

## CRYOPRESERVATION OF EMBRYOGENIC CALLUSES OF TWO COMMERCIAL CLONES OF *HEVEA BRASILIENSIS*

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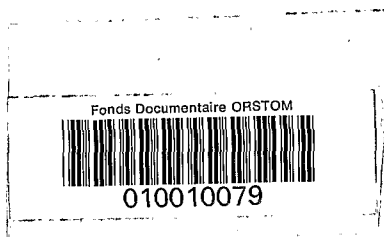
**Summary:** two cryopreservation protocols, one using a classical freezing process and the other a simplified freezing process, were developed for embryogenic calluses of a commercial clone of *Hevea*. After preculture with 1M sucrose and 10% DMSO, embryogenic calluses were frozen in a programmable freezer at  $0.2^{\circ}\text{C}\cdot\text{min}^{-1}$  down to  $-40^{\circ}\text{C}$ , or in a simple device consisting of an isopropanol bath enclosed in a polystyrene box, placed in a  $-80^{\circ}\text{C}$  deep-freezer, thus achieving an average cooling rate of  $0.2^{\circ}\text{C}\cdot\text{min}^{-1}$  down to  $-40^{\circ}\text{C}$ . High survival and rapid regrowth, as well as production of somatic embryos, were obtained with calluses cryopreserved using both freezing protocols. The simple freezing protocol was successfully used with a second commercial clone.

**Keywords:** cryopreservation; *Hevea brasiliensis*; embryogenic callus; classical freezing protocol; simplified freezing protocol.

### Introduction

Multiplication protocols based on somatic embryogenesis, which aim at large-scale production of elite genotypes, are under development for numerous species, including plantation crops such as banana, coffee, sugarcane, oil palm, coconut and *Hevea* (12). Various problems are encountered in the establishment and maintenance of embryogenic cultures, including difficulties in producing embryogenic material, progressive decrease of embryogenic potential over time, risks of somaclonal variation which can occur over extended culture periods and management of a large number of cultures in large-scale production laboratories. Most of these difficulties should be overcome by a rational use of cryopreservation. Cryopreservation protocols have been actively researched for embryogenic cultures of numerous species, including the plantation crops mentioned above (7, 11).

In the case of *Hevea brasiliensis*, the use of somatic embryogenesis would improve intracultural homogeneity as well as vigour and productivity of trees. Somatic embryogenesis for *Hevea* was first reported by Wang *et al.* (22), then by other teams (21, 3). These authors induced the formation of compact calluses from initial explants but more recently, owing to improvements in the protocol leading to the production of friable embryogenic calluses, long-term



embryogenic cultures of several commercial genotypes could be established and routinely maintained on semi-solid medium (14, 15, 9). Improved conditions for the development of somatic embryos and regeneration of plantlets from long-term embryogenic cultures using a temporary immersion system have been described recently (10).

Very limited research only has been performed until now on cryopreservation of *Hevea*. Normah *et al.* (16) demonstrated that embryonic axes could withstand cryopreservation following partial desiccation. More recently, Veisseire *et al.* (20) succeeded in freezing embryogenic cell suspensions of one commercial clone. Their study focused mainly on the effect of pregrowth and preculture conditions on survival.

In the current study, a classical freezing protocol was developed for one commercial clone. A simplified freezing protocol was then established with the same clone and used with a second commercial clone.

## **Materials and Methods**

### Plant material

The material used for cryopreservation studies consisted of a friable embryogenic callus of the commercial clone PB 260 which had been maintained in culture for 2 years. This friable callus was obtained from the internal seed coat of an immature *Hevea* seed (14). Long-term cultured embryogenic callus of a second commercial clone, PR 107, was utilized to evaluate the efficiency of the simplified cryopreservation protocol.

### In vitro culture

Culturing of embryogenic calluses and somatic embryos was performed in the dark at  $27 \pm 1^\circ\text{C}$ .

### *Callus proliferation*

Long-term proliferation was obtained by subculturing the embryogenic calluses every 2 weeks on MH mineral medium (3) with a high  $\text{CaCl}_2$  content (9 mM). The following compounds were added to the proliferation medium: 30 mM  $\text{AgNO}_3$ , 1.34  $\mu\text{M}$  benzyladenine (BA), 1.34  $\mu\text{M}$  3,4-dichlorophenoxyacetic acid (3,4-D, Sigma, St Louis, USA), 0.5  $\mu\text{M}$  abscisic acid (ABA, mixed isomers synthetics N<sup>o</sup>7773-56-0, St Louis, USA), 234 mM sucrose and 2  $\text{g}\cdot\text{l}^{-1}$  Phytigel (Sigma, St Louis, USA).

### *Somatic embryogenesis*

Production of somatic embryos from embryogenic calluses involved the following two successive steps :

1. induction of embryogenesis (day 0 - day 70) during which formation of embryogenic cells and somatic embryos at the globular stage takes place. The embryogenic calluses are cultured on a gelled medium identical to the proliferation medium but with a lower concentration of growth regulators (0.44  $\mu\text{M}$  3,4-D and BA).
2. development of somatic embryos (day 71 - day 130) which is achieved by monthly subcultures in MH liquid medium devoid of growth regulators and with a  $\text{CaCl}_2$  content reduced to 3 mM. Culture is performed using the temporary immersion system developed by Alvard *et al.* (1) and adapted to *Hevea* by Etienne *et al.* (10). At the end of this step,

embryos at the cotyledonary stage are counted to assess the efficiency of the somatic embryo production.

### Cryopreservation

#### *Preculture*

For cryopreservation experiments, fragments of embryogenic calluses ( $\pm 300$  mg fresh weight) were sampled from cultures 12 days after the last transfer on proliferation medium and placed into 2 ml sterile polypropylene cryotubes. They were precultured for 1 h at 0°C in 1 ml of cryoprotective medium containing 0.25 to 1.25 M sucrose and 0 to 15% dimethylsulfoxide (DMSO). DMSO was added progressively over the first 30 min of preculture until the final concentration was reached.

#### *Freezing and thawing*

The classical freezing protocol, performed with a programmable freezer (Minicool LC 40, L'Air Liquide) comprised first slow freezing at various cooling rates (0.2 to 2°C·min<sup>-1</sup>) down to various prefreezing temperatures (-20 to -80°C), followed by direct immersion of cryotubes in liquid nitrogen (LN). For simplified freezing, cryotubes were placed in a simple freezing device consisting of a plastic box filled with isopropanol (Nalgene®, "Mr Freeze") which was itself enclosed, or not, in a polystyrene box. The simple freezing device was placed in a -80°C deep-freezer. Once the temperature of -40°C was reached (measured with a thermocouple placed in a cryotube containing 1 ml of cryoprotective solution), cryotubes were immersed rapidly in LN. Average cooling rates down to -40°C were 0.2 and 0.5°C·min<sup>-1</sup> with and without the polystyrene box, respectively. In both protocols, crystallization in the cryoprotective medium was induced manually at a temperature intermediate between the crystallization and the nucleation temperature of the medium, by briefly pinching the cryotubes with forceps previously cooled in LN. Thawing was performed by immersing the cryotubes in a water-bath maintained by thermostat at 40°C until complete melting of ice.

#### *Recovery*

The contents of the cryotubes were poured on a filter paper placed in a Petri dish containing solid proliferation medium with sucrose concentration equal to that of the cryoprotective medium employed. After 1 h, the filter papers with callus fragments were transferred onto new solid medium with an intermediate sucrose concentration. After 24 h, the filter papers with callus fragments were transferred onto standard proliferation medium in a Petri Dish for recovery. After 15 days, calluses were assessed for regrowth and transferred into test tubes on the same medium for further growth.

### Assessment of survival and recovery

Viability was measured immediately after thawing by staining with fluorescein diacetate (FDA) (23). The survival rate of a sample was assessed by calculating the mean percentage of living cells on a total of 100 cell aggregates (20 cell aggregates chosen randomly on 5 plates observed with a microscope) according to Dussert *et al.* (5). All survival rates are expressed as percentage of the control value. In experiments aiming at optimizing the preculture and freezing parameters (Tables 1-4), recovery of calluses was assessed after 15 days by observing mass increase using a scale reflecting its intensity (0 to 3). In the experiment aiming at observing the production of somatic embryos (Table 5), regrowth rate of control and

cryopreserved calluses was estimated during the 3<sup>rd</sup> subculture on proliferation medium by measuring the fresh weight increase of calluses. Regeneration of somatic embryos was assessed by taking 300 mg of callus and submitting them to the procedure for induction and development of somatic embryos. Regeneration capacity was evaluated by counting the number of embryos at the cotyledonary stage after 2 subcultures in liquid MH medium. For the evaluation of the efficiency of the simplified freezing protocol (Table 7), the number of regrowing calluses was counted at the end of the first subculture on proliferation medium, and the regrowth intensity was evaluated during the first and 3<sup>rd</sup> subcultures by observing mass increase using a scale reflecting its intensity (0 to 3). Results presented in Tables 1-4 and 6 represent the average values of 2-3 independent experiments. Results presented in Tables 5 and 7 were obtained with 6 replicates.

## Results

### 1 - classical protocol

#### *Preculture*

During experiments aiming at determining the optimal cryoprotective medium, viability of preculture controls was generally high (Table 1). However, high concentrations of DMSO had a toxic effect on viability when employed in combination with the lowest sucrose concentrations. Prefreezing down to -40°C induced a drastic drop in viability. The highest viability rates were noted for mixtures of 0.75 to 1.25M sucrose and 0 to 10% DMSO. After cryopreservation, a slight further drop in viability was generally noted. Viability rates higher than 20% were noted with 1 and 1.25M sucrose with 0 to 10% DMSO. The highest viability rate (49%) was obtained with 1M sucrose and 10% DMSO.

**Table 1:** Effect of sucrose and DMSO concentration in the preculture medium on the survival (in %) of control, prefrozen and cryopreserved embryogenic calluses. Embryogenic calluses were frozen at 0.5°C·min<sup>-1</sup> down to -40°C before immersion in liquid nitrogen.

	DMSO (%)	Sucrose (M)				
		0.25	0.50	0.75	1.00	1.25
control	0	97	85	89	94	91
	5	77	58	71	83	62
	10	72	42	63	84	67
	15	2	7	54	69	68
prefrozen	0	0	3	11	32	30
	5	18	37	52	43	69
	10	5	4	21	24	19
	15	1	1	4	13	6
cryopreserved	0	0	2	9	21	23
	5	1	17	17	41	24
	10	1	2	19	49	24
	15	0	0	8	8	8

Regrowth of calluses followed a similar pattern: regrowth of preculture controls was generally high (Table 2). It was drastically reduced after prefreezing down to  $-40^{\circ}\text{C}$  and a further reduction was noted after freezing, especially with the lowest sucrose concentration. Good regrowth (scale = 2) of cryopreserved calluses was obtained with 0.5-0.75 M sucrose and 10% DMSO, and 1M sucrose and 15% DMSO. A combination of 1 M sucrose and 10% DMSO was selected as standard preculture medium for further experiments.

**Table 2:** Effect of sucrose and DMSO concentration in the preculture medium on the regrowth intensity (scaled 0 to 3) of control, prefrozen and cryopreserved embryogenic calluses after 15 days of recovery. Embryogenic calluses were frozen at  $0.5^{\circ}\text{C}\cdot\text{min}^{-1}$  down to  $-40^{\circ}\text{C}$  before immersion in liquid nitrogen.

	Sucrose (M)					
	DMSO (%)	0.25	0.50	0.75	1.00	1.25
control	0	2	3	3	2	1
	5	3	1	3	2	2
	10	2	3	3	3	2
	15	3	2	3	3	2
prefrozen	0	0	0	0	0	1
	5	0	1	1	1	1
	10	2	1	2	2	1
	15	2	1	2	2	1
cryopreserved	0	0	0	0	0	0
	5	0	1	1	1	1
	10	1	2	2	2	1
	15	0	0	1	2	1

### Freezing

Viability of prefrozen controls decreased progressively in line with decreasing prefreezing temperatures for all cooling rates experimented (Table 3). After cryopreservation, no or low viability only was obtained for cooling rates of 1 and  $2^{\circ}\text{C}\cdot\text{min}^{-1}$ . When a cooling rate of  $0.2^{\circ}\text{C}\cdot\text{min}^{-1}$  was applied, viability increased progressively up to 63% for a prefreezing temperature of  $-35^{\circ}\text{C}$ , then decreased progressively and was 6% for a prefreezing temperature of  $-80^{\circ}\text{C}$ . The same pattern was observed with a freezing rate of  $0.5^{\circ}\text{C}\cdot\text{min}^{-1}$ , with the highest survival rate (47%) noted for a prefreezing temperature of  $-45^{\circ}\text{C}$ .

**Table 3:** Effect of freezing rate and prefreezing temperature on the survival (in % of unfrozen control) of prefrozen and cryopreserved embryogenic calluses after 15 days of recovery. Embryogenic calluses were precultured with 1.0M sucrose and 10% DMSO.

	Freezing rate (°C·min <sup>-1</sup> )	Prefreezing temperature (°C)							
		-20	-30	-35	-40	-45	-50	-60	-80
prefrozen	0.2	65	46	65	57	47	27	25	20
	0.5	70	81	76	49	59	18	3	2
	1.0	67	81	68	69	39	37	9	22
	2.0	89	92	90	75	82	50	66	47
cryopreserved	0.2	0	41	63	59	41	25	18	6
	0.5	0	11	45	40	47	38	17	13
	1.0	0	1	3	12	35	46	22	10
	2.0	0	0	0	1	2	1	12	38

Growth recovery of prefrozen controls decreased in line with decreasing prefreezing temperatures (Table 4). After freezing in LN, no regrowth was observed for a freezing rate of 2°C·min<sup>-1</sup> whatever the prefreezing temperature. Regrowth was observed for prefreezing temperatures of -45 and -50°C for a freezing rate of 1°C·min<sup>-1</sup>. The range of prefreezing temperatures ensuring regrowth of cryopreserved calluses was wider for cooling rates of 0.2 and 0.5°C·min<sup>-1</sup>. The highest regrowth intensity (scale = 2) was obtained for calluses frozen at 0.5°C·min<sup>-1</sup> down to -40°C.

**Table 4:** Effect of freezing rate and prefreezing temperature on the regrowth intensity (scaled 0 to 3) of prefrozen and cryopreserved embryogenic calluses after 15 days of recovery. Embryogenic calluses were precultured with 1.0M sucrose and 10% DMSO.

	Freezing rate (°C·min <sup>-1</sup> )	Prefreezing temperature (°C)							
		-20	-30	-35	-40	-45	-50	-60	-80
prefrozen	0.2	2	3	1	2	2	2	1	0
	0.5	3	3	2	2	1	0	1	0
	1.0	1	3	3	1	1	1	0	1
	2.0	2	2	2	2	2	2	1	1
cryopreserved	0.2	0	1	1	1	1	0	0	0
	0.5	0	1	0	2	1	1	1	0
	1.0	0	0	0	0	1	1	0	0
	2.0	0	0	0	0	0	0	0	0

### *Production of somatic embryos*

The regrowth rates of control and cryopreserved calluses during the 3<sup>rd</sup> subculture on proliferation medium were comparable, as was the production of somatic embryos. A tendency to obtain higher numbers of somatic embryos from cryopreserved calluses than from controls was noted consistently during this experiment and confirmed during other experiments (data not shown).

**Table 5:** regrowth rate during the 3<sup>rd</sup> subculture on proliferation medium and production of somatic embryos (No. of cotyledonary embryos produced per g fresh weight) from control and cryopreserved embryogenic calluses. Embryogenic calluses were precultured with 10% DMSO and 1M sucrose, and frozen at 0.5°C·min<sup>-1</sup> down to -40°C before immersion in liquid nitrogen. Production of somatic embryos was carried out as detailed in Materials and Methods. Values presented are means ± standard error from data from 6 replicates.

	regrowth rate	somatic embryos
Control	4.5 ± 1.1	202 ± 57
Cryopreserved	3.87 ± 2.2	250 ± 60

## **2 - simplified protocol**

### *Comparison of classical and simplified protocols*

A progressive decrease in survival in line with decreasing prefreezing temperatures was noted in all experimental conditions (Table 6). Higher survival rates were obtained with both simplified protocols in comparison with equivalent freezing rates performed with a programmable freezer.

**Table 6:** Effect of freezing rate, prefreezing temperature and freezing procedure on the survival of prefrozen and cryopreserved calluses. Freezing was performed using a programmable freezer (cooling rates of 0.2, 0.5 and 1.0 °C·min<sup>-1</sup>), a simple freezing device (N1: average cooling rate 0.5 °C·min<sup>-1</sup>), and a simple freezing device placed in a polystyrene box (N2: average cooling rate 0.2 °C·min<sup>-1</sup>).

	freezing rate (°C·min <sup>-1</sup> )	prefreezing temperature (°C)		
		-35	-40	-45
prefrozen	0.2	65	61	36
	0.5	61	57	12
	1.0	72	44	30
	N1 (±0.5)	82	66	28
	N2 (±0.2)	80	68	58
cryopreserved	0.2	42	51	55
	0.5	40	36	16
	1.0	13	13	7
	N1 (±0.5)	2	21	9
	N2 (±0.2)	28	54	48

After cryopreservation, an important drop in survival occurred for freezing rates of 0.5 (both protocols) and  $1^{\circ}\text{C}\cdot\text{min}^{-1}$ . The decrease in survival was limited for a freezing rate of  $0.2^{\circ}\text{C}\cdot\text{min}^{-1}$  and highest and comparable survival rates were obtained with both protocols for prefreezing temperatures of  $-40$  and  $-45^{\circ}\text{C}$ .

*Cryopreservation of two commercial clones using the simplified freezing protocol*

Good survival was obtained with both clones cryopreserved using the simplified freezing protocol (Table 7). A difference was noted between both clones as regards the number of calluses regrowing after freezing, since the clone displaying higher survival (PB 260) had less calluses regrowing after freezing. Growth recovery of cryopreserved calluses of both clones was rapid since no differences in regrowth intensity in comparison with controls was noted from the 3<sup>rd</sup> subculture onwards. Somatic embryos were regenerated from cryopreserved calluses of both clones (data not shown).

**Table 7:** Survival (in %) of prefrozen (prefr.) and cryopreserved (cryo.) calluses, percentage of control and cryopreserved calluses showing regrowth at the end of the first subculture on proliferation medium, and regrowth intensity (scaled 0-3) of control and cryopreserved calluses during the first (S1) and third (S3) subculture on proliferation medium of two commercial clones cryopreserved using a simplified freezing protocol (simple freezing device placed in a polystyrene box; average cooling rate of  $0.2^{\circ}\text{C}\cdot\text{min}^{-1}$  down to  $-40^{\circ}\text{C}$ ).

	Survival (%)		regrowing calluses (%)		regrowth intensity (S1)		regrowth intensity (S3)	
	prefr.	cryo.	control	cryo.	control	cryo.	control	cryo.
PB260	50±14	40±6	100	40	3	1	3	3
PR107	80±12	30±12	100	60-70	3	1	3	3

**Discussion/conclusion**

The present work allowed the establishment of two efficient cryopreservation protocols for embryogenic calluses of *Hevea* using either a classical or a simplified freezing process. Under optimal conditions, survival was high, regrowth of cryopreserved cultures was rapid since no difference in regrowth intensity was noted in comparison with controls from the 3<sup>rd</sup> subculture on proliferation medium onwards. Somatic embryos could be regenerated in equivalent numbers from cryopreserved and control cultures.

Embryogenic calluses of *Hevea* tolerated exposure to relatively higher concentrations of cryoprotectants than calluses of some other species such as grape, sugarcane and *Citrus* (4, 6, 8) and preculture with high concentrations of cryoprotectants was necessary to obtain regrowth of calluses after freezing. This study also underlined the higher efficiency of binary cryoprotective solutions in comparison with single cryoprotectants and the importance of incorporating DMSO in a cryoprotective solution (24) since no regrowth could be obtained after freezing when employing sucrose alone. This might be due to the properties of DMSO which penetrates cells very rapidly and allows penetration of the other cryoprotectants (19),



thus improving survival by increasing the concentration of intracellular solutes. Another important remark is that the selection of optimal cryopreservation conditions should be based on observation of regrowth, and not on survival data. In this study, several experimental conditions ensuring relatively good survival did not permit regrowth of cryopreserved calluses. Similar observations were made during the establishment of cryopreservation protocols for various materials including embryogenic calluses and suspensions of *Musa*, grape and *Citrus* (17, 5, 8).

The observation that larger numbers of somatic embryos seemed to be regenerated from cryopreserved calluses than from control calluses might be due to the fact that non-embryogenic cells are preferentially destroyed during the freeze-thaw cycle, thus leading to a selection of embryogenic material. Similar observations have been reported notably with *Picea abies* and grape embryogenic calluses (2, 4). These authors suggested that cryopreservation could be used as a tool to "rejuvenate" embryogenic calluses and suspensions when their proliferation potential starts to decrease after extended culture periods.

Simplified freezing protocols have already been developed for various materials including coffee somatic embryos (13), apices and meristematic calluses of white clover (25), cell suspensions of *Citrus* (18, 8). Their obvious advantage is that no sophisticated and expensive equipment is required for freezing, thus making these protocols applicable in the resource-limited environments of most rubber-producing countries. However, the availability of programmable freezers is requested to perform cryopreservation research on a routine basis.

In conclusion, the cryopreservation protocols developed in this study should facilitate the management of embryogenic cultures of commercial clones of *Hevea* in research and production laboratories.

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