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Establishment of a cryopreservation process for long-term storage of embryogenic calli of *Hevea brasiliensis* (Müll. Arg.)

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Summary

A reliable cryopreservation technique was developed for friable embryogenic callus lines of *Hevea brasiliensis*. The technique included a callus pre-culture on a maintenance medium containing 1 mM CaCl₂ prior to cryopreservation to promote post-thaw callus growth recovery. Application of this cryopreservation technique on a sample of 39 callus lines, showed a high percentage of post-thaw lines growth recovery and subsequent maintenance of embryogenic and regeneration competences. Today, all the lines obtained including transformed lines were cryopreserved using this procedure. Data base has been developed for management of cryopreserved stocks.

Keywords: *Hevea brasiliensis*, embryogenic culture, cryopreservation, pre-culture, calcium

Introduction

CIRAD has developed an efficient somatic embryogenesis plant production procedure for the PB 260 rubber tree clone (Carron et al., 1998). This technique is based on regenerating friable embryogenic calli, maintained by regular subcultures at 2-week intervals. However, establishing friable embryogenic callus lines remains a limiting step given 6 to 12 months are needed to obtain a proliferating callus on the one hand, and the frequency of such callus production is low (about 1 per initial thousand explants) on the other hand. In addition, long-term maintenance of friable embryogenic calli lead to loss of callus regeneration competence in rubber tree (Blanc et al., 2006) and increase the risk of somaclonal variations as it was observed in some other species (Sussex and Frei, 1968; Cassells and Morrish, 1987; Yang et al., 1999; Côte et al. 2001). All these problems associated with long-term cell proliferation could be overcome by long-term storage in liquid nitrogen. In this study, we investigated the effect of a callus pre-culture before cryopreservation on various MM CaCl₂ supply to improve post-thaw callus growth recovery and plant regeneration capacity. On a large experiment conducted on 39 lines, we analyzed the effect of cryopreservation on post-thaw growth recovery and subsequent embryogenic and regeneration competences of lines.

Material and methods

Obtention and regeneration of embryogenic friable callus lines

Friable embryogenic lines of clone PB 260 used for the study was obtained at the CIRAD laboratory (Montpellier, France) and at MICHELIN laboratory (Clermont-Ferrand, France). Embryos and plant regeneration was achieved according to the culture conditions defined by Carron et al. (1997).

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Cryopreservation procedure

The cryopreservation procedure was as described by Engelmann et al. (1997). It involved a cryoprotective treatment of callus with a maintenance medium modified with 1 M sucrose and added of dimethylsulphoxide (DMSO). The callus in suspension in the cryoprotective solution was shaken and the callus fraction of about 500 μ m was sampled (1 ml / cryovials). Cryovials containing callus were placed in Nalgene[®] Cryo 1C freezing container (Mr Frosty[®]) filled with isopropanol. The Nalgene[®] Cryo 1C was placed in a -80°C deep-freezer and the temperature was monitored by a thermocouple which was placed in one of the cryovials. At -40°C , the cryovials were rapidly immersed in liquid nitrogen for storage in a cryobiological storage system (LocatorJR Plus, USA).

Thawing was carried out by immersing the cryovials in a warm water-bath maintained at 37°C for 2 min. The content of the cryovials was poured onto Whatman[®] filter paper discs (8.5 cm in diameter) placed in Petri dishes containing 25 ml of MM medium supplemented with 1 M sucrose. After 1h, the filter papers with calli were transferred to fresh MM medium supplemented with 0.5 M sucrose for 24 h after which the filter papers with calli were transferred to another fresh MM medium containing a standard sucrose concentration (234 mM) for a 4-week culture period. Cultures were incubated in the dark as previously described. Post-thaw callus recovery was assessed by weighing growing calli at the end of the 4 weeks of culture in Petri dishes. Then post-thaw callus proliferation was assessed by measuring the calli proliferation rate at the end of a 2 weeks-culture periode on MM medium in glass tubes.

Experimental set-up

Effect of the CaCl_2 supply in the pre-culture medium on post-thaw callus growth recovery and proliferation competence.

In order to assess the effect of CaCl_2 concentration on post-thaw callus regrowth, friable embryogenic calli of lines LF 03-98 and VP 10 were pre-cultured for 12 days on MM containing either 9 (control), 1 or 0 mM of CaCl_2 prior to freezing as previously described.

The experiment was conducted twice on line LF 03-98 (experiment 1 and 2) and once on line VP10 (experiment 3). For experiments 1 and 3, callus tissues from 6 cryovials were thawed and plated for each initial pre-culturing treatment, for experiment 2, calli from 12 cryovials per initial pre-culturing treatment were used.

Impact of cryopreservation on post-thaw callus morphogenetic competence

Using the 1mM CaCl_2 pre-culture medium, we assessed the effect of cryopreservation on line VP10 and on 38 other independent lines obtained in 2003 and 2004. Before cryopreservation, lines were classified according to their morphogenetic competence as “non-embryogenic” when no embryo was regenerated, “embryogenic” when regenerating embryos but no plantlets and “regenerant” when regenerating embryos and subsequent plantlets. Classification was reviewed after cryopreservation according to post-thaw morphogenetic competence of lines. Before cryopreservation, morphogenetic competence of lines was performed on three successive cycles of regeneration and on two after cryopreservation. In this experiment, calli were transferred to the IM medium after two proliferation cycles in glass tubes. Each repetition consisted of 5 flasks each containing 500 mg of callus. After one month of culture on IM medium, calli were then transferred to a RITA[®] system for embryo development. Plantlets were obtained by culturing well-formed embryos on the germination medium.

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Statistical analysis

Each data item was the mean of at least 3 replicates depending on the experiment. Means were tested for significance using the multiple range tests by the Fisher method with a confidence level of 95%. Chi-square test was used to test for embryo conversion into plantlet at a confidence level of 95%.

Results

Effect of the CaCl₂ supply in the pre-culture medium on post-thaw callus growth recovery and proliferation

The same trend in post-thaw callus performance depending on the CaCl₂ concentration in the pre-culture medium was observed in all the three experiments (Table 1). After 2 weeks of culture, small growing clumps of yellow friable calli were developed on calli pre-cultured on 0 or 1 mM CaCl₂. Calli growth was absent on calli pre-cultured on 9 mM CaCl₂ indicating the inhibitory effect of higher concentration of CaCl₂ on cryopreservation of *Hevea brasiliensis* calli (Table 1). After a one-month culture, the clumps uniformly covered the surface of calli pre-cultured on 0 mM CaCl₂ whereas they were sparse on those pre-cultured on 1mM CaCl₂ and absent or rare on those pre-cultured on 9 mM CaCl₂.

Lowering the CaCl₂ concentration from 9 to 1 mM in pre-culture medium led to a significant increase in callus fresh weight of line LF3-98 (Table 1). Further lowering of the calcium concentration to 0 mM led to a significant increase in post-thaw callus fresh weight on line LF3-98 (experiment 1). Similarly, a significant increase in post-thaw callus fresh weight was observed on CaCl₂-free pre-culture medium in line VP10 (experiment 3).

The beneficial effect of lowering the CaCl₂ concentration in the pre-culture medium then persisted during proliferation culture in tubes (Table 1). In all experiments, the number of growing calli increased when calcium concentration was decreased from 9 to 0 mM. Interestingly, with line LF3-98, only callus pre-cultured on 1 or 0 mM CaCl₂ were able to grow after transfer to tubes. The best proliferation rate was obtained with pre-culturing on 0 mM CaCl₂. With line VP10, although calli grew in tubes, the proliferation rate was significantly higher when calli were pre-cultured on calcium-free medium than on medium enriched with calcium (Table 1).

For the three experiments, lowering the CaCl₂-concentration in the pre-culture medium resulted in a significant increase in the percentage of callogenesis recovery, in callus fresh weight and in the proliferation rate compared to the control treatment maintained on 9 mM CaCl₂.

Impact of cryopreservation on post-thaw callus morphogenetic competence

Out of 39 independent cryopreserved callus lines, 36 showed post-thaw growth recovery; 14 out of 20 regenerant callus lines before cryopreservation have maintained this capacity after cryopreservation (Table 3). Hence, for a majority of lines, cryopreservation did not affect morphogenetic competence in terms of plant regeneration capacity. Interestingly, from 9 non-embryogenic lines before cryopreservation, 2 became embryogenic and 3 became regenerant after cryopreservation. Concerning the 10 initially embryogenic lines, 2 lost the embryogenic competence and 2 acquired the regeneration competence after cryopreservation.

Discussion

Role of calcium in cell cryotolerance

Montoro et al. (1993) have shown that a high CaCl₂ concentration (12 mM) in the callogenesis medium has appeared as the factor inducing callus friability without damaging subsequent embryogenic competence. Then, they concluded that calcium undeniably appeared to be the active element in the calcium chloride inducing friability Montoro et al. (1995).

Callus friability and embryogenic competence can be maintained for a long-term period on a maintenance medium containing 9 mM CaCl₂ (Etienne et al. 1997). More recently, it was shown that pre-culturing calli which have been maintained on 9 mM CaCl₂ on a medium without calcium prior to genetic transformation increased transformation efficiency (Montoro et al. 2000). In this study we reported that it was necessary to pre-culture calli on a medium with a reduced CaCl₂ concentration (0 or 1 mM) prior to cryopreservation to enable post-thaw growth recovery. Etienne et al. (1997) and (Blanc et al. 1999) have independently observed that friable embryogenic calli of *Hevea* maintained on 9 mM CaCl₂ consisted of undifferentiated active cells (meristematic cells and embryogenic cells), together with differentiated largely vacuolized cells. Transferring calli to a maintenance medium with little or no calcium therefore increased the proportion of active cells (meristematic and embryogenic cells) (Montoro et al., 2000) with a high nucleo-cytoplasmic ratio and high soluble protein content which are cryotolerant cell types (Grout, 1995; Mathur et al. 2003; Winkelmann et al. 2004).

This result supports the hypothesis that endogenous calcium content of calli cultured on 0, 1 or 9 mM CaCl₂ are mediators of cell modifications enabling callus growth after cryopreservation. Calcium also plays a fundamental role in cryotolerance, through its involvement in preserving membrane integrity. Indeed, cryopreservation causes many changes and much damage to cell membranes (Benson et al. 1992; Mari et al. 1995). Fleck et al. (2003) showed that cryotolerance was closely correlated to cell protection mechanisms against oxidative stress. As a secondary message of external stimuli, notably of environmental stress factors (Yang and Poovaiah, 2003), calcium is known to stimulate cell detoxification mechanisms by triggering catalase activity (Yang and Poovaiah, 2002) and limits lipid peroxidation phenomena by maintaining a high level of intracellular glutathione (Umeshita et al. 1988).

Influence of cryopreservation on post-thaw morphogenetic competence

Initial results on cryopreservation of *Hevea* embryogenic tissues indicated possible growth recovery from cell suspensions (Veisseire et al. 1993). Then, Engelmann et al. (1997) developed a simplified cryopreservation technique for friable embryogenic *Hevea* calli and succeeded on post-thaw calli regrowth and embryo regeneration but without plant regeneration. In this study, we have demonstrated both post-thaw embryogenic competence and subsequent plant regeneration from embryogenic calli of *Hevea brasiliensis* for the first time.

Post-thaw growth recovery from cryopreserved material is the main limiting factor when developing cryopreservation techniques. Concerning post-thaw growth recovery, we achieved a successful result by reducing the calcium concentration in the pre-culture medium. Interestingly, our study showed a tendency towards both greater morphogenetic competences for cryopreserved callus in some initial poor-competent lines (non embryogenic lines). Increased embryogenic competence in cryopreserved calli has already been reported for *Hevea brasiliensis* (Engelman et al. 1997) as well as cell suspensions of *Cyclamen persicum* (Winkelmann et al. 2004). Preferential preservation of active cells during cryopreservation might be an explanation for this

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result (Mathur et al. 2003, Winkelmann et al. 2004). We also showed that some non-regenerant lines became regenerant after cryopreservation indicating a better conversion competence of embryos derived from cryopreserved callus. This result is not common in the literature and the rate of plants regenerated from cryopreserved or non-cryopreserved tissues is often identical (Danso and Ford-Lloyd, 2004; Valladares et al. 2004). During cryopreservation of *Hevea* callus, cell selection made it possible to eliminate both vacuolized cells and embryogenic cells displaying physiological disruptions likely to lead to abnormal embryo development.

For regenerant lines, both maintenance and loss of embryogenic competence was recorded after cryopreservation. This observation indicates different post-thaw physiological status of cryopreserved callus. Several subcultures on maintenance medium after thawing might be a prerequisite to restore callus regeneration competence.

Conclusion

Our study showed that pre-treating calli before cyropreservation on a medium with little (1 mM) or no calcium is essential for post-thaw callus growth recovery. Lowering the CaCl₂ content of the medium modified the physiological and structural characteristics of the cells. Those modifications might explain the greater cryoresistance of the cells. Data from this study complete the cryopreservation procedure defined by Engelman *et al.* (1997) by adding a pre-culture step of 12 days on a MM medium containing 1mM CaCl₂ before cryopreservation. More, results led to a better knowledge on post-thaw morphogenetic competence of rubber tree callus. This new cryopreservation procedure is integrated into our somatic embryogenesis plant production scheme, for long-term preservation of the different embryogenic lines. Today, more than 225 lines were cryopreserved including transformed lines.

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Table 1: Effect of CaCl₂ concentration in pre-culture medium on post-thaw callus growth recovery and proliferation competence. For "callus fresh weight" and "proliferation rate", values are means \pm SD (the number of replicates corresponds to the number of platings or tubes with callus growth recovery). Values with different letters are significantly different according to Fisher's Multiple range test, P < 0.05. Callogenesis intensity quantified as follows: absent (-), rare (+), common (++).

CaCl ₂ concentration in preculture medium (mM)	Post thaw callus growth recovery in Petri dishes				Post-thaw callus proliferation (2 weeks' culture in glass tubes on MM)		
	Number of callus platings	Number of grown calli	Callogenesis intensity (2 weeks of culture)	Callus FW (mg) (1 month of culture)	Number of calli ¹	Number of proliferating calli	Proliferation rate ²
Experiment 1 (Line LF 03-98)							
9	6	2	-	150 \pm 14 ^c	2	0	0 ^c
1	6	4	+	750 \pm 28 ^b	10	10	4.7 \pm 1.2 ^b
0	6	4	++	1270 \pm 106 ^a	24	24	8.1 \pm 1.9 ^a
Experiment 2 (Line LF 03-98)							
9	12	9	-	137 \pm 60 ^b	11	0	0 ^b
1	12	12	+	367 \pm 150 ^a	19	17	5.2 \pm 7.2 ^{ab}
0	12	12	+	465 \pm 260 ^a	35	35	6.9 \pm 8.0 ^a
Experiment 3 (Line VP10)							
9	6	2	-	9 \pm 0 ^b	2	2	6.5 \pm 3.7 ^b
1	5	4	+	130 \pm 80 ^{ab}	22	22	5.4 \pm 2.6 ^b
0	6	5	+	310 \pm 100 ^a	69	69	18.2 \pm 4.6 ^a

¹ "Number of calli obtained at the end of the regrowth phase in Petri dishes

² "Proliferation rate" is: fresh weight of calli at the end of the proliferation phase / fresh weight of calli on day 0 of the proliferation phase

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Table 2: Evolution of morphogenetic competence of callus lines before and after cryopreservation

Before cryopreservation		After cryopreservation		Names of lines
Morphogenetic competence of lines	Number of lines	Morphogenetic competence of lines	Number of lines	
Non embryogenic	9	No post-thaw recovery	0	
		Non embryogenic	4	A3, 1-5(5), 1-3(15), 1-2(2)
		Embryogenic	2	A9, 1-59(5)
		Regenerant	3	A7, A18, A21
Embryogenic	10	No post-thaw recovery	2	A19, 4-1(20)
		Non embryogenic	2	A14, A32
		Embryogenic	4	A26, A36, A38, A39
		Regenerant	2	VP10, A41
Regenerant	20	No post-thaw recovery	1	A24-2
		Non embryogenic	1	1-21(15)
		Embryogenic	4	A1, A2, A6, 1-2(15)
		Regenerant	14	A4, A5, A8, A17, A20, A22, A27, A28, A30, A37, A40, G1, G6, 1-212(15)
Total Non embryogenic	9	Total No post-thaw recovery	3	
Total Embryogenic	10	Total Non embryogenic	7	
Total Regenerant	20	Total Embryogenic	10	
		Total Regenerant	19	