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Alginate Encapsulation Technique and Cryogenic Procedures for Long-Term Storage of the Tropical Forest Tree *Guazuma crinita* Mart. *In Vitro* Cultures

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Abstract

Long-term storage of the tropical forest tree *Guazuma crinita* Mart. Following alginate encapsulation technique and cryogenic procedures was attempted. Shoot tips excised from *in vitro* plantlets were encapsulated in calcium alginate beads and stored on different substrates at 12, 20, and 25°C. Viability was retained when encapsulated shoot tips were stored on substrate containing only water solidified with 1% (w/v) agar. Percentage of viability 12 months after storage was 90% at 25°C and 70% at 20°C. For cryogenic experiments, shoot tip, nodal segment, root tip. And adventitious bud cluster explants were stored in liquid nitrogen following 4 cryopreservation methods: (1) simple freezing, (2) rapid freezing, (3) slow pre-freezing. and (4) encapsulation/dehydration method. High survival rates (about 80%) were achieved when the adventitious bud cluster explants were exposed to a cryoprotectant mix solution containing (w/V), 25% glycerol, 15% sucrose, 15% ethylene glycol, 13% dimethyl sulfoxide, and 2% polyethylene glycol(4,000), at 25°C for 15-60 min prior to storage in liquid nitrogen.

Discipline: Biotechnology/Forestry and forest products/Genetic resources
Additional key words: artificial seed. bolaina Blanca. Cryopreservation, germplasm conservation, vitrification

Introduction

Bolaina blanca (*Guazuma crinita* Mart., Sterculiaceae) is a useful fast-growing tree endemic in the Amazon forest region[8]. This tree is a light hardwood species with good working properties that has been used for light construction, panelling, interior joinery, mouldings, cases, matches, packing, etc. *G. crinita* has a fast initial growth rate of up to 3 m in height per year (final rotation age of 10 to 15 years) and shows an excellent adaptability to a wide range of sites including areas with poorly drained heavy clay soils which are widespread throughout the tropics. It has been used for reforestation in the Peru-Amazon[17].

Germplasm conservation has become necessary for future sustainable harvesting systems, and as a means of maintaining species diversity to prevent genetic erosion.

The propagation and conservation of this species traditionally take place by seeds. However, propagation and conservation by vegetative means are desirable for better preservation of true-to-type genetic characteristics. The conservation of desirable genetic materials using conventional *in situ* or *ex situ* methods such as a field genebank is costly and prone to possible catastrophic losses due to diseases and pest attacks, and is liable to environmental and political threats. Although the use of *in vitro* genebanks alleviates these problems, the maintenance of large collections under conventional *in vitro* storage systems requires much handling and is expensive because most cultures need subculturing at regular intervals to prevent browning and loss of viability, hence the increasing risk of contamination.

Alginate encapsulation technique and cryogenic procedures may be reliable methods for long-term storage of plant genetic resources without apparent risk of genetic instability using minimum space and with lower labor and maintenance costs.

This paper describes the storage of shoot tips

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encapsulated in calcium alginate beads under temperature conditions above freezing (12, 15, and 20°C), and the cryogenic procedures for the long-term storage of *G. crinita* using *in vitro*-cultured plant materials.

Materials and methods

1) Storage following alginate encapsulation technique

(1) Plant material

Shoot tips, about 3–4 mm long, were aseptically excised from *in vitro*-cultured plants regenerated by the method described by Maruyama et al. (1996)[17].

(2) Encapsulation

Calcium alginate beads were produced by encapsulation according to the method of Kinoshita and Saito (1990)[12]. Shoot tips were immersed in autoclaved standard woody plant medium (WPM)[15] supplemented with 1 μM kinetin containing 4% (w/v) sodium alginate and 2% (w/v) sucrose. Next, the shoot tips in the alginate medium were picked up individually with tweezers and dropped into a sterile solution of 1.4% (w/v) calcium chloride where they remained for 30 min. The calcium chloride solution was then decanted and the alginate beads, each containing 1 shoot tip, were rinsed 3 times with the same autoclaved medium.

(3) Storage

Encapsulated shoot tips were transferred to 200 mL Erlenmeyer flasks containing about 75 mL of the following substrates: water, water containing 2% (w/v) sucrose, and sucrose and plant growth regulator-free WPM. All the substrates were solidified with 1% (w/v) agar (Wako Pure Chem. Ind.). Storage lasted for 3, 6, and 12 months at 25 to 20°C (16 h photoperiod under photon flux density of about 35 μmol m^{-2} s^{-1}) and 12°C (in the dark).

(4) Plant regeneration

Stored encapsulated shoot tips were transferred to germination media (WPM with 1 μM KIN) and incubated in a culture room at 25°C under a photon flux density of about 65 μmol m^{-2} s^{-1} for 16 h daily. About 60 days after the transfer to the germination media, encapsulated shoot tips regenerated to plantlets were counted.

(5) Data and analysis

Five encapsulated shoot tips were employed for each of 2 replicates per treatment, at 3 storage temperatures, on 3 storage substrates, over 3 storage periods. Data were analyzed quantitatively by calculating rates of plant regeneration from stored encapsulated shoot tips. The standard error of means was calculated according to Snedecor (1957)[25].

2) Storage following cryogenic procedures

(1) Plant material

Shoot tips, nodal segments and root tips, excised from 2 to 3-month-old plantlets regenerated *in vitro* by the method described by Maruyama et al. (1996)[17], were cut into segments about 2 mm long. Adventitious bud clusters obtained from petiole culture by the method described by Maruyama et al. (1997)[18] were cut into small (1.0–1.5 mm^3) or large (3.0–4.0 mm^3) cubic segments.

(2) Cryoprotectant mix

The following cryoprotectant mix solutions, modified from Sakai et al. (1991)[23] and Towill (1990)[27], were tested containing (w/v), Mix A: 20% glycerol and 15% sucrose; Mix B: 30% glycerol, 15% sucrose, 15% ethylene glycol, and 15% dimethyl sulfoxide (DMSO); Mix C: 25% glycerol, 15% sucrose, 15% ethylene glycol, 13% DMSO, and 2% polyethylene glycol (PEG); Mix D: 35% ethylene glycol, 10% DMSO, and 5% PEG. The aqueous volume added consisted of WPM without sucrose and growth regulators. All the cryoprotectant mix solutions were filter-sterilized.

(3) Cryopreservation methods

a) Simple freezing

Explants were treated with cryoprotectant mix solution A at 25°C for 5, 10, 15, 20, 30, 45, and 60 min and then cooled in a freezer at −30°C for 1 h prior to immersion in liquid nitrogen (LN) and kept there for at least 1 h.

b) Rapid freezing

Explants were treated with cryoprotectant mix solutions A, B, C, D, alone or in succession at 25°C for different periods of time (0 to 90 min) and then directly immersed in LN and kept there for at least 1 h.

c) Slow pre-freezing

Explants were treated with cryoprotectant mix solutions A, B, C, alone or in succession at 25°C for different periods of time (0 to 45 min) and then cooled to −40°C at a rate of 0.5°C/min prior to immersion in LN and held there for at least 1 h.

d) Encapsulation/dehydration

Explants were progressively pre-cultured at 5°C by successive daily transfer onto solidified WPM containing 5, 10, and 20% (w/v) sucrose. Then, they were encapsulated or not, in 3% (w/v) alginate-coated beads containing 20% (w/v) sucrose. Encapsulated (constructed beads about 5 mm in

diameter containing one shoot tip or nodal segment by the same method as that described previously) and non-encapsulated explants were treated with the same medium supplemented with 30% (w/v) sucrose for 16 h at 5°C. After treatments with sucrose they were subjected to dehydration at 25°C for 0 to 24 h in the laminar flow cabinet or inside petri dishes (9 cm in diameter) containing about 50 g silica gel sterilized by heating at 110°C for 16 h, and then cooled in LN by the slow pre-freezing or by the rapid freezing method.

(4) Survival and plant regeneration

Explants were thawed by rapid transfer of cryotubes in a water bath at 37°C. After thawing, cryoprotectant mix solutions were drained from the cryotubes and replaced with a medium containing 40% (w/v) sucrose and kept for 20 min. Then, shoot tip and nodal segment explants were transferred onto solidified WPM containing 10 μM zeatin and cultured at 25°C under a photon flux density of 65 μmol m^{-2} s^{-1}. The root tip and adventitious bud cluster explants were transferred into liquid WPM containing 10 μM zeatin and cultured on a bio-shaker at 73 rpm under a photon flux density of 25 μmol m^{-2} s^{-1}. All the explants were cultured at 25°C and under a 16 h photoperiod provided by cool white fluorescent lamps (100 V, 40 W; Toshiba Co.). Examination of the explants for survival was performed at weekly intervals. Survival was recorded when explants had turned green and produced leaves or adventitious buds. Plant regeneration was considered to have occurred when explants had developed into plantlets.

(5) Acclimatization of plantlets

Plantlets regenerated from cryopreserved explants were transplanted into plastic pots filled with vermiculite after washing the roots with tap water to free any adhering agar. They were acclimatized in a growth cabinet at 25–30°C at a photon flux density of 35 μmol m^{-2} s^{-1} under a 16 h photoperiod, and were irrigated with water for the first 2 weeks and then with 0.1% (v/v) Hyponex plant food solution (The Hyponex Co., Inc.) containing (w/v): 5.00% N, 4.36% P, and 4.15% K. During the first 2 weeks plantlets were kept in a closed container with a transparent cover inside the growth cabinet. After that, the cover was opened gradually during another 2 weeks and removed completely about 1 month after transplanting.

Results and discussion

1) Storage following alginate encapsulation technique

Attempts at growth suppression using alginate encapsulation techniques in combination with temperature reduction and/or omission of some nutrients essential for normal growth were made in order to develop an efficient method for germplasm conservation under temperature conditions above freezing. Table 1 shows the results of plant regeneration from alginate-encapsulated shoot tips after 3, 6, and 12 months of storage under various conditions. When the encapsulated shoot tips were stored on a water substrate, the growth process was suppressed or reduced at all the storage temperatures tested. However, the percentage of plant regeneration varied with the temperature and period of storage.

Although the addition of 2% sucrose into the water substrate promoted rooting (not shown) in beads stored at 25°C, it did not induce conspicuous growth under the other storage conditions. However,

Table 1. Effects of substrates and storage temperature on plant regeneration from stored alginate-encapsulated shoot tips of *G. crinita*

Months of storage	25°C			20°C			12°C		
	W	W+S	M	(W)	W+S	M	W	W+S	M
	Percentage of plant regeneration (Mean ± SE)			Percentage of plant regeneration (Mean ± SE)			Percentage of plant regeneration (Mean ± SE)		
3	90 ± 10	10 ± 10	10 ± 10	90 ± 10	50 ± 10	20 ± 0	80 ± 0	70 ± 10	30 ± 10
6	100 ± 0	0 ± 0	0 ± 0	100 ± 0	0 ± 0	0 ± 0	20 ± 20	50 ± 10	0 ± 0
12	90 ± 10	0 ± 0	0 ± 0	70 ± 10	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Data were calculated from 5 beads for each of 2 replicates per treatment. After 3, 6, and 12 months of storage, respectively, beads were transferred to WPM supplemented with 1 μM kinetin for inducing germination and shoot growth. The results are expressed as percentage of beads that produced plants. W: Water. W+S: Water containing 2% (w/v) sucrose. M: Sucrose and plant growth regulator-free WPM. All the substrates were solidified with 1% (w/v) agar.

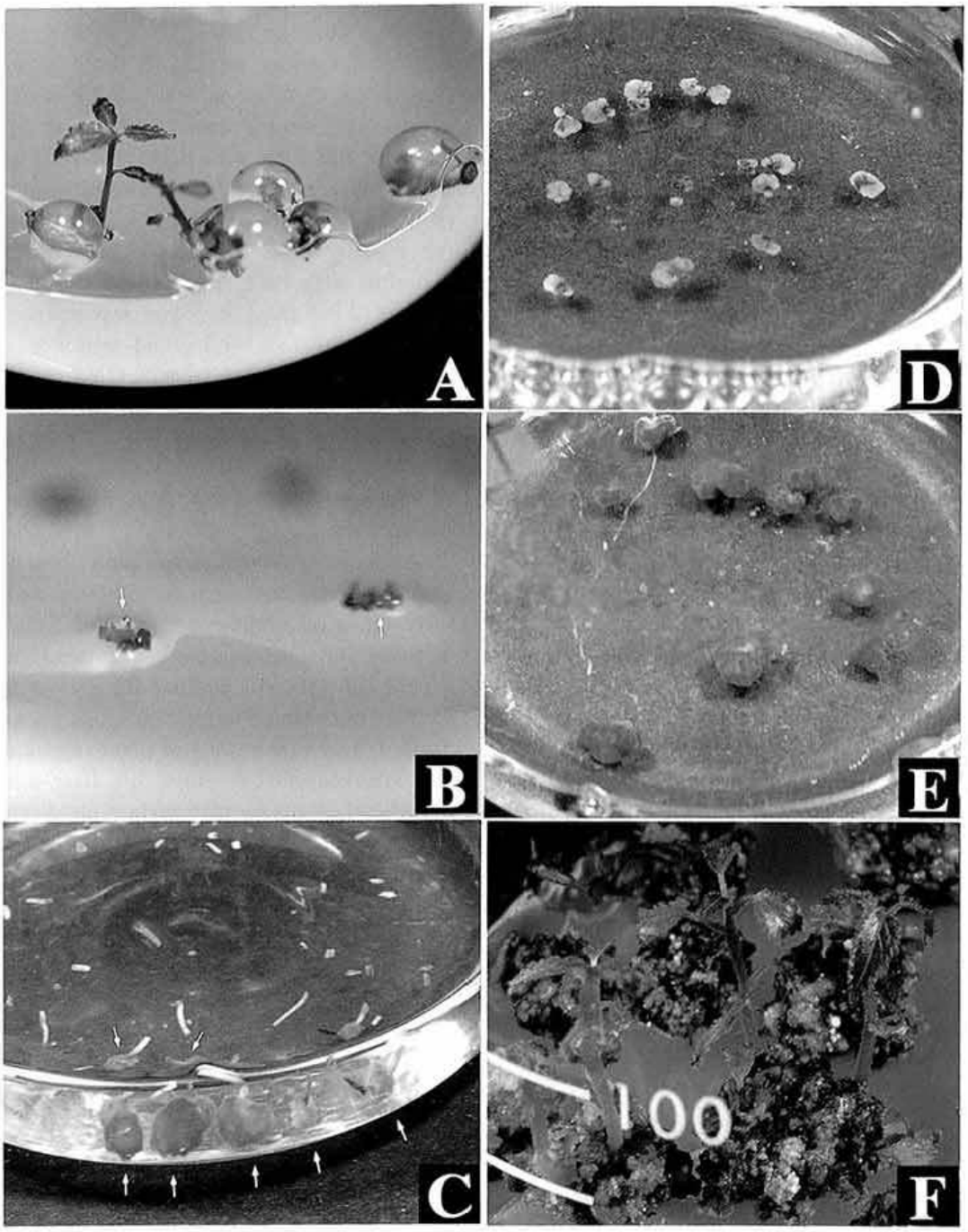

Fig. 1. Storage of *G. crinita in vitro* cultures
(A) Germination of shoot tips encapsulated in calcium alginate beads after storage for 12 months at 25°C on water substrate solidified with 1% (w/v) agar.
(B) Survivors (arrows) of shoot tips after storage in LN by slow pre-freezing method.
(C) Adventitious bud cluster formation (arrows) on root tips after storage in LN by rapid freezing.
(D) Cryopreserved adventitious bud cluster segments after 15 days of culture in recovery growth medium.
(E) Cryopreserved adventitious bud cluster segments after 45 days of culture in recovery growth medium.
(F) Shoot development from cryopreserved adventitious bud cluster segments after 60 days of culture onto WPM supplemented with 1 μM zeatin.

the effect of sucrose on the stored beads was not beneficial because plant regeneration 6 months after storage was only achieved at 12°C. These results suggest that (1) although conspicuous growth did not occur in stored beads, metabolic processes for germination were induced, (2) the addition of sucrose into the agar substrate enhanced the deterioration of stored beads at storage temperatures of 25 and 20°C, and (3) the beads deteriorated least at a storage temperature of 12°C.

The results may be summarized as follows: (1) in general, water was the optimum substrate for suppressing growth in alginate-encapsulated shoot tips, (2) the optimum temperature for storage of encapsulated shoot tips was 25°C, (3) 20°C was the lowest temperature recommendable for storage of alginate-encapsulated shoot tips of *G. crinita* under temperature conditions above freezing, and (4) shoot tips encapsulated in alginate beads could be stored for more than 12 months without considerable loss of viability (Fig. 1A).

Storage of alginate-encapsulated shoot tips under temperature conditions above freezing was found to be a suitable method for conservation of *in vitro*-cultured germplasm. After storage for 12 months, the rate of plant regeneration was up to 90%. Development of germplasm conservation techniques, based on the storage of shoot tips under temperature conditions above freezing, is an important alternative for many species that do not tolerate conventional reduced temperature storage systems (generally at about 4°C).

The alginate bead effect on growth immobilization or minimal growth storage may be attributed to a reduction in the respiration process in encapsulated cells[4]. Alginate is one of the most commonly used polymers for immobilization of plant cells and production of manufactured seeds because it is available in large quantities, is inert, non-toxic, cheap, and easily handled[7]. In addition, while the conventional conservation of *in vitro*-cultured germplasm requires about 4 to 6 subcultures in fresh media within a year to prevent browning and loss of viability, with the method of storage using alginate encapsulation techniques, the handling, cost of maintenance, and risk of contamination, can be reduced four to sixfold.

In vitro germplasm conservation methods based on conditions that permit minimal rates of growth, generally achieved by reducing the temperature of culture or using growth retardants, have been published for many species[6,9,28]. However, studies on *in vitro* germplasm conservation using alginate encapsulation techniques have been reported for only a few species. Embryogenic tissues of *Santalum album*[2], axillary buds of *Morus indica*[1], and adventitious buds of *Morus alba*[16] have been encapsulated in alginate or agar beads and then stored for 45–80 days at 4°C without loss of viability. Kinoshita and Saito (1992)[13] reported that the encapsulated axillary buds of *Betula platyphylla* var. *japonica* could be stored at 4°C for more than 80 days without loss of viability. Micro-cuttings of *Eucalyptus grandis* × *E. urophylla*, encapsulated in

Table 2. Effects of different cryopreservation methods on the survival (S) and plant regeneration (PR) rates of *G. crinita* explants after cooling in LN

Explants	Simple freezing method[a]		Rapid freezing method[b]		Slow pre-freezing method[c]		Encapsulation/ dehydration method[d]	
	S(%)	PR(%)	S(%)	PR(%)	S(%)	PR(%)	S(%)	PR(%)
Shoot tip	0	0	0	0	50	15	0	0
Nodal segment	0	0	0	0	5	0	0	0
Root tip	0	0	30	5	30	5	0	0
Adv. bud cluster			85	25				

a): Explants were treated with cryoprotectant mix and then cooled in a freezer at −30°C for 1 h prior to immersion in LN.
b): Explants were treated with cryoprotectant mix and then cooled by direct immersion in LN.
c): Explants were treated with cryoprotectant mix and then cooled to −40°C at a rate of 0.5°C/min prior to immersion in LN.
d): Explants, with or without alginate encapsulation, were treated with a medium enriched with sucrose before dehydration in a laminar flow cabinet or inside petri dishes containing silica gel, and then cooled in LN by slow pre-freezing or by the rapid freezing method.
Shoot tip, nodal segment, and root tip explants were cut into segments about 2 mm long. Adventitious bud cluster explants were cut into about 1.0–1.5 mm^3 cubic segments.

alginate beads and kept on a nutrient-free agar medium, were stored for 10 months at 30/25°C with a plant regeneration rate of at least 52% after storage[30].

Although more studies to determine the optimum temperature, photon flux density, concentration of alginate in gel matrix, concentration of essential nutrients into substrate, and other conditions for minimal growth storage, are necessary to further improve the efficiency of the system, the method developed here can be used as a good alternative for the germplasm conservation of many tropical forest trees species that do not tolerate conventional reduced temperature storage systems.

2) Storage following cryogenic procedures

The effects of cryopreservation methods on the survival and plant regeneration of different explants are shown in Table 2. Survival of explants after immersion in LN was not achieved in any of the treatments tested by the simple freezing and encapsulation/dehydration method. Only the use of the rapid freezing and the slow pre-freezing method resulted in plant recovery after storage in LN. These results suggest that the explants tested were not suitable materials for cryopreservation by simple freezing since this method had been successfully applied to cultured cells[24], and that treatments in media with a high concentration of sucrose by the encapsulation/dehydration method that has been reported as a successful cryopreservation method for some species[21,24,26], caused damage to explants apparently due to the fact that this species does not tolerate drastic dehydration processes.

Slow pre-freezing was the optimum method for cryopreservation of shoot tip explants (Fig. 1B). Rates of 50% for survival and 15% for plant regeneration were achieved when shoot tips were treated with cryoprotectant mix A and B for 20 and 10 min, respectively, and then cooled to −40°C at a rate of 0.5°C/min prior to storage in LN. For root tip explants no difference between the rapid freezing method and the slow pre-freezing method was found (Table 2). As in the case of shoot tip explants, plant regeneration from cryopreserved root tips was obtained through adventitious bud formation (Fig. 1C) and subsequently shoot differentiation on medium supplemented with 10 and 1 μM ZEA, respectively. Although this method is time-consuming and laborious, and requires controlled freezing equipment and complicated procedures, it is the most commonly used method for the cryopreservation of meristems/shoot tips, cell cultures and somatic embryos. Several species have been successfully cryopreserved following slow pre-freezing methods[5,9–11,24,29].

In the present study, although rapid freezing failed to cryopreserve the shoot tip and nodal segment explants, this method was effective for the cryopreservation of adventitious bud clusters of *G. crinita*. Survival of adventitious bud clusters after storage in liquid nitrogen varied depending on the size of explant, cryoprotectant mix and on the duration of the cryoprotectant mix treatment. High survival rates (73–85%) were achieved for small cubic segments (1.0–1.5 mm^3) pretreated with cryoprotectant mix B or C. In contrast, large cluster explants (3.0–4.0 mm^3) and cryoprotectant mix D-treated bud clusters did not survive after storage in liquid nitrogen (Fig. 2A). These results suggest that (1) the large size of the explants is not favorable for the dehydration-cryoprotective action of the vitrification solution, or that (2) the duration of the cryoprotectant mix treatments for large cluster explants was insufficient and that (3) glycerol and sucrose are necessary as components of the cryoprotectant mix solution for cryopreservation of bud clusters of *G. crinita* by the rapid freezing method. Based on these results, large-sized cluster explants and cryoprotectant mix D were not used for further experiments.

Survival of small cluster explants did not differ when they were treated with either mix B or C at 25°C for various periods of time prior to direct immersion into liquid nitrogen. The highest rate of survival was obtained with the explants treated with mix B for 15–45 min or with mix C for 15–60 min, respectively. In both cryoprotectant mix treatments, a pretreatment of 5 min in a cryoprotectant solution was insufficient, and pretreatment for 90 min was inhibitory to the survival of explants (Fig. 2B). The original green color of the bud cluster segments was evident immediately following cooling but was lost within 24–48 h after warming. However, the successfully vitrified bud cluster segments regained their green color within 1 to 2 weeks after transfer to recovery growth medium (Fig. 1D). After 45 days of culture, clumps of numerous bulbous structures about 5 mm in diameter were formed (Fig. 1E). When transferred onto agar-solidified WPM containing 1 μM zeatin, after 60 days, about 30% of the surviving cryopreserved explants formed shoots (Fig. 1F). No differences were observed among the rates of shoot development from untreated control and surviving cryopreserved explants. Regenerated

plantlets were successfully acclimatized and all survived and grew well. No morphological abnormalities were observed in the plants regenerated from cryopreserved explants.

Rapid freezing is the simplest method of cryopreservation and it does not require sophisticated and expensive controlled freezing equipment and complicated cryoprotective procedures, but allows cells and meristems to be cryopreserved by direct immersion into liquid nitrogen[14,19,24,27,29].

Although several authors have indicated that cold hardening and/or pre-culturing with a high concentration of sugar medium are essential to successful cryopreservation of *in vitro*-cultured plant

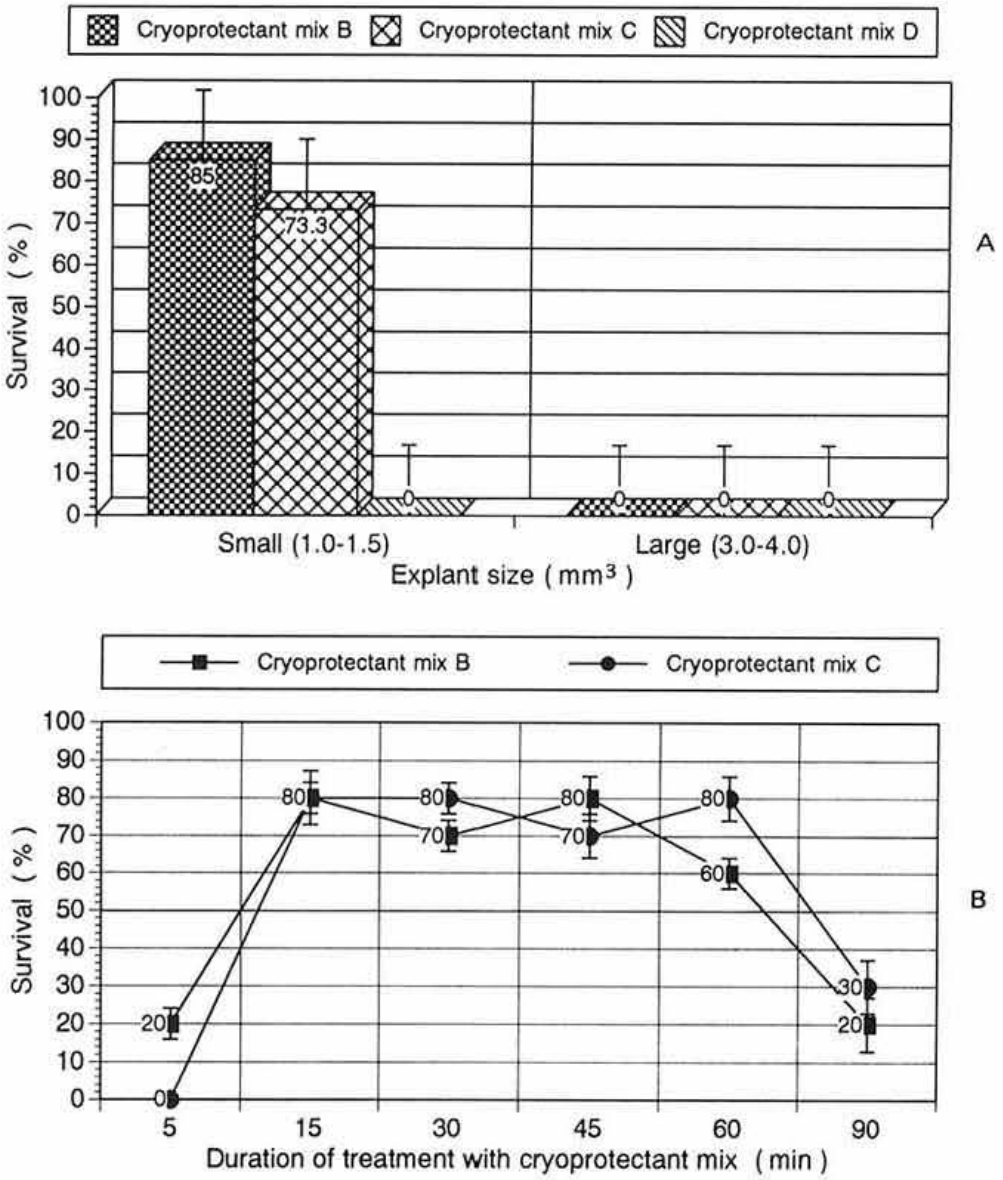

Fig. 2. Cryopreservation of adventitious bud cluster explants of *G. crinita* cultured *in vitro*
A: Effects of explant size and cryoprotectant mix solution on survival after storage in liquid nitrogen (LN). Explants were treated with a cryoprotectant mix solution for 45 min at 25°C and then inmersed into LN.
B: Effects of duration of treatment with cryoprotectant mix solution prior to cooling on survival after storage in LN. Explants placed in a 1.5 mL cryotube were treated with a cryoprotectant mix solution for different periods of time at 25°C and then directly immersed into LN.
Ten segments were treated for each of 4 replicates. Bars indicate standard error.

materials[11,20,22], adventitious bud clusters of *G. crinita* cryopreserved by the rapid freezing method showed high survival rates without any cold-hardening and/or pre-culturing treatments. Similar results were reported in the vitrified bud clusters of asparagus[14]. On the other hand, cold-hardening and/or pre-culturing with sugar-enriched medium were not found to be effective to induce dehydration tolerance in this species. Attempts at using several cold-hardening and/or pre-culturing treatments modified from Niino & Sakai (1992)[21], Suzuki et al. (1994)[26], Matsumoto et al. (1994)[19], and Brison et al. (1995)[3], to enhance the survival and plant regeneration rate (data not presented) were not successful.

The results of the cryopreservation experiments may be summarized as follows: (1) slow pre-freezing was the optimum method for cryopreservation of shoot tips, (2) rapid freezing was not effective for the cryopreservation of the shoot tip and nodal segment explants, however this method was found to be effective for the cryopreservation of adventitious bud clusters, (3) for root tip explants, there was no difference between the rapid freezing and slow pre-freezing method, (4) the simple freezing and encapsulation/dehydration method failed to cryopreserve all explants tested, and (5) cold-hardening and/or pre-culturing treatments were not effective in enhancing the survival or plant recovery rate after storage in LN.

Although the plant recovery rates should be improved in the near future for a more effective cryopreservation system, in our opinion, these results can be used at present without inconvenience because, considering that more than 823,000 shoots can be obtained in a year from only one shoot tip explant[17], further propagation of *G. crinita* from a few cryopreserved surviving explants is really possible. Thus, cryopreservation by using both the slow pre-freezing and rapid freezing methods can be considered to be a suitable alternative for the long-term storage of *G. crinita* germplasm. Cryopreserved germplasm of tropical forest tree species will be the major source for planting stocks in the so-called biotechnology-assisted reforestation in the tropics soon.

Storage of selected germplasm following the alginate encapsulation technique and/or cryogenic procedures described here is an attractive possibility for the conservation of genetically superior tropical forest trees for many decades.

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