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CRYOPRESERVATION OF OIL PALM SOMATIC EMBRYOS: IMPORTANCE OF THE FREEZING PROCESS.

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SUMMARY

Cryopreservation of oil palm somatic embryos was carried out using one-step rapid freezing (200°C.min^{-1}) or two-step freezing, i.e. programmed cooling from +20°C to -100°C with cooling rates ranging between 0.5 and 40°C.min^{-1} followed by immersion of samples in liquid nitrogen. For both clones studied, the survival and recovery rates, as well as the recovery intensity, were greater for cooling rates equal to or higher than 5°C.min^{-1}. Moreover, when a slow cooling rate (0.5°C.min^{-1}) was employed, the recovery rate in one clone increased when

the prefreezing temperature was decreased from -20°C to -100°C.

KEY-WORDS

Cryopreservation, cooling rate, prefreezing temperature, survival rate, recovery rate, somatic embryos, oil palm, germplasm storage.

INTRODUCTION

The cryopreservation techniques used for most plant species have been applied to protoplast, cell, callus or meristem cultures (1). Fewer publications have dealt with the application of these processes to somatic, pollinic or zygotic embryo preservation (2).

The first successful cryopreservation of oil palm somatic embryos was achieved in 1985 (3) using rapid freezing obtained by direct immersion of the samples in liquid nitrogen. This technique ensures reproducible resumption of adventive embryogenesis of frozen material (4) and the production of plantlets. Plantlet development in the nursery has, so far, been comparable to that of clonal plantlets originating from non-frozen material (5).

In this paper, the results obtained by using a different technique, i.e. two-step freezing, are compared with those noted in the rapid freezing method. The first step involves programmed cooling to a given prefreezing temperature. The second step is performed by plunging the samples into liquid nitrogen. In the experiments described, each of the two main parameters (cooling rate and prefreezing temperature) was held constant while the other was varied.

MATERIALS AND METHODS

Tissue culture procedures

Two embryoid clones of oil palm (*Elaeis guineensis* Jacq.),obtained according to a method developed in France by the ORSTOM/IRHO Research Team (6) were used : one (clone BC 068) was obtained from a nursery seedling, the other (clone BC 156) from an elite palm.

The embryoids were subcultured monthly on modified Murashige and Skoog medium (7), devoid of growth regulators and containing 0.1 M sucrose. Cultures were maintained in a controlled-growth room at 27°C with a 12-hour diurnal period and lighting of 20 $W.m^{-2}$ (True Lite type Durotest fluorescent tubes).

The embryoid clumps used for the cryopreservation experiments were obtained after two-month culture on a medium enriched with 0.3 M sucrose (8). Their average weight was about 50 mg.

Cryopreservation process

The cryopreservation method comprises 4 successive stages : pretreatment, freezing, thawing and post-treatment. Only the freezing method was modified during the experiments.

Pretreatment : the embryoid clumps excised from two-month old cultures were precultured for 7 days in Petri dishes, on the standard semi-solid medium containing 0.75M sucrose.

Freezing : the embryoid clumps were transferred into sterile 2 ml cryotubes, without any liquid medium.

- For rapid freezing (200°C.min^{-1}), the cryotubes were plunged directly into liquid nitrogen.
- Two-step freezing was achieved in the chamber of a programmable freezer (Minicool type, by l'Air Liquide). The cryotubes were cooled first at a given

temperature drop rate from +20°C to a pre-set prefreezing temperature. They were then immersed in liquid nitrogen.

The effects of the different cooling rates (0.5, 1, 5, 10, 20, 40°C.min^{-1}) were studied for the same prefreezing temperature (-100°C) and subsequent cooling in liquid nitrogen. Importance of the prefreezing temperature (-20°C, -40°C, -100°C) was studied with embryoid clumps cooled at 0.5°C.min^{-1} to the chosen prefreezing temperature and then plunged into liquid nitrogen or thawed immediately.

Thawing : after 1 hour in liquid nitrogen, the samples were thawed by plunging the ampoules for 1 min into a water bath kept at +40°C.

Post-treatment : the embryoid clumps were cultured for 3 weeks in Petri dishes on media enriched with 10^{-6}M 2,4-dichlorophenoxyacetic acid (2,4-D), with progressive decreases in sugar content. Embryoid clumps were then transferred to the standard medium without auxin (3).

Somatic embryogenesis recovery

The survival rates were determined after the three weeks of post-treatment and the somatic embryogenesis potentiality recovery after two months of subculture on the standard medium.

In order to determine the intensity of the resumption of embryoid adventive somatic embryogenesis, as a function of cooling rate, subsequent development of embryoid clumps was observed for 11 weeks after thawing. Two parameters were selected: the number of test tubes used at every subculture (7 and 11 weeks after thawing), which depends on the mass of plant material regenerated from the frozen clumps,

and the number of embryoid clumps showing resumption of adventive embryogenesis, 5, 7 and 11 weeks after thawing.

RESULTS

1) Effects of cooling rate

Survival rate and recovery rate.

The survival and recovery rates for embryoid clumps frozen in liquid nitrogen are presented in figure 1. The survival rate measured 3 weeks after thawing corresponds to the percentage of embryoid clumps which showed measurable growth during post-treatment. The recovery rate, measured 11 weeks after thawing, corresponds to the percentage of embryoid clumps for which resumption of adventive embryogenesis was noted.

The embryoid clumps of clone BC 068 gave survival rates ranging between 58 and 80%, whatever the cooling rate. In contrast, the greater differences noted for clone

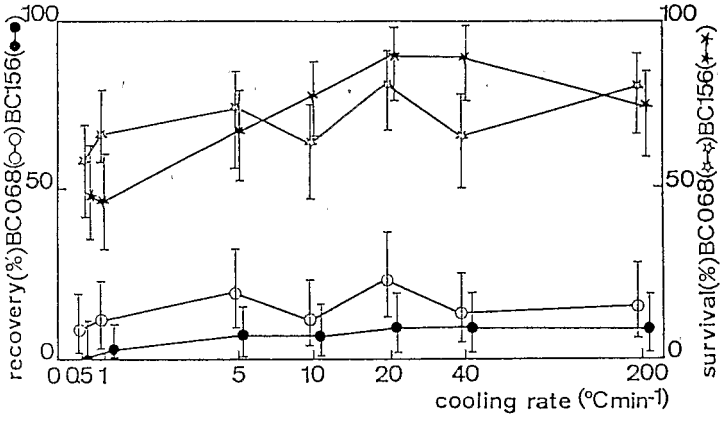

Figure 1 : Effect of cooling rate (°C.min^{-1}) on survival (☆,★) and recovery (○,●) of embryoid clumps from clone BC 068 (☆,○) and clone BC 156 (★,●). 43 to 48 embryoid clumps were used for each set of conditions. Vertical bars indicate standard deviation.

			cooling rate (°C.min-1)						
			0.5	1	5	10	20	40	200
average recovery rate (%)	clone	BC 068	7.8 (2-18)	7.8 (2-18)	20.3 (10-34)	12.2 (7.5-22)	23.8 (14-26)	15.2 (7-26)	18.2 (10-28)
		BC 156	1.2 (0-4)	2.1 (0-10)	7.6 (2-17)	6.3 (1-16)	8.3 (2-20)	8.6 (3-18)	8.1 (3-15)

Table 1 : Average proliferation recovery rates for embryoid clumps of clone BC 068 and BC 156 after freezing in liquid nitrogen, as a function of cooling rate. Each value is given with its standard deviation (in brackets).

		cooling rate (°C.min-1)						
		0.5	1	5	10	20	40	200
number of frozen embryoid clumps		43	44	47	46	48	46	46
number of embryoid clumps showing proliferation recovery and, in brackets, number of test tubes used for subculture as a function of culture duration after thawing (in weeks)	LN + 5 weeks	2 (2)	1 (1)	3 (3)	3 (3)	3 (3)	1 (1)	4 (4)
	LN + 7 weeks	4 (4)	4 (4)	7 (11)	4 (4)	9 (14)	2 (2)	6 (6)
	LN + 11 weeks	4 (5)	5 (5)	9 (13)	5 (7)	11 (18)	6 (10)	7 (10)

Table 2 : Changes in the number of embryoid clumps of clone BC 068 in which adventive embryogenesis resumed 5, 7 and 11 weeks after thawing, as a function of cooling rate. The intensity of resumption of adventive embryogenesis is represented by the number T of test tubes (in brackets) used for every subculture.

BC 156 between the results obtained with slow cooling rates (0.5°C. min^{-1}) and those obtained with faster cooling rates were statistically significant (X^2-test). For every cooling rate, the differences between the two clones were never statistically significant (X^2-test).

Proliferation resumed under every set of conditions with clone BC 068, and embryogenesis recovery rates varied between 9.3% (0.5°C.min^{-1}) and 22.9% (20°C.min^{-1}). The differences observed were never statistically significant. In the case of clone BC 156, no recovery was noted when the embryoid clumps were frozen at 0.5°C.min^{-1}. The differences

		cooling rate (°C.min-1)						
		0.5	1	5	10	20	40	200
number of frozen embryoid clumps		48	48	48	48	48	48	48
number of embryoid clumps showing proliferation recovery and, in brackets, number of test tubes used for subculture as a function of culture duration after thawing (in weeks)	LN + 5 weeks	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (1)	1 (1)
	LN + 7 weeks	0 (0)	0 (0)	0 (0)	2 (2)	3 (3)	4 (4)	1 (1)
	LN + 11 weeks	0 (0)	1 (1)	3 (3)	3 (3)	4 (5)	4 (6)	3 (3)

Table 3 : Changes in the number of embryoid clumps of clone BC 156, in which adventive embryogenesis resumed, 5, 7 and 11 weeks after thawing, as a function of cooling rate. The intensity of resumption of adventive embryogenesis is represented by the number T of test tubes (in brackets) used for every subculture.

observed between the highest recovery rates (8.33%), which were observed for cooling rates of 20, 40 and 200°C.min^{-1},

and values noted for the slowest cooling rate ($0.5°C.min^{-1}$), were statistically significant.

The experiments were repeated several times using different cooling rates, in order to evaluate reproduci-

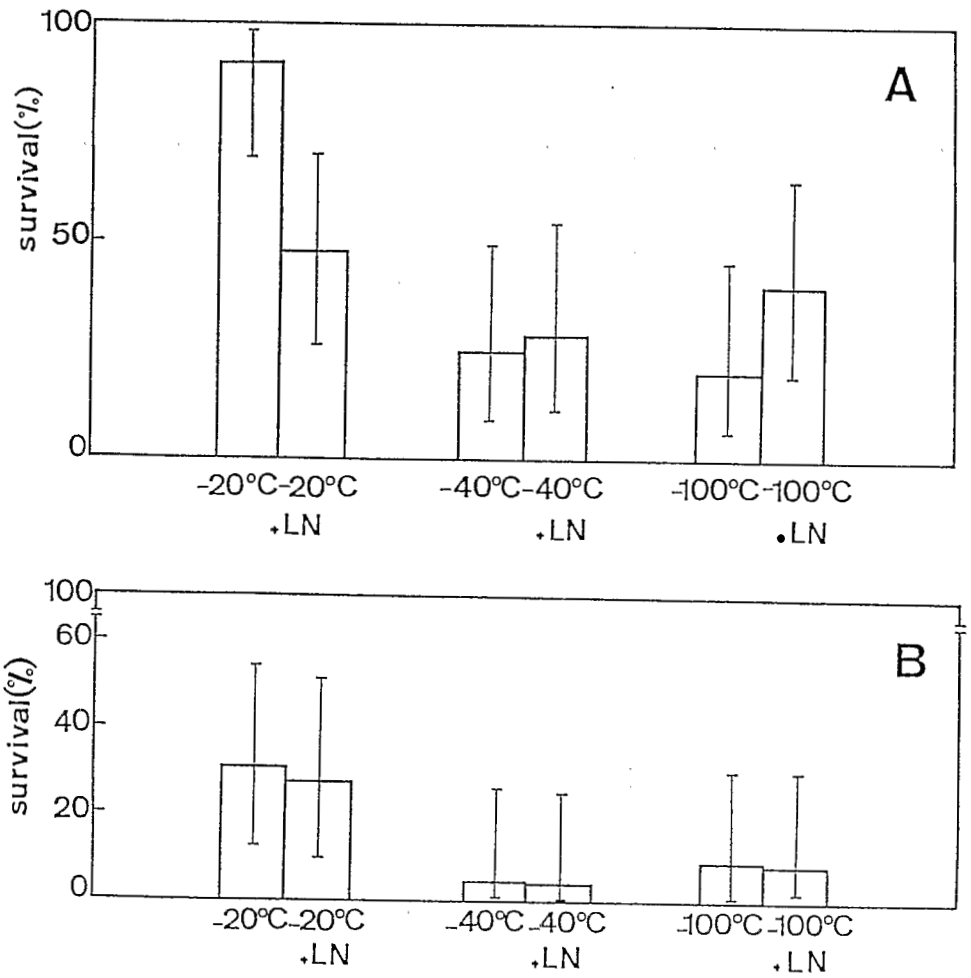

Figure 2 : Survival rates for embryoid clumps of clone BC 068 (A) and clone BC 156 (B) cooled to various temperatures (-20°C, -40°C or -100°C) at 0.5°C.min-1, and then immersed in liquid nitrogen (+LN) or thawed directly. Vertical bars represent standard deviation. 20 to 30 embryoid clumps were used for every set of conditions.

bility (table 1). The results obtained for both clones when using slow cooling rates (0.5 and 1°C.min^{-1}) were lower than those noted for cooling rates equal to or higher than

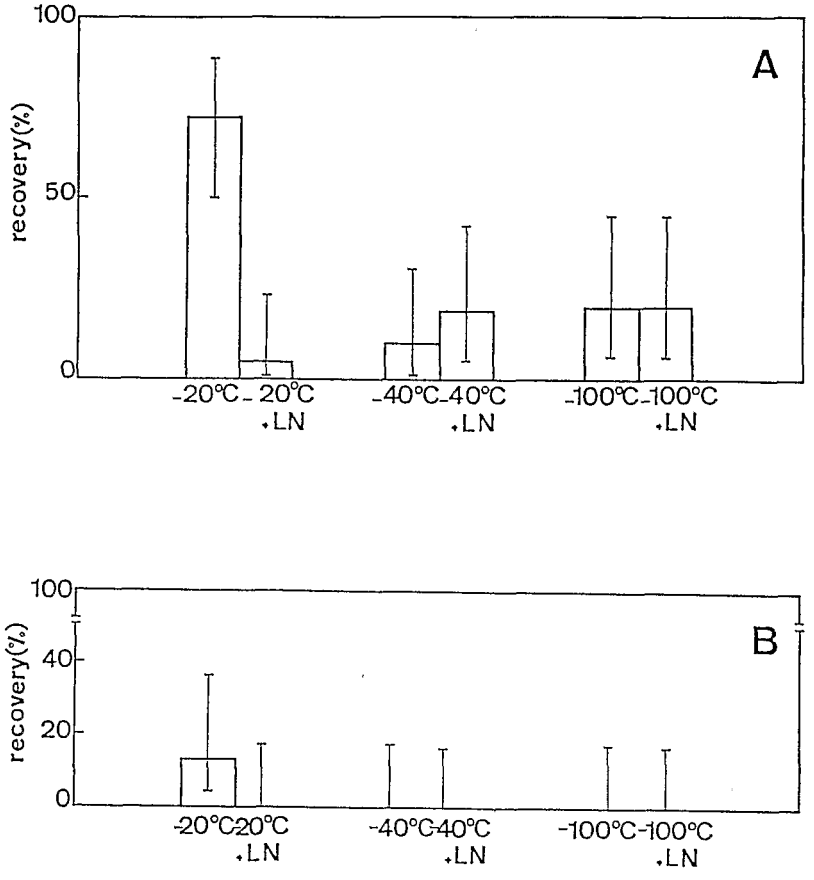

Figure 3 : Somatic embryogenesis recovery rates for embryoid clumps of clone BC 068 (A) and clone BC 156 (B) cooled to various temperatures (-20°C, -40°C or -100°C) at 0.5°C.min^{-1}, and then immersed in liquid nitrogen (+LN) or thawed directly. Vertical bars represent standard deviation. 20 to 30 embryoid clumps were used for every set of conditions.

5°C.min^{-1}. Clone BC 156 seemed more sensitive to freezing than clone BC 068, whatever the conditions. Hence, the

embryoid clump recovery rate in both clones seemed to depend on cooling rate.

Intensity of recovery.

With clone BC 068 (table 2), recovery of adventive embryogenesis was observed 5 weeks after thawing, whatever the cooling rate. Even if the recovery rate did not differ significantly as a function of the cooling rate during this experiment (cf figure 1), its intensity varied with the conditions: it was greater for cooling rates equal to or higher than 5°C.min^{-1}. In the case of clone BC 156 (table 3), resumption of proliferation was observed 5 weeks after thawing, but only for high cooling rates (20, 40, 200°C.min^{-1}). When recovery of adventive embryogenesis occurred, it was observed only 11 weeks after thawing.

Intensity of recovery was very low, since 11 weeks after thawing, under optimal conditions (i.e. cooling rate of 40°C.min^{-1}), the 4 embryoid clumps which had proliferated required only 6 test tubes for subculture. Proliferation resumed sooner and at a higher intensity in clone BC 068, compared with clone BC 156.

2) Effects of prefreezing temperature

The survival rates for non-frozen embryoid clumps from clone BC 068 (figure 2A) were higher than, equal to or lower than those of frozen material for respective prefreezing temperatures of -20°C, -40°C and -100°C. Survival rates for clone BC 156 (figure 2B) were lower under all conditions than those noted for clone BC 068, and were not modified by immersion of embryoid clumps in liquid nitrogen before rewarming.

The recovery rate for clone BC 068 embryoids frozen and immediately thawed (without immersion in liquid nitrogen) decreased with the prefreezing temperature from

72.7% (prefreezing to -20°C) to 10 or 20% (prefreezing to 40°C or -100°C) (Figure 3A). In contrast, the recovery rate for embryoids frozen and immersed in liquid nitrogen increased with the prefreezing temperature from 4.7% (prefreezing to -20°C) to 19 or 20% (prefreezing to -40°C or -100°C). The resumption of adventive embryogenesis in clone BC 156 was only noted for embryoids frozen to -20°C without immersion in liquid nitrogen (figure 3B).

DISCUSSION AND CONCLUSIONS

The survival rates obtained with the embryoid clones tested were relatively high, whatever the cooling rate, since they varied from 58 to 81% for clone BC 068 and from 46 to 79% for clone BC 156. In the period between determination of survival rate, (3 weeks after thawing) and evaluation of resumption of adventive embryogenesis (11 weeks after thawing), necrosis was noted in some embryoid clumps, in almost equivalent proportions for all cooling rates. Seibert and Wetherbee (9) observed an identical phenomenon after freezing carnation meristems : 35 to 40% of frozen meristems did not develop into plantlets, whatever the cooling rate used. It is likely that the living meristematic tissue in embryoid clumps that did not develop was inadequate for subsequent proliferation. This phenomenon was particularly obvious in slow-freezing experiments : proliferation recovery, when observed, occurred only from confined zones of frozen embryoid clumps.

The differences in resistance to liquid nitrogen which appeared between the two clones should be compared with similar observations in potato and carnation shoot tips (10, 11), cell cultures of periwinkle (12) or alfalfa callus (13). The better results obtained with clone BC 068 could be due to faster resumption of cell multiplication and cellular functions transitorily altered by freezing, e.g. plasmalemma-associated active transport (14).

The most original result observed with oil palm embryoids was the possibility of obtaining resumption of proliferation using very different cooling rates, ranging from 0.5°C.min^{-1} to 40°C.min^{-1} to the temperature of -100°C before immersion in liquid nitrogen, or a rate of about 200°C.min^{-1} in the case of rapid freezing. Nevertheless, resumption rates were lower for slow cooling rates (0.5 or 1°C.min^{-1}) than for faster cooling rates. The following hypothesis could be formulated in order to explain these results: ice-forming mechanisms could be basically different for these two freezing modes. Rapid freezing induces the formation of intracellular, non-lethal ice micro-crystals, whereas during slow freezing, the first ice crystals formed are extracellular. When the temperature decreases, cells dehydrate progressively : the internal water flows to the external medium. When the samples are immersed in liquid nitrogen intracellular ice crystals form and damage cellular structures (15).

For a given material, there is usually a relatively narrow range of cooling rates that ensures high resumption rates. For example, in the case of strawberry meristems, the optimal cooling rate ensuring 95% survival was 0.84 °C.min^{-1}. Survival dropped to 33% when the cooling rate was increased to 0.95°C.min^{-1} (16). In the case of embryos, only rapid cooling has until now ensured survival of maize or oilseed rape zygotic embryos (17, 18). Carrot somatic embryos, on the other hand, have only been frozen successfully when slow or intermediary cooling rates were used (19).

The good results obtained with oil palm somatic embryos, whatever the cooling rate, could be due to the fact that they well tolerated pronounced partial dehydration ,a vital pre-condition for freezing of oil seed rape zygotic embryos according to Withers (18). During pretreatment, the moisture of the oil palm embryoids decreased from 80% to about 60% (wet basis). Turnham and

Northcote (20, 21) have pointed out that, at the beginning of oil palm somatic embryo development, two main types of lipid are synthesized in large amounts : triacylglycerols (storage lipids) and polar lipids. The presence of such lipids, which are components of cellular membranes, could be of major importance for the repair of membrane structures injured during freezing, and lipids may therefore contribute to the high survival rates of the embryoids after thawing. The presence of lipids could also result in improved resistance due to increased intracellular osmotic pressure.

The importance of the prefreezing temperature during two-step freezing has been pointed out. Under these conditions, resumption of adventive embryogenesis was only noted in clone BC 068. The best results were obtained after prefreezing to -40°C or -100°C. The importance of the prefreezing conditions has already been emphasized by many authors. Optimal prefreezing temperatures are often noted : -100°C for *Lavandula* cells (22), -40°C for *Catharanthus* cells (23). Towill (24) has indicated that this optimum varies with the cultivar considered. Lastly, Kartha et al. (25) have shown that the prefreezing temperature of cassava meristems could modify the morphogenetical response of frozen explants.

It may be that the low resumption rates obtained when oil palm embryoids were prefrozen to -20°C before freezing in liquid nitrogen were due to insufficient cell dehydration. Dehydration would become sufficient only when the prefreezing temperature reached -40°C. It is possible that all the embryoids cooled to -20°C and rewarmed immediatly have not frozen. In effect, oil palm embryoids are capable of supercooling to temperatures around -20°C (8). This might account for the high recovery rates found in this treatment with both clones. Cooling to -40°C and lower would inevitably cause freezing.

The thawing rate can be of great importance for survival of the frozen material. With a few exceptions, increases in thawing rate ensured higher rates of resumption of adventive embryogenesis, as observed in the case of carrot cells (26). Similar results were obtained with growth recovery of carnation (9), strawberry (27) and pear tree (28) meristems.

The difference observed between the survival and resumption rates for embryoids thawed without previous immersion in liquid nitrogen could result from different thawing rates under these two sets of conditions. The following hypothesis can be proposed: the thawing rate was probably proportionally higher between -196°C and +40°C (temperature of the thermostated water-bath), than between -40°C and +40°C. This could be particularly important when passing through the critical temperature zone where recrystallization detrimental to cellular integrity can occur (15).

In conclusion, oil palm somatic embryos are an original material for plant tissue and organ cryopreservation studies since resumption of adventive embryogenesis is noted over a wide range of cooling rates (0.5 to 200°C.min^{-1}). However, greater and more intense recovery is obtained with cooling rates higher than or equal to 5°C.min^{-1}. This value could represent a threshold between the two different ice-formation mechanisms described above. The results presented here emphasize the importance of the prefreezing temperature during slow-freezing experiments. Finally, on a practical level, the possibility of employing a programmable apparatus for freezing oil palm embryoids could ensure that conditions are well-defined and reproducible from one experiment to another, and also from one laboratory to the another. Moreover, the large range of usable cooling rates should allow suitable cooling regimes to be defined for every

clone, should this phase of the cryopreservation process prove critical for cryopreservation of a given clone.

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