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EFFECTS OF PREGROWTH AND FREEZING CONDITIONS ON THE RESISTANCE OF MERISTEMS OF DATE PALM (*Phoenix dactylifera* L. var. Bou Sthammi Noir) TO FREEZING IN LIQUID NITROGEN

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SUMMARY: Optimal cryoprotection and freezing conditions were studied for date palm meristems. A 1- or 3-day culture period on standard medium, immediately after their dissection, prior to pregrowth on medium containing 0.5 M sucrose, was necessary in order to obtain the survival of meristems after freezing in LN. The maximal survival was reached after cryoprotection with 0.5 M sucrose and 5 to 15 % DMSO, followed by freezing at 1°C.min^{-1} to -30°C before immersion in LN.

KEY WORDS: *Phoenix dactylifera*; date palm; cryopreservation; meristems; pregrowth; *in vitro* culture.

INTRODUCTION

The needs for young trees of date palm (*Phoenix dactylifera* L.) increases constantly. Many plantations have to be renewed, and planted areas increase

constantly. Moreover, the Maghreb plantations are threatened by a fungal disease, bayoud (*Fusarium oxysporum* Schlect. var. *albedinis*). Date palm is traditionally propagated using the sprouts it produces, but the rate is not sufficient to fulfil current needs (2 to 20 sprouts in the life of a palm).

Various *in vitro* culture techniques have been developed for multiplying this species (1). In France, the GRFP (French Group For Date Palm Research), using the axillary bud proliferation technique (2), is experimenting with industrial scale plantlet production from apices and axillary buds taken from sprouts (3). This technique should ensure the mass production of elite plants from palms selected for their resistance to bayoud. The *in vitro* culture of numerous genotypes poses practical management problems. The maintenance of cultures necessitates regular transfers and the continuous introduction of plant material *in vitro* places increasing demands on culture room space, as well as labour and material costs. Cultures are exposed to risks of contamination and somaclonal variation, which increase with time and the number of transfers.

Today, only cryopreservation (freezing at the temperature of liquid nitrogen (LN), -196°C) ensures the long-term conservation of plant material without alterations and with reduced maintenance costs. This technique has been tested with more than 70 different species (4). One of its major uses is for conservation of the genetic resources of recalcitrant seed producing species. With *Palmaceae*, which include many such species, resistance to freezing in LN has been obtained with various materials : zygotic embryos of oil palm (5), coconut (6) and of the ornamental palms *Howea* and *Veitchia* (7). Cryopreservation is currently being applied to the storage of oil palm somatic embryos (8, 9). In the case of date palm, freezing experiments have already been carried out using calluses (10, 11).

In this paper, we examine the influence of pregrowth and freezing conditions on the resistance of date palm meristems to freezing in LN.

MATERIALS AND METHODS

Plant material and culture conditions

Meristems were taken from date palm var. Bou Sthammi noir plantlets, at the end of a 6-week culture period on a multiplication medium containing 0.1 M sucrose (3). Cultures were placed under a light intensity of 42 $\mu E.m^{-2}.s^{-1}$, with a 16h lighted and 8h dark period at a temperature of 28°C (day) alternating with 20°C (night). The experiments were carried out using 25 to 30 meristems per treatment.

Cryopreservation

Pregrowth/cryoprotection

Before pregrowth, the meristems could be maintained for 1 to 7 days, immediately after their dissection, on an induction medium, favouring the growth of the meristems (3). For their pregrowth, the meristems were placed for 24 hours on media containing 0.3 or 0.5 M sucrose. These pregrowth conditions had been determined as optimal during preliminary experiments.

For treatment with cryoprotectants, the meristems were suspended in 5 ml liquid induction medium in a 10 ml conical centrifuge tube and placed at 0°C. The cryoprotective mixture, at twice the final concentration, was added at the rate of 0.5 ml every 3 min., until the desired concentration was reached. The meristems were kept in contact with the cryoprotective solution for 2 hours.

Freezing/thawing

The meristems, suspended in 1 ml of cryoprotective medium, were placed in 2 ml sterile polypropylene cryovials. Freezing was carried out using a programmable freezer (Minicool, from L'Air Liquide). The ampoules were cooled at $1°C.min^{-1}$ to various prefreezing temperatures (end of programmed freezing). Once the prefreezing temperature was reached, the ampoules were either thawed immediately or immersed in LN, where they were kept for 1 hour before being thawed. Crystallisation of the cryoprotective medium was induced manually at a temperature intermediate between the crystallisation and nucleation temperatures of the medium, by briefly pinching the ampoules with forceps previously cooled in liquid nitrogen. For thawing, the ampoules were plunged in a water-bath thermostated at 40°C, until melting of ice was complete.

Post-treatment

For post-treatment, the meristems were transferred to Petri dishes of solid induction medium containing 0.5 M sucrose. They were then transferred at 24-hour intervals onto media with progressively lower sucrose concentrations (0.3 and 0.1 M). The survival rate of the meristems was recorded 3 weeks after their transfer to standard medium containing 0.1 M sucrose.

RESULTS

Table 1 presents the survival rate of meristems as a function of the duration of their culture, after dissection, on the induction medium. These meristems were pregrown for 1 day with 0.5 M sucrose, cryoprotected with the same sucrose concentration plus 15 % DMSO (dimethylsulfoxide), then frozen to -30°C before

immersion in LN. With control meristems, the duration of culture on the induction medium had no effect on survival. After freezing in LN no survival was observed for meristems which had not been submitted to this culture on the induction medium, or which had been cultured on this medium for 5 or 7 days. Only a 1- or 3-day culture period allowed growth recovery. The development of the frozen meristems, 6 weeks after thawing, was satisfactory.

Table 2 indicates the survival of meristems as a function of the sucrose and DMSO concentrations used during cryoprotective treatment. In this experiment, the meristems were pregrown for 1 day with the same sucrose concentration as that used during the cryoprotective treatment. For unfrozen control meristems, there was no effect of the DMSO concentration, whatever the sucrose level. For meristems frozen in LN, survival was optimal for 0.5 M sucrose and 5 to 15 % DMSO.

The change in survival rate of the meristems as a function of the prefreezing temperature is shown in Fig. 1. For this experiment, the meristems were pregrown for 1 day on a medium containing 0.5 M sucrose, then cryoprotected with 0.5 M sucrose and 15 % DMSO. Crystallisation was induced at -13°C. The survival of the prefrozen meristems decreased progressively with the lowering of the prefreezing temperature. There was no survival for a prefreezing temperature of -40°C. After freezing in LN, survival was observed only for a prefreezing to -30 and -35°C. The optimal value (17 %) was reached for -30°C.

DISCUSSION/CONCLUSIONS

In this paper, an original technique is proposed for the cryopreservation of date palm meristems. It resulted in 17 % survival of the frozen meristems, after 1-day culture on induction medium, 1-day pregrowth on 0.5 M sucrose, cryoprotection with 0.5 M sucrose plus 15 % DMSO followed by freezing at $1°C.min^{-1}$ to -30°C before immersion in LN. The conditions differed greatly from those used with date palm calluses (7, 8): no pregrowth, cryoprotection with a mixture of polyethyleneglycol, glucose and DMSO (10 %/ 8 %/ 10 %). Only the freezing conditions were similar ($1°C.min^{-1}$ to -30°C), although prefreezing temperatures of -17 or -13°C led to the same results.

In this study, the importance of various parameters of a cryopreservation process was emphasized. The culture of meristems on induction medium for 1 or 3 days before pregrowth on medium containing increased levels of sucrose seems essential for survival. This has already been observed with potato meristems by Benson *et al.* (12). It seems that healing of the meristems before freezing is necessary to survival. This culture could have the effect of "synchronising" the explants, thus reducing their physiological heterogeneity. With date palm, it was important not to

extend this culture for more than 3 days. Beyond this period, increase in size of the meristems will certainly have contributed to their failure to withstand freezing.

With meristems of date palm, DMSO had no toxic effects at the concentrations tested (5 to 15 %), which are commonly employed for the cryoprotection of meristems (13). Its addition was necessary in order to obtain survival after freezing in LN. An improvement in the survival rates after freezing in LN may be obtained by using higher DMSO concentrations.

During programmed freezing, the survival of control meristems decreased progressively with the prefreezing temperature. Freezing in LN was lethal after prefreezing between -13 and -25°C, due to an insufficient dehydration of the tissues. At -40°C, excessive dehydration during prefreezing was responsible for the death of the meristems. Only a very narrow range of prefreezing temperatures (-30 to -35°C) ensured survival after freezing in LN. Towill (14) as well as Kartha *et al.* (15) observed the same results with potato and cassava meristems, respectively. In the latter case, the type of organogenesis was also modified, callogenesis being favoured by the lowest prefreezing temperatures, which is not observed with date palm meristems.

The results presented are still preliminary and will have to be improved. Research is focusing on the optimization of the freezing rate, which can be decisive (16), and on modifications of the post-treatment medium, its hormonal balance (17), and mineral (18) or organic elements (19), which can have a positive effect on the recovery of the material. This technique will have to be extended to other varieties. It should thus be possible, in the near future, to ensure the long-term preservation of date palm germplasm.

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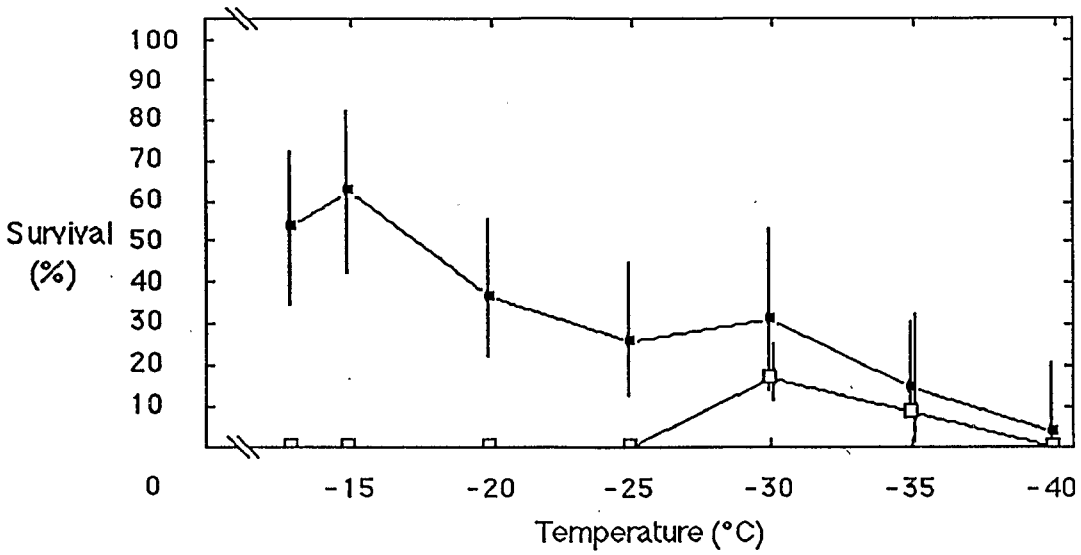

Fig. 1 : Effect of the prefreezing temperature on the survival rate of control (■) or frozen (□) meristems. Vertical bars represent the standard deviation.

Table 1 : Effect of the duration of culture on induction medium on the survival rate of control (-LN) or frozen (+LN) meristems.

		Duration of culture (days)				
		0	1	3	5	7
Survival (%)	- LN	57.1	79.1	86.6	86.6	87.0
	+ LN	0	22.0	15.1	0	0

Table 2 : Effect of sucrose and DMSO concentration during the cryoprotective treatment on the survival rate of control (-LN) or frozen (+LN) meristems.

		DMSO (%)			
	Sucrose (M)	0	5	10	15
- LN	0.3	62.5	62.9	62.9	61.5
	0.5	66.6	56.6	56.6	57.1
+ LN	0.3	0	0	3.6	7.5
	0.5	0	18.9	14.0	17.1