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EFFECT OF THAWING AND RECOVERY CONDITIONS ON THE REGROWTH OF MERISTEMS OF DATE PALM (*PHOENIX DACTYLIFERA* L.) AFTER CRYOPRESERVATION IN LIQUID NITROGEN.

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SUMMARY: During this study, the effect of thawing and recovery conditions were observed on cryopreserved meristems of date palm. A thawing temperature of 40°C was optimal compared with 60 and 80°C or slow rewarming at room temperature. Recovery was improved by placing the thawed meristems in the dark for ten days and then under attenuated light on a medium containing 1 $g.l^{-1}$ activated charcoal. In these conditions, up to 30% of the cryopreserved meristems could withstand freezing in liquid nitrogen. This technique was successfully applied to two other varieties of date palm.

KEY WORDS: *Phoenix dactylifera* L. ; cryopreservation ; thawing ; activated charcoal ; lighting conditions.

INTRODUCTION

A cryopreservation process was set up recently with meristems of date palm var. Bou Sthammi noir (1). However, only 17% of recovery could be obtained after freezing in liquid nitrogen. Improvements to a freezing procedure can be achieved by manipulating various parameters of the process. Notably, several studies showed that results could be increased by modifying the thawing and recovery conditions.

The effect of the thawing rate on recovery after freezing has been only rarely studied. However, changes in the thawing regime can greatly modify the results. In most cases, rapid thawing is preferable to slow thawing (2). However, the survival of cell suspensions of carrot and maize was independent of the thawing rate (3, 4). On the contrary, Reuff *et al.* (4) observed a two-fold increase in the recovery of a cell suspension of *Coleus blumei* by increasing the thawing temperature to 60°C.

Withers (5) obtained the survival of carrot somatic embryos after slow thawing carried out in the air current of a laminar flow cabinet.

Activated charcoal is commonly employed in tissue culture. For cryopreservation, Dussert *et al.* (6) showed that including activated charcoal in the recovery medium had a beneficial effect in the case of grape embryogenic cell suspensions, whereas, with sugarcane embryogenic suspensions, Gnanapragasam and Vasil (7) observed a negative influence of this substance.

Recovery of plant tissues can also be improved by modifying the lighting conditions during regrowth. Thus, post-treatment in the dark proved to be beneficial in the case of meristems of potato (8) and embryogenic calluses of sugarcane (9).

In this paper, we studied the effect of thawing and recovery conditions (activated charcoal and lighting conditions) on the regrowth of cryopreserved date palm meristems. The modified process was then applied to two additional varieties.

MATERIALS AND METHODS

Plant material

Meristems of *in vitro* plantlets of the variety Bou Sthammi noir were used for the experiments on the effects of thawing and post-treatment conditions. The cryopreservation method was then tested on meristems of plantlets of the varieties Zahidi and Nabut Seif, taken from the *in vitro* collection of GRFP.

Methods

In vitro culture

The plantlets from which the meristems used in this study were sampled were cultured according to the method described by Ferry *et al.* (10).

Cryopreservation

Pregrowth and freezing: The process used was that described by Bagniol and Engelmann (1). After their dissection, the meristems were cultured for 24 hours on standard medium containing 0.1 M sucrose. They were then transferred for 24 hours on a medium enriched with 0.5 M sucrose and submitted to a cryoprotective treatment for 2 hours at 0°C in a liquid medium containing 0.5 M sucrose and 10% dimethylsulfoxide (DMSO). They were prefrozen to -30°C at a rate of $1°C.min^{-1}$ before their immersion in liquid nitrogen where they were kept for 24 hours.

Thawing: The meristems were thawed either rapidly by immersion of the cryotubes in a water-bath thermostated at 40, 60 or 80°C, or slowly by placing the cryotubes at room temperature in the air current of a laminar flow cabinet.

The thawing curves were recorded by placing a Cu/Co thermocouple in 1 ml of cryoprotective medium contained in a 2 ml cryotube previously frozen in liquid nitrogen. The cryotube was plunged in a water-bath thermostated at 40, 60 or 80°C or placed in the air current of the laminar flow cabinet.

Recovery: The thawed meristems were transferred at 24 hours intervals in Petri dishes containing 10 ml of media with progressively lower sucrose concentrations (0.5 M, 0.3 M) until the standard concentration of 0.1 M was reached. These media were or not supplemented with 1 $g.l^{-1}$ activated charcoal. The cultures were either placed directly under the standard lighting conditions (photoperiod of 16 hours/24, 34 $\mu E.m^{-2}.s^{-1}$) or in the dark during the first 10 days following thawing and then under attenuated light (7 $\mu E. m^{-2}.s^{-1}$, same photoperiod).

Expression of the results: The survival rate of the meristems was recorded 3 weeks after thawing (2 weeks for the experiment concerning the effect of the lighting conditions). The regrowth of the meristems was followed under the binocular by measuring their size. Immediately after thawing, all the meristems bleached but living meristems progressively recovered their original pigmentation, whereas dead meristems remained completely white. Were considered as surviving meristems which had shown measurable regrowth and had recovered their original pigmentation.

The results correspond to the mean value of two sets of independent experiments. Twenty to thirty meristems were used in each condition.

RESULTS

The thawing curves recorded for water-bath temperatures of 60 and 80°C were similar for temperatures under 0°C (Fig. 1). In these conditions, the temperature of 0°C was reached after 70 s and after 110 s with a water-bath thermostated at 40°C. In the case of slow thawing at room temperature, 15 min were necessary to reach 0°C. No survival was obtained after slow thawing (Table 1). The highest rewarming temperatures had a negative effect on survival. It was not significantly different between these conditions. The optimal thawing temperature was 40°C.

The presence of activated charcoal in the recovery medium improved the survival rate of frozen meristems (Table 2). Regrowth of control meristems was delayed on a medium containing activated charcoal (Fig. 2). However, after freezing in liquid nitrogen, activated charcoal did not modify the regrowth of frozen meristems in comparison with controls placed on the same medium (Fig. 3).

The response of frozen meristems to the lighting conditions to which they were submitted depended on the composition of the recovery medium (Table 3). The determining factor was the presence of activated charcoal, since a minimal survival rate of 19.6% was obtained, whatever the lighting conditions if the medium contained activated charcoal. Without charcoal, light totally inhibited the recovery of frozen meristems.

The cryopreservation method set up proved to be successful with the two additional varieties tested (Table 4). The positive effect of activated charcoal in the recovery medium was also observed with these materials. Indeed, survival of 0 and 13.3% were obtained with Zahidi without and with activated charcoal respectively, and 11.8 and 48.2% with Nabut Seif.

DISCUSSION/CONCLUSION

The refinement of the recovery conditions allowed to significantly improve our cryopreservation method, since the survival of meristems of the variety Bou Sthammi noir was almost doubled, compared to our previous experiments (1).

The importance of several parameters of a cryopreservation process were underlined. With date palm meristems, rapid thawing in a water-bath at 40°C was preferable to slow thawing and higher thawing temperatures. Indeed, rapid rewarming allows to avoid recrystallization phenomena which induce lethal damages of the cells (11). However, too high thawing rates can also be detrimental to cell survival. They may provoke a too rapid cellular rehydration which creates an important osmotic shock, detrimental to the cells (9).

Activated charcoal is widely used in tissue culture. It has an effect of adsorption of the growth regulators, thus decreasing the level of hormones available to the cultures (12, 13). This could explain the fact that the growth of control date palm meristems was lower on a medium containing activated charcoal. After freezing in liquid nitrogen, only groups of cells remain alive, and not the whole meristems (14). When these meristems are transferred on a medium without activated charcoal, the quantity of hormones available in the medium is too high compared with the number of living cells and becomes toxic. With activated charcoal, the level of free hormones may become sufficiently low to be compatible with the number of living cells and allow the regrowth of the frozen meristems.

We observed with date palm that growth recovery in the dark improved the survival of cryopreserved meristems. Chemiluminescence analyses showed an increase of oxygen singlets in cryopreserved tissues submitted to light immediately after thawing (15). Light has an oxidative effect which induces browning and necroses which can lead to the death of the cells (8). This was observed in the case of date palm meristems. The presence of activated charcoal in the recovery medium could also allow the adsorption of the oxided substances which are toxic to the cells, and thus increase the recovery rate, even under lighted conditions. Indeed, activated charcoal also adsorbs toxic compounds produced by the cells, as it has been shown by various authors (16, 17).

For many plant materials, the recovery rates vary among varieties of a same species, as observed with cryopreserved meristems of apple tree (18), potato (19) and mint (20). This was also observed in the case of date palm, with the two additional varieties tested.

This study allowed to set up a protocol ensuring the cryopreservation of date palm meristems of three different varieties with survival rates ranging from 11.8% (Zahidi) to 48.2%(Nabut Seif), which are satisfactory values when compared to that generally obtained in the literature. However, additional studies are needed concerning the application of the present technique to a broader range of varieties and the extension of the storage duration. The development of collections of date palm meristems in liquid nitrogen may thus be foreseen in a near future.

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Table 1: Effect of thawing temperature on the survival rate (± S.D.) of meristems frozen in liquid nitrogen, after three weeks on standard medium.

	Thawing temperature (°C)			
Survival	25	40	60	80
(%)	0	30.3±3.0	13.4±0.9	15.0±5.0

Table 2: Effect of activated charcoal in the recovery medium on the survival rate (±S.D.) of cryopreserved meristems, after three weeks of culture.

	Activated charcoal ($g.l^{-1}$)	
Survival	0	1
(%)	10.5±0.5	29.0±6.3

Table 3: Effect of lighting conditions and activated charcoal on the survival rate (±S.D.) of cryopreserved meristems after two weeks of culture.
After thawing, meristems were exposed directly to a photon dose of 34 $\mu E.m^{-2}.s^{-1}$ or placed in the dark for 10 days and then exposed to a photon dose of 7 $\mu E.m^{-2}.s^{-1}$.

		Activated charcoal ($g.l^{-1}$)	
		0	1
Lighting conditions	34 $\mu E.m^{-2}.s^{-1}$	0	19.6±5.3
	0/7$\mu E.m^{-2}.s^{-1}$	9.1±0	25.9±11.6

Table 4: Effect of activated charcoal on the survival rate (±S.D.) of cryopreserved meristems of two varieties of date palm, after three weeks of culture.

		Variety	
		Zahidi	Nabut Seif
survival rate (%)	+activated charcoal	11.8±0.7	48.2±11.8
	-activated charcoal	0	13.3±0

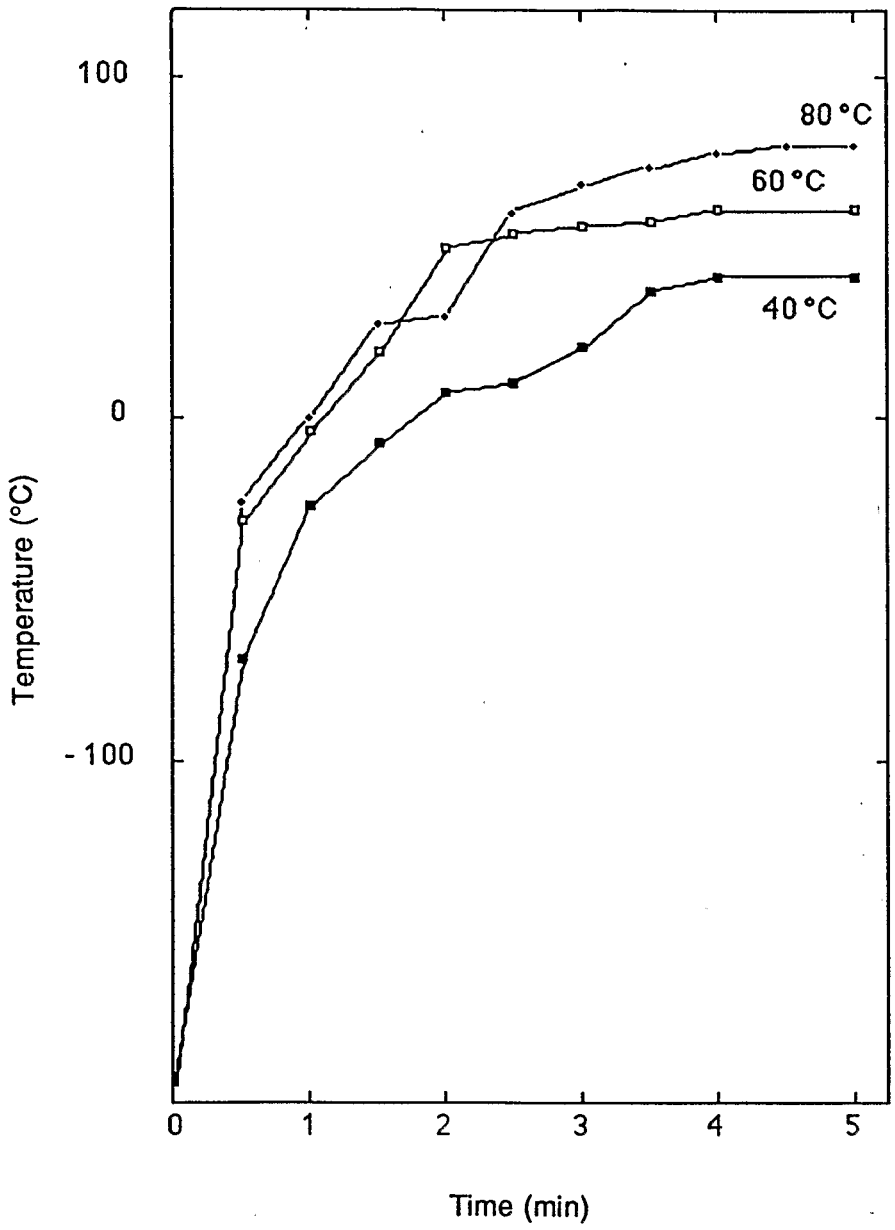

Figure 1: Thawing curve of 1 ml cryoprotective medium as a function of the thawing temperature.

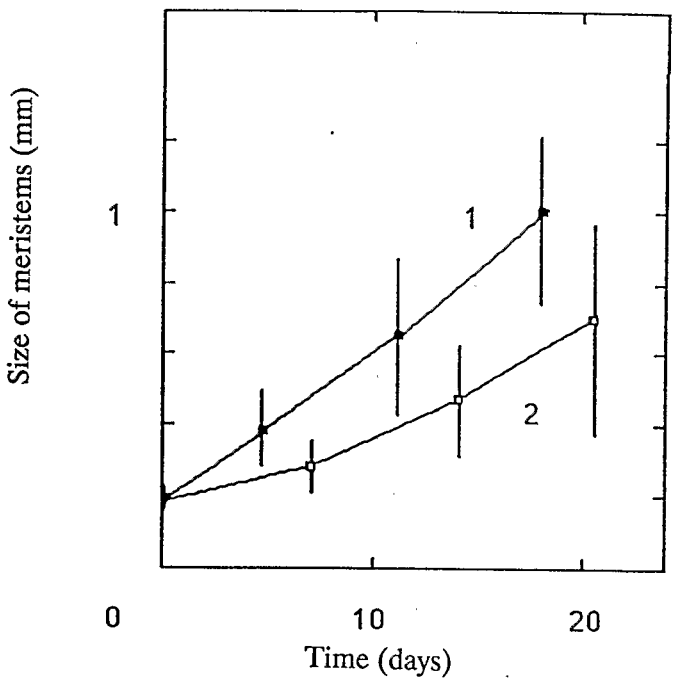

Figure 2: Effect of activated charcoal in the culture medium on the growth of control meristems. (1: medium without charcoal; 2: medium with charcoal). Vertical bars represent the standard deviation.

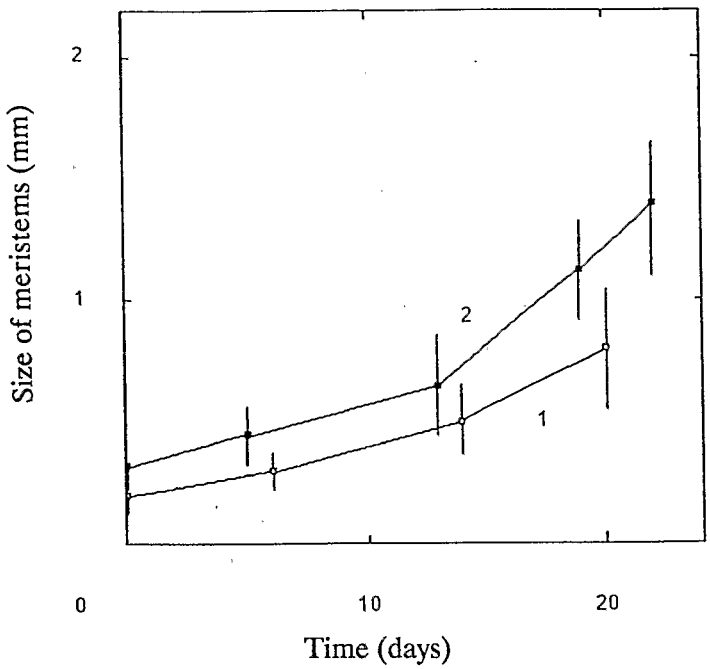

Figure 3: Effect of freezing in liquid nitrogen on the regrowth of the meristems. (1: control meristems; 2: cryopreserved meristems). Vertical bars represent the standard deviation.