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Cryopreservation Approach for the Germplasm Conservation of the Tropical Forest Tree Species: *Cedrela odorata* L., *Guazuma crinita* Mart., and *Jacaranda mimosaefolia* D. Don.

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Cryopreservation approach for the germplasm conservation of three useful tropical forest tree species (*Cedrela odorata* L., *Guazuma crinita* Mart., and *Jacaranda mimosaefolia* D. Don.) were carried out using shoot-tip or root-tip explants from *in vitro*-grown plantlets by four cryopreservation methods (① simple freezing, ② rapid freezing, ③ slow pre-freezing, and ④ dehydration method). The effects of cold hardening treatments (5, 10, 15, and 20/10°C) and pre-culture (dehydration treatments) were also studied.

The best results were achieved when the shoot-tips were cooled by slow pre-freezing before immersion in liquid nitrogen (LN). Survival and plant recovery rates of 50 and 20%; and, 50 and 15%; were obtained in cryopreserved shoot-tips of *C. odorata* and *G. crinita*, respectively. Although the effects of cold hardening and/or pre-culture treatments are not clear, apparently they were not effective in enhancing the survival or plant recovery rate after immersion in LN because these species may be intolerant of low temperature treatments of long duration and/or to drastic dehydration treatments.

The cryopreservation of shoot-tips from tissue-cultured plants can be considered as a feasible alternative for the long-term storage of *C. odorata, G. crinita*, and *J. mimosaefolia* germplasm.

Introduction

Several important valuable tropical tree species have become seriously deteriorated in terms of amount and genetic resources due to extensive and selective cutting, and burning for shifting cultivation or raising livestock.

Cedrela odorata L. (cedro) is one of the most important forest tree species in the tropics and, in Peru, the price of its wood is only exceeded by that of the mahogany[1]. Cedro wood is greatly valued for its good quality, ductility and durability and is used for making fine furniture and cigar-boxes and for decorative veneer. This species belongs to the Meliaceae family which includes mahogany (*Swietenia macrophylla* King.), andiroba (*Carapa guianensis* Aubl.) and bead-tree (*Melia azederach* L.). Due to the continuous selective felling of good-quality cedro, the resources of this tree are on a steady decline. The seeds of cedro have a small range of dispersal and so, once it is

felled, there is little possibility that it will grow naturally in the area again. In the tropical forest of Peru-Amazon, *C. odorata* and other Meliaceae species have been used for reforestation[1,2]. However, they have been damaged severely by the Meliaceae shoot borer, *Hypsipyla grandella* Zeller (Lepidoptera, Pyralidae)[1-5]. To date, a suitably economical control method for the insect has not been found. Mechanical, chemical, and biological control methods have been investigated but without success on significant reforested areas[1,2].

Guazuma crinita Mart. (bolaina blanca) is a medium-sized fast-growing tree species, native of South America in the Amazon forest region of Peru, Brazil and Ecuador[6,7]. This species belonging to the Sterculiaceae family and has a soft-light wood with good working properties which has been used for light construction, panelling, interior joinery, mouldings, cases, matches, packing and various other miscellaneous uses. Since *G. crinita* has a fast initial growth rate of up to 3 m in height per year, with a final rotation age of 10 to 15 years, and shows excellent adaptability to a wide range of sites including degraded areas of exploited bush and areas of poorly drained heavy clay-soils, it is considered as one of the potential species for reforestation in low lands of the Peru-Amazon region. In fact it has already been used for reforestation in the tropical forest of Peru-Amazon[8].

Jacaranda mimosaefolia D. Don. (jacaranda), which belongs to the Bignoniaceae family, is a medium-sized tree species with a dense crown and with attractive lilac-coloured flowers[9]. According to Encarnación[6] and Macbride[10], *J. mimosaefolia* is a synonym of *J. acutifolia* H. *et* B., and is endemic in the Peru-Amazon. This species is one of the most beautiful ornamental trees which are planted on a large scale in the gardens, parks, avenues and in house gardens in tropical and subtropical countries. No exact data are available, but numbers of trees planted run into millions every year[11]. Besides an ornamental tree, this species is used as saw-timber[6] and, in Bolivia, it has been commercialized as one of the most important tree species[12]. In addition, its dried fruits are used for decoration. Actually other *Jacaranda* species have been considered marketable species for many purposes such as furniture, interior joinery, mouldings, packing cases, plywood or blockboard, broomsticks, matches, and toys[13].

The germplasm conservation of cultivated species and their wild relatives is necessary for sustainable exploitation systems and as a means of maintaining species diversity and genetic diversity to prevent genetic erosion. 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 disease and pest attack, and is liable to environmental and political threats. Although seed storage is the most popular and economical means of germplasm conservation, this is not always feasible for long-term, because many species produce seeds that lose their viability in a short time under natural conditions and are recalcitrant to storage by a reduction of moisture content and temperature. In addition, some species do not produce viable seeds for several years, while seeds of some species are very heterozygous and, therefore, not suitable for maintaining true-to-type genotypes. *In vitro* genebanks have been developed for a large number of plant species, and they reduce these problems. However, the maintenance of large *in vitro* collections under conventional storage systems requires much handling and is expensive because most cultures need subculturing at regular intervals to prevent browning and loss of viability. This creates a continual demand for skilled labour, culture vessels, media, and space in controlled-environment growth chambers. In addition, subculturing also increases the risk of contamination and somaclonal variation.

Due to long-term seed storage of *C. odorata, G. crinita*, and *J. mimosaefolia* is difficult under

conventional methods, and *in vitro* storage of plantlets requires about 4 to 6 subcultures in fresh media per year, cryopreservation can be a reliable method for long-term conservation of plant genetic resources without apparent risk of genetic instability using a minimum space and with less labour and maintenance costs. In this study, we report a cryopreservation approach for the germplasm conservation of three useful tropical forest tree species, using tissue-cultured plant materials.

Materials and Methods

1. In vitro *plantlet regeneration*

Cedrela odorata. Shoot-tips of five-month-old potted seedlings grown in the greenhouse were successfully surface sterilized by rinsing in 70% (v/v) ethyl alcohol for 1 min. and then 0.1% (w/v) mercuric chloride solution for 10 min. The shoot-tip segments were washed three times in sterile distilled water and cut into lengths of about 1-1.5 cm. Shoot multiplication was induced on woody plant (WP) medium[14] supplemented with 0.89-8.89 μM 6-benzylaminopurine (BAP). This achieved three to four-fold multiplication rate in one month. High rooting percentages (up to 100%) were obtained on a half-strength of the same initial medium containing 2.46 μM indole-3-butiric acid (IBA), 0.27 μM α-naphthaleneacetic acid (NAA), and 0.93 μM 6-furfurylaminopurine (KIN).

Guazuma crinita. Seeds were surface sterilized with 70% (v/v) ethyl alcohol for 3 min. and then with 5% (v/v) hydrogen peroxide solution for 10 min. After surface sterilization, seeds were washed three times in sterile distilled water and then placed individually onto 0.8% (w/v) agar medium (15 m*l*) containing 2% (w/v) sucrose in 1.8 cm × 18 cm test tubes. Shoot multiplication was obtained by the subculturing of about 1.5 cm long shoot-tips, excised from aseptically germinated seedlings, on WP medium supplemented with 10 μM *trans*-6-(4-hydroxy-3-methylbut-2-enylamino) purine (ZEA). After six weeks of culturing, a seven-fold multiplication rate was achieved. Multiplicated shoots were simultaneously elongated and rooted on WP medium containing 1 μM KIN. After two months of culturing, a high rooting rate (more than 90%) and good growth were obtained.

Jacaranda mimosaefolia. Seeds were surface sterilized by agitating them in 70% (v/v) ethyl alcohol for 3 min. and then in 5% (w/v available chlorine) sodium hypochlorite solution for 10 min. After three rinses with sterile distilled water, seeds were placed on 0.8% (w/v) agar medium in 300 m*l* Erlenmeyer flasks. Shoot multiplication was obtained by the subculturing of 1-2 cm long shoot-tips, excised from 1 to 2-month-old aseptically germinated seedlings. After six months of culturing on Gamborg's B5 (B5) medium[15] supplemented with a high concentration of KIN (100 μM) and solidified with 0.2% (w/v) Gelrite, an eight-fold multiplication rate was achieved. Rhizogenesis frequency was 100% on 1/2 B5 medium containing IBA (0.49-4.9 μM) alone, or in combination with NAA (0.27 μM).

2. *Plant materials for cryopreservation*

Shoot-tips and root-tips cut into about 2 mm lengths from about 2 to 3-month-old *in vitro* regenerated plantlets were used as plant material for cryopreservation experiments.

3. *Cryoprotectant mix*

In the present study the following cryoprotectant mix solutions, modified from Sakai *et al.*[16,17] and Towill[18], containing (w/v), Mix A[16]: 20% glycerin+15% sucrose, Mix B[17]: 30% glycerin+15% sucrose+15% ethylene glycol+15% dimethyl sulfoxide (DMSO), Mix C[17]: 25% glycerin+15% sucrose+15% ethylene glycol+13% DMSO+2% polyethylene glycol 4,000 (PEG), Mix D[18]: 35%

ethylene glycol+10% DMSO+5% PEG, were tested. The aqueous volume added was medium without sucrose and growth regulators. WP medium was used for *C. odorata* and *G. crinita*, and B5 medium for *J. mimosaefolia*. All cryoprotectant mix solutions were filter sterilized.

4. *Cold hardening*

In an attempt to increase the resistance to dehydration stress and freezing, some experiments were carried out using explants excised from cold hardened plantlets. The following cold hardening treatments, modified from Niino and Sakai[19], Kohmura *et al.*[20], Suzuki *et al.*[21], Kuranuki and Sakai[22], and Brison *et al.*[23] were tested: HA[19-21]: 1 week at 5°C with photoperiod of 8 h, HB[19-21]: 2 weeks at 5°C with photoperiod of 8 h, HC[19-21]: 4 weeks at 5°C with photoperiod of 8 h, HD[22,23]: 6 weeks at alternating temperature of 20°C for 8 h (light) following 10°C for 16 h (dark), HE[22,23]: 6 weeks at 10°C with photoperiod of 8 h, HF[22,23]: 6 weeks at 15°C with photoperiod of 8 h, and HG[22,23]: 12 weeks at 15°C with photoperiod of 8 h.

5. *Pre-culture*

Several pre-culture treatments, modified from Kohmura *et al.*[20], Kuranuki and Sakai[22], Brison *et al.*[23], Matsumoto *et al.*[24,25], and Yoshimatsu *et al.*[26], were tested: PA[20,22,24-26]: on solidified medium with 10% sucrose for 16 h at 5°C, PB[20,22,24-26]: on solidified medium with 20% sucrose for 16 h at 5°C, PC[20,22,24-26]: on solidified medium with 10% sucrose for 16 h at 25°C, PD[20,22,24-26]: on solidified medium with 20% sucrose for 16 h at 25°C, PE[23]: on solidified medium with 5% DMSO+2% proline for 16 h at 5°C, PF[23]: on solidified medium with 5% DMSO+2% proline for 16 h at 25°C. WP medium was used for *C. odorata* and *G. crinita*, and B5 medium for *J. mimosaefolia*.

6. *Cryopreservation methods*

Simple freezing

Shoot-tip 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 LN and held there for at least 1 h.

Rapid freezing

Shoot-tip or root-tip explants, excised from cold hardened or non-cold hardened plantlets, were pre-cultured or not, exposed to cryoprotectant mix solutions at 25°C for different lengths of time and then directly immersed in LN and held there for at least 1 h.

Slow pre-freezing

Shoot-tip or root-tip explants, excised from cold hardened or non-cold hardened plantlets, were pre-cultured or not, treated with cryoprotectant mix solutions at 25°C for different lengths of time 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.

Dehydration

Shoot-tip explants, excised from cold hardened or non-cold hardened plantlets, were progressively pre-cultured at 5°C by successive daily transfer of the explants onto solidified medium 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. Shoot-tips or encapsulated shoot-tips (constructed beads about 5 mm in diameter containing one shoot-tip), were treated in medium supplemented with 30% (w/v) sucrose for 16 h at 5°C. After treatments with sucrose, shoot-tips or encapsulated shoot-tips 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 hours, and then cooled in LN by the slow pre-freezing or by the rapid freezing method.

7. *Survival and plant recovery*

Explants were thawed by rapidly transferring of cryotubes in a water bath at 37°C. After thawing, cryoprotectant mix solutions were drained from the cryotubes and replaced with medium containing 40% (w/v) sucrose and held for 20 min. Then, shoot-tip explants were transferred onto sterilized filter paper discs over solidified WP medium containing 1 μM BAP for *C. odorata* or 10 μM ZEA for *G. crinita*, and solidified B5 medium supplemented with 1 μM KIN for *J. mimosaefolia* and cultured at 25°C under photon flux density of 65 μmol $m^{-2}s^{-1}$ (about 3, 800 lux). The root-tip explants were transferred into liquid WP medium containing 10 μM ZEA and cultured on a bio-shaker at 73 rpm under photon flux density of 25 μmol $m^{-2}s^{-1}$ (about 1, 500 lux). All the explants were cultured at 25°C and 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 recovery was considered when they had developed into plantlets.

Results and Discussion

1. *Simple freezing*

The main advantage of the simple freezing method is to reduce or eliminate the need for cellular dehydration during slow freezing by osmotically dehydrating cells or meristems prior to cooling, without the necessity of controlled-rate freezing equipment. Keeping the cells or meristems into ordinary freezers (temperature of −30°C) for 30 to 60 min. before their storage in LN, the complex cryoprotective and freezing procedures are simplified. This method has been successfully applied to cultured cells such as orange, rice, carrot, asparagus, and meristemoids of white clover[27]. However, in this study, the simple freezing method was not effective for the shoot-tip cryopreservation of *C. odorata, G. crinita*, and *J. mimosaefolia*. Survival of explants after immersion in LN was not achieved in any of the treatments tested. This result suggests that possibly the shoot-tip explants (about 2 mm long) was not a proper material for cryopreservation by simple freezing, apparently, this method is more recommendable for cultured cell explants[16,28].

2. *Rapid freezing*

Rapid freezing method does not require controlled freezing equipment or sophisticated and expensive apparatus, because samples are cryopreserved by direct immersion in LN. In this condition, the cells do not have sufficient time to equilibrate with the external ice by effusion of water as explained for the slow pre-freezing method, and to prevent the growth of intracellular ice crystals formed during rapid freezing, the cells must be sufficiently dehydrated prior to storage in LN. In recent years, the conventional rapid freezing technique has been modified to evolve a vitrification process based on the ability of highly concentrated cryoprotectant solutions to super-cool to very low temperatures upon imposition of rapid cooling rates, and to become viscous at sufficiently low temperatures and solidify into metastable glass without the formation of ice[29]. However, chemical toxicity or dehydration stress caused by the high concentration levels of many cryoprotective agents required for vitrification, can be the main limitation in a number of species. Cryopreservation of cultured cells and meristems of woody plants by vitrification, and subsequent plant regeneration, are reported by Sakai[27].

In the present study, the best result with the rapid freezing method was achieved in shoot-tips of *C. odorata*. But, although survival rates up to 60% were achieved after storage in LN, the maximum percentage of explants that developed into plantlets was 10% (**Table 1**). On the other hand, this method was not effective to cryopreserve the shoot-tips of *G. crinita* (**Table 2**) and

J. mimosaefolia (**Table 3**). No cryopreserved-survivor shoot-tips were achieved in all treatments tested in both species. However, the rapid freezing method was found to be feasible for the cryopreservation of root-tips of *G. crinita*. Survival rates of up to 30% were achieved after

Table 1. Effects of cryoprotectant mix treatments, cold hardening, and pre-culture on survival and plant recovery rates of *C. odorata* shoot-tips after cooling in LN by the rapid freezing method.

Treatment	Cold hardening	Pre-culture	Holding time (min.) in cryoprotectant mix			Survival (%)	Plant recovery (%)
			mix A	mix B	mix C		
1	—	—	0	5	0	0	0
2	—	—	0	15	0	0	0
3	—	—	0	30	0	0	0
4	—	—	0	60	0	0	0
5	HA	—	0	15	0	0	0
6	HA	PA	0	15	0	0	0
7	—	—	10	15	0	0	0
8	HA	PA	10	15	0	60	0
9	—	PC	10	15	0	30	0
10	—	PD	10	15	0	10	10
11	HC	—	10	15	0	0	0
12	HC	PA	10	15	0	0	0
13	—	—	10	30	0	0	0
14	—	—	15	15	0	31	6
15	—	PB	15	15	0	0	0
16	—	PE	15	15	0	0	0
17	HD	PB	15	15	0	0	0
18	—	—	20	10	0	20	0
19	—	—	20	20	0	10	0
20	—	—	30	15	0	0	0
21	—	—	30	20	0	13	0
22	—	—	30	30	0	0	0
23	—	—	45	45	0	0	0
24	—	—	60	60	0	0	0
25	—	—	0	0	10	0	0
26	—	—	0	0	20	0	0
27	—	PB	0	0	20	0	0
28	—	PE	0	0	20	0	0
29	HD	PE	0	0	20	0	0
30	—	—	0	0	40	0	0

Ten to 20 shoot-tips about 2 mm long were treated with cryoprotectant mix at 25°C and then immersed in LN.
HA cold hardening : 1 week at 5°C with photoperiod of 8 h.
HC cold hardening : 4 weeks at 5°C with photoperiod of 8 h.
HD cold hardening : 6 weeks at alternating temperature of 20°C for 8 h (light) following 10°C for 16 h (dark).
PA pre-culture : On solidified medium with 10% sucrose for 16 h at 5°C.
PB pre-culture : On solidified medium with 20% sucrose for 16 h at 5°C.
PC pre-culture : On solidified medium with 10% sucrose for 16 h at 25°C.
PD pre-culture : On solidified medium with 20% sucrose for 16 h at 25°C.
PE pre-culture : On solidified medium with 5% DMSO+2% proline for 16 h at 5°C.
Cryoprotectant mix A : 20% glycerin+15% sucrose.
mix B : 30% glycerin+15% sucrose+15% ethylene glycol+15% DMSO.
mix C : 25% glycerin+15% sucrose+15% ethylene glycol+13% DMSO+2% PEG.
— : No treatment.

Table 2. Effects of plant material, cryoprotectant mix treatments, cold hardening, pre-culture, and freezing method on survival of *G. crinita* explants after cooling in LN.

Treatment	Plant material	Cold hardening	Pre-culture	Freezing method	Holding time (min.) in cryoprotectant mix			Survival (%)
					mix A	mix B	mix C	
1	Shoot-tip	—	—	Rapid*1	20	10	0	0
2	Shoot-tip	—	—	Slow2)	20	10	0	50
3	Shoot-tip	HA	—	Rapid	20	10	0	0
4	Shoot-tip	HA	—	Slow	20	10	0	20
5	Shoot-tip	HB	—	Rapid	20	0	0	0
6	Shoot-tip	HB	—	Slow	20	10	0	0
7	Shoot-tip	HC	—	Rapid	20	0	0	0
8	Shoot-tip	HC	—	Slow	20	10	0	0
9	Shoot-tip	—	PA	Slow	20	10	0	20
10	Shoot-tip	—	PB	Slow	20	10	0	10
11	Shoot-tip	HA	PA	Slow	20	10	0	30
12	Shoot-tip	—	—	Slow	0	0	20	17
13	Root-tip	—	—	Rapid	0	45	0	10
14	Root-tip	—	—	Rapid	20	20	0	30
15	Root-tip	—	—	Slow	20	10	0	30
16	Root-tip	—	PA	Slow	0	10	0	10

Shoot-tips and root-tips were cut into about 2 mm lengths. Ten to 20 explants were tested in each treatment.

*1 Plant materials were treated with cryoprotectant mix at 25°C and then immersed in LN.

*2 Plant materials were treated with cryoprotectant mix at 25°C and then cooled to −40°C at a rate of 0.5°C/min. prior to immersion in LN.

HA cold hardening : 1 week at 5°C with photoperiod of 8 h.

HB cold hardening : 2 weeks at 5°C with photoperiod of 8 h.

HC cold hardening : 4 weeks at 5°C with photoperiod of 8 h.

PA pre-culture : On solidified medium with 10% sucrose for 16 h at 5°C.

PB pre-culture : On solidified medium with 20% sucrose for 16 h at 5°C.

Cryoprotectant mix A : 20% glycerin+15% sucrose.

Cryoprotectant mix B : 30% glycerin+15% sucrose+15% ethylene glycol+15% DMSO.

Cryoprotectant mix C : 25% glycerin+15% sucrose+15% ethylene glycol+13% DMSO+2% PEG.

— : No treatment.

storage in LN (**Table 2**). Cryopreserved-survivor root-tips developed multiple bud clusters (clumps of green bulbous structures containing small adventitious buds) in liquid WP medium containing 10 μM ZEA (**Fig. 1-A**). Subsequently shoot differentiation was achieved onto solid WP medium containing 1 μM ZEA (**Fig. 1-B**). Multiple bud cluster formation on the cut-end part(s) of the cryopreserved-survivor root-tips was evident within two to three weeks of culturing. After 45 days of culturing, developed bud clusters, about 5 mm in diameter, were transferred onto shoot differentiation medium and cultured for 60 days. The plant recovery rate was about 15% of the cryopreserved-survivor root-tips.

3. *Slow pre-freezing*

Protective dehydration of cells by regulated slow cooling, avoiding the chances of intracellular ice formation, is the basis of the slow pre-freezing method. Addition of cryoprotectants alters the freezing point of cells and may reduce it down to −10°C. If the temperature is reduced to below the freezing point of water, ice formation occurs in the extracellular space of the medium. Since the cells remain unfrozen but supercooled, and their aqueous vapour pressure exceeds that of the frozen exterior, the cells equilibrate by water condensation on the external ice (loss of water from

Table 3. Effects of cryoprotectant mix treatments, cold hardening, pre-culture, and freezing method on survival of *J. mimosaefolia* shoot-tips after cooling in LN.

Treatment	Cold hardening	Pre-culture	Freezing method	Holding time (min.) in cryoprotectant mix				Survival (%)
				mix A	mix B	mix C	mix D	
1	—	—	Rapid*1	0	30	0	0	0
2	—	—	Slow*2	0	30	0	0	20
3	—	—	Rapid	15	15	0	0	0
4	—	—	Slow	15	15	0	0	0
5	—	—	Rapid	0	0	20	0	0
6	—	—	Slow	0	0	20	0	0
7	—	—	Rapid	0	0	0	20	0
8	—	—	Slow	0	0	0	20	0
9	—	PB	Rapid	15	15	0	0	0
10	—	PB	Slow	15	15	0	0	0
11	—	PB	Rapid	0	0	20	0	0
12	—	PB	Slow	0	0	20	0	0
13	—	PE	Rapid	15	15	0	0	0
14	—	PE	Slow	15	15	0	0	20
15	—	PE	Rapid	0	0	20	0	0
16	—	PE	Slow	0	0	20	0	0
17	HA	PA	Rapid	20	10	0	0	0
18	HA	PA	Slow	20	10	0	0	17
19	HB	PA	Slow	20	10	0	0	0
20	HC	PA	Slow	20	10	0	0	0
21	HD	PB	Slow	15	15	0	0	0
22	HD	PE	Slow	0	0	20	0	0
23	HE	PB	Slow	15	15	0	0	0
24	HE	PE	Slow	0	0	20	0	0
25	HG	—	Slow	15	15	0	0	0

Ten to 12 shoot-tips about 2 mm long were tested in each treatment.

*1 Shoot-tips were treated with cryoprotectant mix at 25°C and then immersed in LN.

*2 Shoot-tips were treated with cryoprotectant mix at 25°C and then cooled to −40°C at a rate of 0.5°C/min. prior to immersion in LN.

HA cold hardening : 1 week at 5°C with photoperiod of 8 h.

HB cold hardening : 2 weeks at 5°C with photoperiod of 8 h.

HC cold hardening : 4 weeks at 5°C with photoperiod of 8 h.

HD cold hardening : 6 weeks at alternating temperature of 20°C for 8 h (light) following 10°C for 16 h (dark).

HE cold hardening : 6 weeks at 10°C with photoperiod of 8 h.

HG cold hardening : 12 weeks at 15°C with photoperiod of 8 h.

PA pre-culture : On solidified medium with 10% sucrose for 16 h at 5°C.

PB pre-culture : On solidified medium with 20% sucrose for 16 h at 5°C.

PE pre-culture : On solidified medium with 5% DMSO+2% proline for 16 h at 5°C.

Cryoprotectant mix A : 20% glycerin+15% sucrose.

Cryoprotectant mix B : 30% glycerin+15% sucrose+15% ethylene glycol+15% DMSO.

Cryoprotectant mix C : 25% glycerin+15% sucrose+15% ethylene glycol+13% DMSO+2% PEG.

Cryoprotectant mix D : 35% ethylene glycol+10% DMSO+5% PEG.

— : No treatment.

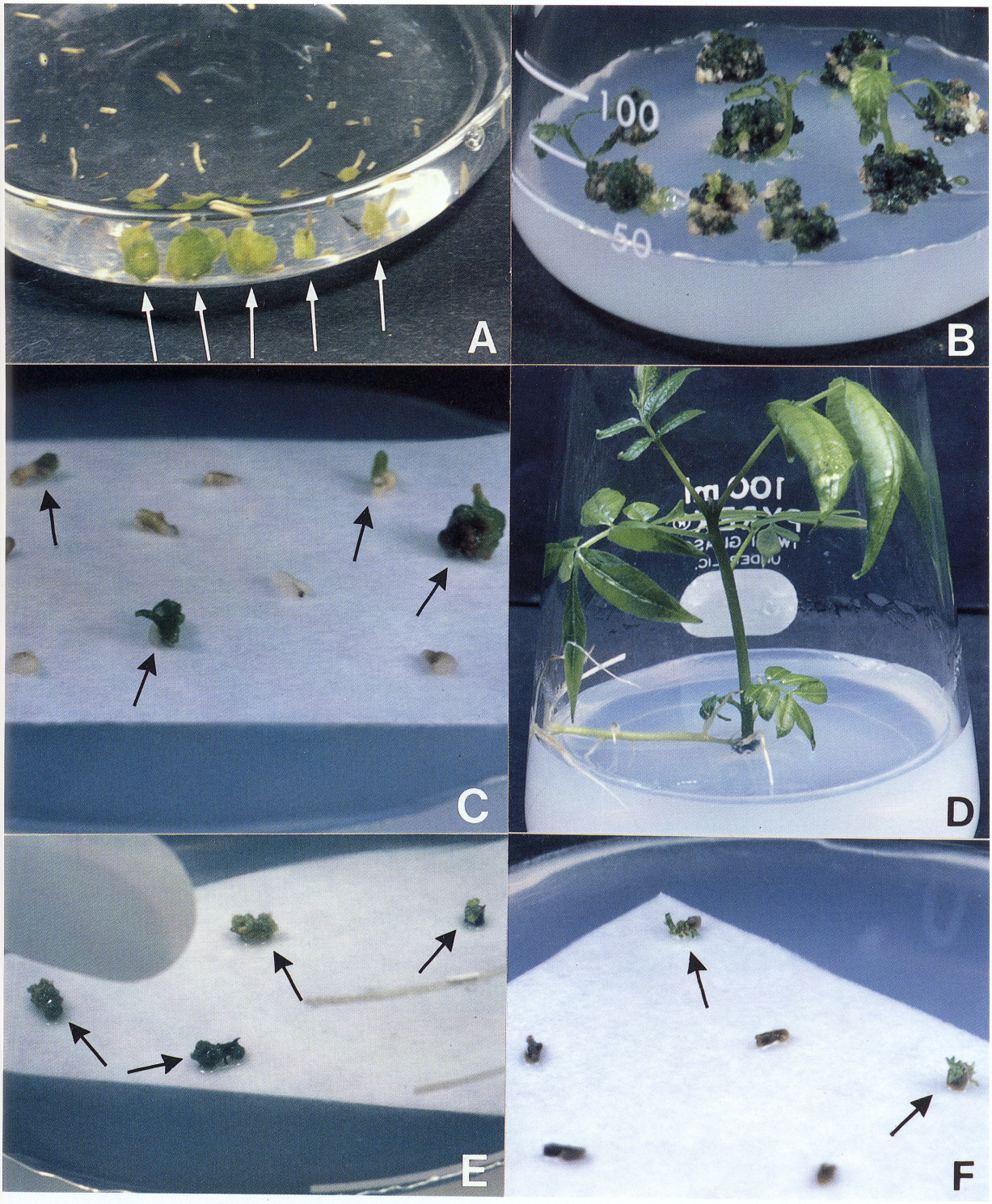

Fig. 1 (**A**) Adventitious bud formation (arrows) on cryopreserved root-tips of *G. crinita*, and (**B**) later shoot development. (**C**) Survivors (arrows) and (**D**) a plantlet developed from cryopreserved shoot-tips of *C. odorata*. Survivors (arrows) of cryopreserved shoot-tips of (**E**) *G. crinita* and (**F**) *J. mimosaefolia*.

the cell), resulting in a slow dehydration process[30,31]. 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[27,31-36]. The results of the cryopreservation experiments with shoot-tips of *C. odorata, G. crinita*, and *J. mimosaefolia*, and with root-tips of *G. crinita*, using the slow pre-freezing method, are shown in **Tables 2~4**; and **Fig. 1-C~F**. Although, using slow pre-freezing, no difference in survival rate of cryopreserved root-tips of *G. crinita* by the rapid freezing method was found, this method was the best for cryopreservation of shoot-tips (**Table 5**). Survival rates up to 80%, 50%, and 20% were achieved in *C. odorata* (**Table 4**), *G. crinita* (**Table 2**), and *J. mimosaefolia* (**Table 3**),

Table 4. Effects of cryoprotectant mix treatments, cold hardening, and pre-culture on survival and plant recovery rates of *C. odorata* shoot-tips after cooling in LN by the slow pre-freezing method.

Treatment	Cold hardening	Pre-culture	Holding time (min.) in cryoprotectant mix			Survival (%)	Plant recovery (%)
			mix A	mix B	mix C		
1	—	—	20	10	0	40	0
2	—	PC	20	10	0	0	0
3	HA	—	20	10	0	8	0
4	HA	PC	20	10	0	80	10
5	HF	—	0	5	0	0	0
6	—	—	15	15	0	50	20
7	—	PB	15	15	0	0	0
8	—	PE	15	15	0	0	0
9	HD	PB	15	15	0	0	0
10	HD	PE	15	15	0	0	0
11	HE	PB	15	15	0	0	0
12	HE	PE	15	15	0	33	8
13	—	—	0	0	10	0	0
14	—	—	0	0	20	30	10
15	—	—	0	0	40	7	0
16	—	PC	0	0	20	10	0
17	—	PD	0	0	20	30	0
18	—	PB	0	0	20	0	0
19	—	PF	0	0	20	0	0
20	—	PE	0	0	20	10	10

Ten to 20 shoot-tips about 2 mm long were treated with cryoprotectant mix at 25°C and then cooled to −40°C at a rate of 0.5°C/min. prior to immersion in LN.
HA cold hardening : 1 week at 5°C with photoperiod of 8 h.
HD cold hardening : 6 weeks at alternating temperature of 20°C for 8 h (light) following 10°C for 16 h (dark).
HE cold hardening : 6 weeks at 10°C with photoperiod of 8 h.
HF cold hardening : 6 weeks at 15°C with photoperiod of 8 h.
PB pre-culture : On solidified medium with 20% sucrose for 16 h at 5°C.
PC pre-culture : On solidified medium with 10% sucrose for 16 h at 25°C.
PD pre-culture : On solidified medium with 20% sucrose for 16 h at 25°C.
PE pre-culture : On solidified medium with 5% DMSO+2% proline for 16 h at 5°C.
PF pre-culture : On solidified medium with 5% DMSO+2% proline for 16 h at 25°C.
Cryoprotectant mix A : 20% glycerin+15% sucrose.
Cryoprotectant mix B : 30% glycerin+15% sucrose+15% ethylene glycol+15% DMSO.
Cryoprotectant mix C : 25% glycerin+15% sucrose+15% ethylene glycol+13% DMSO+2% PEG.
— : No treatment.

respectively. Plant regeneration of *C. odorata* were achieved by direct growth from the cryopreserved-survivor shoot-tips(**Fig. 1-C, D**), however, the maximum plant recovery rate obtained was 20%. Similar to the result with the rapid freezing method, although high survival rates were achieved after storage in LN, most of the cryopreserved-survivor shoot-tips of *C. odorata* developed leaves only without the subsequent apical growth of shoots, and after several weeks of culture, only the growth of leaves was observed. In *G. crinita*, same to the case of plant regeneration from cryopreserved root-tip explants, the plant regeneration of cryopreserved shoot-tips was obtained through adventitious bud formation(**Fig. 1-E**) and subsequently shoot differentiation on medium supplemented with 10 and 1 μM ZEA, respectively. The plant recovery rate was about 30% of the cryopreserved-survivor shoot-tips. Cryopreserved-survivor shoot-tips of *J. mimosaefolia* turned green and produced 1-2 leaves within one to two weeks after thawing(**Fig. 1-F**), however, after that subsequent growth was not achieved and they failed to regenerate into plantlets.

4. *Dehydration*

Recently dehydration methods by air-drying have been developed for the cryopreservation of several species. In this method, resistance to desiccation and to freezing was induced by pre-culturing in medium enriched with sucrose before dehydration. Sufficiently dehydrated samples vitrify during cooling in LN. Pre-culture in sucrose solutions may eliminate the use of other cryoprotectants such as DMSO and glycerin. Successful cryopreservation after air-drying treatments have been reported for mulberry[19], apple[19], kiwifruit[21], pear[19,37], asparagus[38], potato[39], grape[40], sugarcane[41], and coffee[42].

Shoot-tips of *C. odorata, G. crinita*, and *J. mimosaefolia*(alginate-encapsulated or not) were dehydrated following the procedures described previously, before cryopreserving by the slow pre-freezing method or by direct immersion in LN. After rapid thawing, all the cryopreserved samples transferred to fresh medium failed to survive. The results suggest that the three species can not be tolerant to drastic dehydration processes. Although, in this method, the induction or modification of dehydration tolerance by cold hardening treatments has been reported to be the key to successful cryopreservation, it was not effective in *C. odorata, G. crinita*, and *J. mimosaefolia*.

Table 5. Effects of different cryopreservation methods on plant recovery rate of *C. odorata, G. crinita*, and *J. mimosaefolia* explants after cooling in LN.

Species	Simple freezing method Plant recovery(%)	Rapid freezing method Plant recovery(%)	Slow pre freezing method Plant recovery(%)	Dehydration method Plant recovery(%)
C. odorata	0*1	10*1	20*1	0*1
G. crinita	0*1	0*1	15*1	0*1
		5*2	5*2	
J. mimosaefolia	0*1	0*1	0*1	0*1

Simple freezing method : Explants were treated with cryoprotectant mix and then cooled in a freezer at −30°C for 1 h prior to immersion in LN.

Rapid freezing method : Explants were treated with cryoprotectant mix and then cooled by direct immersion in LN.

Slow pre-freezing method : 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.

Dehydration method : Explants, with or without alginate-encapsulation, were treated in 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.

*1 Shoot-tip explants were cut into about 2 mm lengths.

*2 Root-tip explants were cut into about 2 mm lengths.

In the present study, four different cryopreservation methods: simple freezing, rapid freezing, slow pre-freezing, and dehydration method, were tried in an attempt to develop an effective system for the germplasm conservation of the useful tropical forest trees *C. odorata, G. crinita*, and *J. mimosaefolia*, by the cryopreservation of shoot-tip and/or root-tip explants. Only the rapid freezing and the slow pre-freezing method resulted in plant recovery after storage in LN (**Table 5**). Shoot-tips of *C. odorata* shown high survival rates in both methods but with subsequent low plant recovery rate because most of the survivor explants developed leaves only. However, although plant recovery rates of 10-20% 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 177,000 shoots can be obtained in a year from only one shoot-tip explant, the further propagation of *C. odorata* from a few cryopreserved-survivor shoot-tips is feasible. In like manner, more than 823,000 shoots can be obtained in *G. crinita*.

Cold hardening[19-23,27,31] and pre-culture treatments[20-27,31,43-45] before cryoprotectant exposure and cooling have been reported as beneficial to enhance the survival rate in many species. However, the results achieved in this study, regarding to the effects of cold hardening and pre-culture treatments on survival or plant recovery rate of explants after storage in LN, are not clear in *C. odorata* (**Table 1** and **4**), and apparently did not enhance the survival rates in *G. crinita* (**Table 2**) and *J. mimosaefolia* (**Table 3**). Thus, although high survival rates (60-80%) were achieved in shoot-tips of *C. odorata* with cold hardening and pre-culture treatments, these did not enhance the plant recovery rates, and the best result (20%) was obtained without any treatments. In *G. crinita* (both shoot-tip and root-tip explants) and in *J. mimosaefolia*, the best survival rates were also obtained with non-treated explants. In the three species tested, cold hardening treatments at 5°C for more than one week resulted in a feebleness of the plantlets and in some cases the browning of shoots was evident. Neither did temperatures higher than 5°C for cold hardening of plantlets enhance the survival rates. Drastic pre-culture treatments in media with a high concentration of sucrose (to attempt induce resistance to desiccation and to freezing, such as in the dehydration method) apparently caused damage to most explants in the three species tested as low survival and plant regeneration rates were achieved before to the storage in LN (data not presented).

Plant regeneration by direct growth from cryopreserved-survivor shoot-tips of *G. crinita* was attempted but without success. After storage in LN, the thawed shoot-tips were transferred onto solid WP medium containing 1 μM KIN (medium for growth of non-cryopreserved shoots) and cultured for several weeks. Survivor shoot-tips developed leaves only without apical growth of shoots, failing to regenerate into plants. Further plant regeneration from cryopreserved shoot-tips of *G. crinita* was obtained same as the plant regeneration from root-tip explants, through adventitious bud formation and subsequent shoot differentiation. No morphological abnormalities were observed in the plants regenerated from cryopreserved explants.

The results obtained in the cryopreservation experiments may be summarized as follow: ①slow pre-freezing was the best method for cryopreservation of shoot-tips, ②rapid freezing was not effective in cryopreserving the shoot-tips of *G. crinita* and *J. mimosaefolia*, however this method was found to be feasible for the cryopreservation of root-tips of *G. crinita*, ③although the effects of cold hardening and/or pre-culture treatments are not clear, apparently they were not effective in enhancing the survival or plant recovery rate after storage in LN because the studied species may be intolerant to cold hardening at low temperatures for a long time and/or to drastic dehydration treatments, ④the simple freezing and dehydration method failed to cryopreserve shoot-tips in the three species, ⑤although further studies are necessary to improve the plant recovery rate, the

cryopreservation by the slow pre-freezing method can be considered as a feasible alternative for the conservation of genetic resources of *C. odorata, G. crinita*, and *J. mimosaefolia*, and the results of this study can be used as reference for further studies on cryopreservation in other tropical forest tree species.

Cryopreserved germplasm of tropical forest tree species will be the major sources for planting stocks in the so-called biotechnology assisted reforestation in the tropics soon. Cryopreservation of selected trees will be realized, resulting in the storage of genetically superior germplasm for many decades.

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《和文要約》

熱帯林森林樹木の遺伝資源保全のための凍結保存方法: *Cedrela odorata* L., *Guazuma crinita* Mart., と *Jacaranda mimosaefolia* D. Don.

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3種の有用な熱帯林森林樹木(*Cedrela odorata* L., *Guazuma crinita* Mart., と *Jacaranda mimosaefolia* D. Don.)の培養苗の茎頂や根端を用いての凍結保存を行った．冷却方法は，①簡易冷却，②急速冷却，③緩速予備冷却，④乾燥後冷却を試験した．低温でのハードニングや前培養処理も試みた．茎頂を緩速予備冷却し

たのち，液体窒素に浸漬した場合が最も植物片の生存率が高かった．*Cedrela* では，生存率と植物体再生率は 50%，20% であり，*Guazuma* では 50% と 15% であった．低温のハードニングや前培養処理はこれらの樹種では効果がなかったが，これはこれらが熱帯樹種で低温や乾燥に耐性がなかったからだと思われる．組織培養した頂端を凍結保存することは，*C. odorata, G. crinita* と *J. mimosaefolia* の遺伝資源を保存する一つの方法として考えられる．