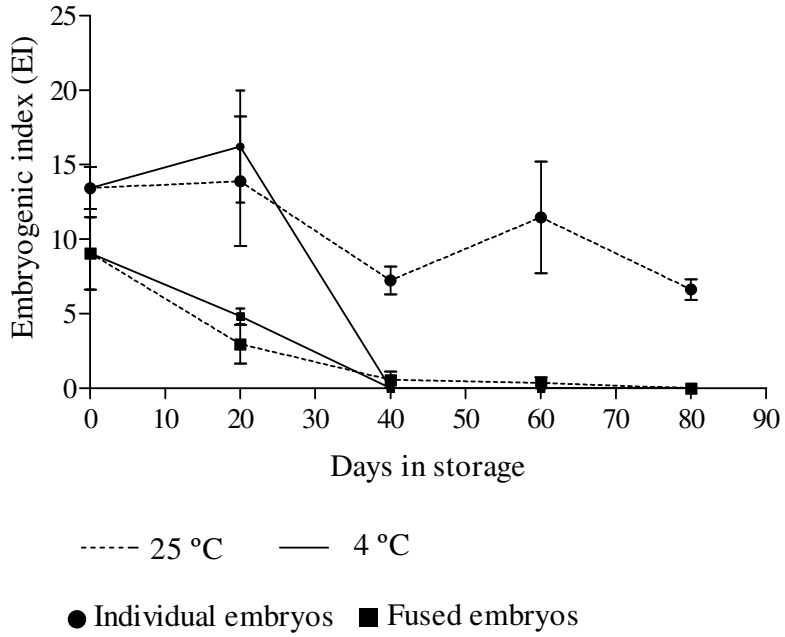

Figure 4. Effect of storage temperature and of storage duration of *M. azedarach* immature fruits on the embryogenic index of individual (EII) and of fused (EIF) somatic embryos.

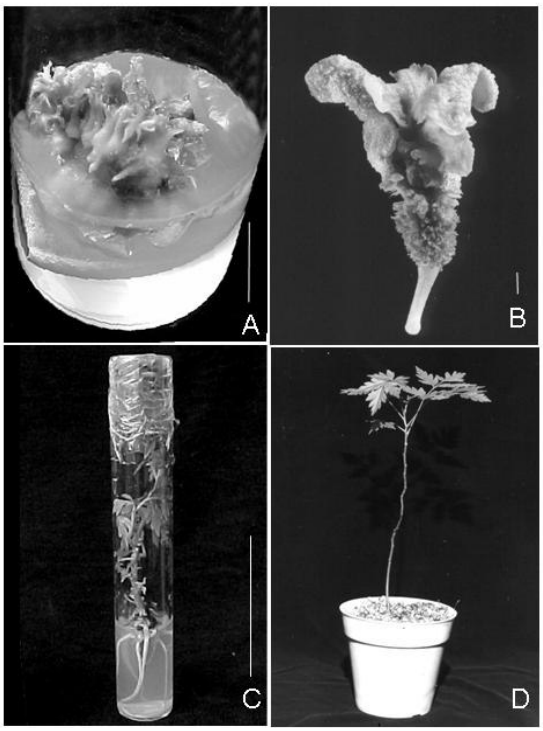

Figure 5: Somatic embryogenesis and plant regeneration from cultures of zygotic embryos of paradise tree.
A, Somatic embryos induced on zygotic embryo cultured after 20 days under storage at 25°C (bar = 1 cm).
B, Somatic embryos induced on zygotic embryo cultured after 60 days under storage at 25°C (bar = 1 mm).
C, Individual somatic embryos converted into *in vitro* plantlet (bar = 5 cm).
D, Plantlet produced from somatic embryo.

No survival was achieved with the desiccation technique, whatever the freezing protocol (rapid or slow freezing) employed (Table 1). When using the encapsulation-dehydration technique, the MC of pretreated embryos decreased more rapidly than that of non-pretreated embryos with increasing dehydration periods. Survival of desiccated pretreated controls was higher than that of desiccated non-pretreated controls since after 4 h desiccation, 43% pretreated embryos were still alive compared to only 10% non pretreated ones. No survival was obtained after rapid freezing and only pretreated embryos displayed survival after slow freezing, reaching 7% after 2 h desiccation and 36% after 3 h.

Table 1. Effect of cryopreservation technique and of dehydration duration (h) on the MC (%, fresh weight basis) and survival of control (-LN) and cryopreserved (+LN) *Melia azedarach* somatic embryos. Embryos were frozen slowly (SF) or rapidly (RF).

Cryopreservation technique				
Desiccation of naked embryos		Survival (%)		
Dehydration duration (h)	MC (%)	-LN	+LN/SF	+LN/RF
0	83.5	66±5	0	0
2	22.0	40±10	0	0
3	14.0	16±6	0	0
4	9.3	3±3	0	0
Encapsulation-dehydration		Survival (%)		
(without sucrose pre-treatment) Dehydration duration (h)	MC (%)	-LN	+LN/SF	+LN/RF
0	98.3	70±10	0	0
2	54.5	43±3	0	0
3	26.5	27±6	0	0
4	22.4	10±10	0	0
Encapsulation-dehydration		Survival (%)		
(with sucrose pre-treatment) Dehydration duration (h)	MC (%)	-LN	+LN/SF	+LN/RF
0	91.7	73±6	0	0
2	32.1	67±5	7±3	0
3	21.0	54±5	36±5	0
4	17.4	43±3	0	0
Pregrowth-dehydration		Survival (%)		
Dehydration duration (h)	MC (%)	-LN	+LN/SF	+LN/RF
0	90.5	60±10	0	0
2	25.4	50±10	3±5	0
3	19.0	44±5	30±10	0
4	13.5	27±6	0	0

Surviving cryopreserved somatic embryos germinated rapidly and gave rise to *in vitro* plantlets which grew into phenotypically normal plants after transfer into pots (Fig. 6 A-E, G). Around 10% of cryopreserved embryos gave rise to secondary embryogenesis originating at the root pole (Fig. 6F), a phenomenon which was not observed on non-frozen control embryos.

The MC of pregrown-dehydrated embryos varied between 91% without desiccation and 14% after 4 h dehydration (Table 1). Survival of control embryos varied from 60% without dehydration to 27% after 4 h dehydration. No survival was achieved after rapid freezing. When using slow freezing, limited survival (3%) was achieved after 2 h desiccation and intermediate survival (30%) after 3 h desiccation.

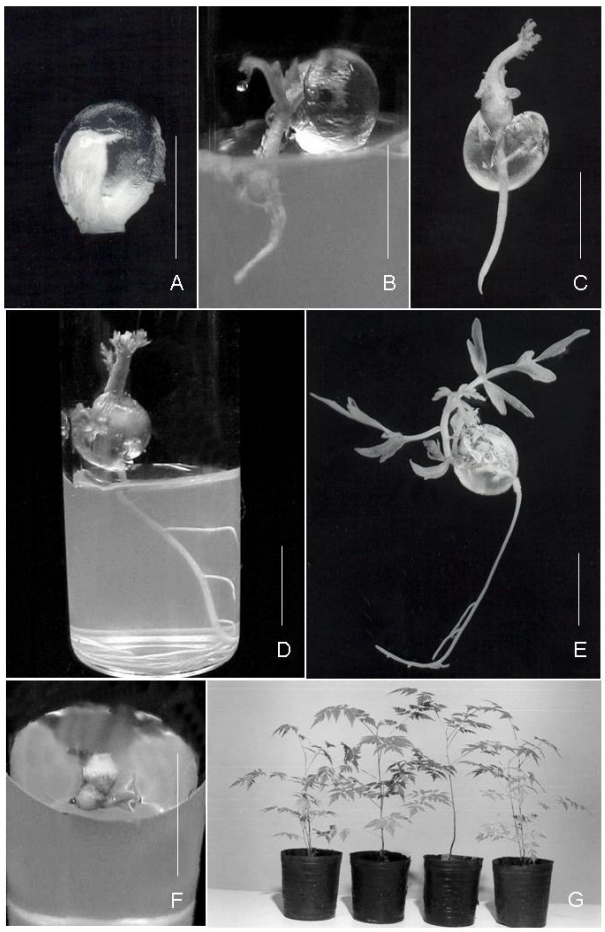

Figure 6: **A** to **E**- Sequential development of somatic embryos and plantlet regeneration after cryopreservation using the encapsulation – dehydration technique.
A, Somatic embryo after 15 days.
B and **C**, Germinated somatic embryos after 21 days.
D, Germinated somatic embryo after 35 days.
E, *In vitro* plantlet regenerated after 45 days.
F, Secondary embryogenesis occurring on a cryopreserved embryo.
G, Phenotypically normal plants regenerated from cryopreserved somatic embryos. (bars = 1cm).

DISCUSSION

Paradise tree seeds have been classified as displaying orthodox storage behavior (18). However, immature fruits did not withstand storage at negative temperatures (-20°C and -196°C) because of their high water content at the developmental stage used, which led to freezing injury (19), resulting in rapid death of the fruits. Thus, for paradise tree it was impossible to establish the same strategy as those developed for coffee and cocoa, where primary explants are conserved under cryo-storage (15, 16). In a similar way, immature fruits stored at 4°C lost viability rapidly because of the chilling injury they suffered at this temperature (23). There is a progressive transition from desiccation intolerance to tolerance during the development of orthodox seeds (20), which enables subsequent freezing treatments to be applied successfully. By contrast, survival of fruits stored at 25°C was still high after 80 days. It would be worth checking which maximal storage duration of immature fruits for somatic embryo induction can be reached at this temperature and what would be the effect of storage at other temperatures, comprised between 4°C and 25°C. Another parameter to study might be the effect of developmental stage of fruits/zygotic embryos on production of somatic embryos.

From a practical point of view, the period during which somatic embryos can be induced on zygotic embryos has been increased four-fold in the current study compared to previous results (32), and a further extension of this period is highly likely, as no decrease in the somatic embryo induction potential has been noted between 40 and 80 days. It would be interesting to define the conditions leading to maximal storage duration of immature fruits, as this would further broaden the window of utilization of immature zygotic embryos for

induction of somatic embryos employed for cryopreservation. An interesting observation was made during this study, which is that the number of fused embryos decreased in line with increasing duration of storage of immature fruits at 25°C. This might be due to the fact that zygotic embryos continued to grow during storage, thereby providing more "space" on the primary explants for the development of isolated somatic embryos. Maturation of zygotic embryos during storage, with the consequence of a decrease in their potential to produce somatic embryos, could also explain this result.

In this study, with both freezing techniques employed which provided positive results, *i.e.* encapsulation-dehydration and pregrowth-dehydration, sucrose pretreatment was necessary to achieve recovery after cryopreservation. Similar results are generally observed with other plant materials frozen using these techniques (12, 14, 17). Sucrose preculture has two main effects: it reduces the moisture content of the explants through an osmotic effect; sucrose is also absorbed by the explants, thereby increasing the concentration of internal solutes (7, 9).

An original result of the current study is that, with the two cryopreservation techniques used which gave positive results, encapsulation-dehydration and pregrowth-desiccation, recovery was obtained only when a two-step freezing protocol was employed. This is contrary to what is usually observed with these techniques, for which rapid freezing is generally employed (12, 14, 17). Another exception is grape, for which slow freezing of excised encapsulated shoot tips produces higher recovery than rapid freezing (26). This result might imply that there is still some free water in the somatic embryos, part of which is extracted from the explants during the slow cooling phase. Upon rapid freezing, either vitrification of internal solutes (if all remaining free water has been removed) or non detrimental intracellular freezing (formation of small ice crystals) occurs (27). Differential scanning calorimetry (DSC) studies should be performed to give us precise information on the thermal events which take place during freezing/rewarding of paradise tree somatic embryos, as has been performed in various other cases (e.g. 4, 8, 9, 30).

The plantlets regenerated from cryopreserved somatic embryos were phenotypically normal and identical to those produced from non-frozen embryos. These preliminary results will have to be confirmed by a larger scale and more in-depth comparison of plants regenerated from control and cryopreserved material.

In conclusion, this paper has highlighted the importance of the manipulation/storage of primary explants, *i.e.* immature fruits, from which the material to be cryopreserved is produced. Various additional experiments have been suggested to explore the possibilities of increasing the storage duration of the immature fruits. This paper also reports for the first time the cryopreservation of paradise tree somatic embryos. The recovery percentages currently achieved are still intermediate. It might be possible to further improve the results by refining the pretreatment conditions, e.g. by increasing the duration of treatment with the successive sucrose media employed, or by employing higher final sucrose concentrations, or by testing other techniques such as vitrification, encapsulation-vitrification or droplet-vitrification (28). Finally, the protocol established will have to be validated using additional paradise tree accessions.

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