DOI: 10.1007/s10535-012-0284-y BIOLOGIA PLANTARUM 57 (2): 375-379, 2013

BRIEF COMMUNICATION

Cold hardening and sucrose treatment improve cryopreservation of date palm meristems

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Abstract

Date palm (*Phoenix dactylifera* L.) cv. Khenizi caulogenic meristems were initiated from achlorophyllous leaves excised from *in vitro* shoot cultures and then proliferated on a specific culture medium supplemented with 70 g dm^{-3} sucrose. Regeneration rates obtained when using standard vitrification, droplet-vitrification, and encapsulation-vitrification protocols reached 26.7, 60.0, and 40.0 %, respectively. Only explants smaller than 3 mm in diameter were found to survive cryogenic treatments. Sucrose preculture, cold hardening and loading solution pretreatments showed significant effects on regeneration rates. Moreover, our results indicate that both sucrose preculture and cold acclimation of explants increased proline content. Cryopreservation of date palm tissue with high proliferation capacity can directly benefit large scale micropropagation projects.

Additional key words: caulogenesis, *in vitro*, micropropagation, *Phoenix dactylifera*, proline, vitrification.

Date palm (*Phoenix dactylifera* L.) is one of the most important perennial plants in sub-Saharan and hot regions of the globe. Extensive efforts have been undertaken in order to overcome constraints hampering the extension of date palm plantations (Fki *et al.* 2003). Biotechnology has been successfully used in order to propagate, improve, and preserve plant genetic resources (Vasanth and Vivier 2011). In the case of date palm, biotechnological techniques have already been employed for *in vitro* propagation (Fki *et al.* 2011c). However, very few commercial tissue culture laboratories are now able to provide sizeable quantities of plants because of the known recalcitrance of this species to *in vitro* regeneration (Drira *et al.* 1985). In fact, date palm tissue cultures grow very slowly: the initiation phase may require more than 24 months, especially when low amounts of plant growth regulators are used in order to avoid somaclonal variation (Cohen *et al.* 2011, Fki *et al.* 2011*a*). The presence of uncontrollable endophytic bacteria is another serious constraint hampering the large scale micropropagation of date palm (Fki *et al.* 2010).

Cryopreservation refers to the storage of a living tissue at ultra-low temperature (-196 °C) such that it can be revived and restored to the same living state as before it was stored in liquid nitrogen. All living cells suffer from severe osmotic stress and/or ice crystal damage during the freezing and thawing processes. The most effective ways to minimize such lethal effects are *1*) to use cryoprotective compounds in the culture medium prior freezing and *2*) to control the transient cooling and warming rates during preservation. Cryopreservation is a smart strategy to efficiently manage large scale micropropagation of date palm. Several studies have already been published although research is still needed before this

Received 28 March 2012, accepted 22 August 2012.

Abbreviations: 2,4-D - 2,4-dicholorophenoxyacetic acid; LS - loading solution; MS - Murashige and Skoog; PGR - plant growth regulator; PVS - plant virification solution; RS - recovery solution.

Acknowledgments: This research work was supported by the Ministry of Higher Education of Tunisia, IAEA (TC Project RAF/5/049), ALECSO (Arab League Educational Cultural and Scientific Organisation) and COST (European Cooperation in Science and Technology, Action 871).

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technology can reach a routine stage (Fki *et al.* 2011*b*). The present research is aimed at generating and cryopreserving highly proliferating meristems from the date palm cv. Khenizi using standard vitrification, droplet-vitrification, and encapsulation-vitrification protocols. In the longer term, we plan to create a cryobank of proliferating tissues which can constitute a helpful tool for the large scale micropropagation of date palm.

In vitro cultures of date palm (*Phoenix dactylifera* L.) cv. Khenizi shoots were established in our laboratory according to our previously described protocol (Fki *et al.* 2011*a*) which is based on the utilization of low concentration of 2,4-D (0.1 mg dm^{-3}) with fragmented juvenile leafs excised from offshoots as primary explants. Hypertrophied achlorophyllous leaves were excised from shoot cultures, cut in 4 mm size pieces, and cultivated onto solid culture medium for the initiation of adventitious meristems. The culture medium consisted of Murashige and Skoog (1962; MS) basal solution to which 30, 50, or 70 g dm^{-3} sucrose, and 2 g dm^{-3} *Phytage*l were added. The medium was adjusted to pH 5.7 before autoclaving at 120 °C for 15 min. Cultures were maintained by subculturing every month.

For shoot multiplication, the temporary immersion of cultures in liquid medium were used. The RITA bioreactor (Alvard *et al.* 1993) is made of two compartments: the explants are cultivated in the upper compartment and the lower one holds the liquid medium. Six bud clusters per bioreactor were cultivated using 200 cm^3 of MS medium supplemented with 70 g dm^{-3} sucrose. The immersion cycle was 15 min every 24 h and the culture medium was renewed once every 4 months. Stock cultures were incubated in a growth chamber at temperature of 28 °C, a 16-h photoperiod, and irradiance of 30 μmol m^{-2} s^{-1}.

Three different cryopreservation protocols were assessed: standard (tube) vitrification, droplet-vitrification, and encapsulation-vitrification. Three replicates of ten samples were used for each experiment. Explants (< 3 mm) were excised from *in vitro* grown leaves showing bud initiation. Such explants bearing caulogenic meristems were subcultured on MS medium supplemented with 180 g dm^{-3} sucrose (sucrose preculture) or incubated at 4 °C (cold hardening) for 2 d. In the first two protocols (standard vitrification and droplet-vitrification), explants were transferred into 15 cm^3 loading solution (LS) containing 2 M glycerol and 0.4 M sucrose in MS medium for 20 min (Panis *et al.* 2005). In the third protocol, explants were placed into previously autoclaved 3 % (m/v) sodium alginate dissolved in MS medium with 7 % (m/v) sucrose and no $CaCl_2$; then they were sucked up with a micropipette and gently dropped into 75 mM $CaCl_2 \cdot 2\ H_2O$ in MS medium supplemented with 7 % sucrose (Lakshmana and Singh 1990) and kept for 15 min. Encapsulated plant tissues were then transferred into the loading solution for 20 min.

The loading solution was then replaced by ice-cooled plant vitrification solution (PVS) 2 solution (Sakai *et al.* 1990). This solution consisted of 3.26 M glycerol, 2.42 M ethylene glycol, and 1.9 M DMSO in MS medium containing 0.4 M sucrose. The pH was adjusted to 5.8 and the solution was filter sterilized. Both naked and encapsulated explants were treated with PVS2 solution at 0 °C for 15, 30, 60, or 120 min.

Explants were transferred into 2 cm^3 cryotubes containing 0.5 cm^3 of PVS2 and then plunged into liquid nitrogen (standard vitrification protocol). Alternatively, explants were transferred to a droplet of PVS2 on a strip of aluminium foil and then plunged into liquid nitrogen (droplet-vitrification protocol). For permanent cryostorage, frozen foil strips were quickly transferred to 2 cm^3 cryotubes filled with liquid nitrogen and then closed. For encapsulated explants, alginate beads were transferred into 2 cm^3 cryotubes filled with 0.5 cm^3 of PVS 2 solution and then plunged in liquid nitrogen (encapsulation-vitrification protocol).

After one hour in liquid nitrogen, strips of aluminium foil were transferred to recovery solution (RS) containing 1.2 M sucrose dissolved in MS medium at room temperature (25 °C) for 15 min. Cryotubes containing the meristems or alginate beads were thawed in a water bath at 40 °C for 2 min and then treated by RS at room temperature for 15 min. Explants were then placed onto two sterile filter papers on top of MS medium containing 180 g dm^{-3} sucrose and then incubated in the dark. After 2 d, tissues were transferred onto MS medium containing 50 g dm^{-3} sucrose and 0.1 mg dm^{-3} 2,4-D. Survival rates were estimated using growth measurement at 4 - 6 weeks after thawing. Proline content was estimated according to Bates *et al*. (1973) in fresh leaf tissue

Statistical analyses of data were performed using one-way *ANOVA* and Duncan's test using *SPSS 13* software. $P < 0.05$ was considered as statistically significant. Experiments were replicated three times. Data expressed in percentage were transformed by arcsin transformation and then analyzed.

We found that low sucrose concentrations (30 and 50 g dm^{-3}) were inadequate for the initiation of caulogenic meristems from *in vitro* cultivated chlorophyll-free leaves. MS medium supplemented with 70 g dm^{-3} sucrose enabled the generation of highly proliferating meristems (data not shown). Hypertrophied achlorophyllous leaves showed a high morphogenetic capacity as they produced high numbers of caulogenic meristems after 3 months in culture (Fig. 1*A*). Our previous studies showed that plant growth regulators (PGRs) such as 2,4-D were essential to induce organogenesis on primary explants in date palm and that the culture period required to observe the first adventitious buds was much longer (17 months), especially when low concentrations of 2,4-D were used (Fki *et al.* 2011*a*). Removing PGRs from culture media can minimise the risk of both somaclonal variation and loss of morphogenetic capacity (Bairu *et al.* 2011). Indeed, LoSchiavo *et al*. (1989) showed that auxins impact global DNA methylation rates which might disturb gene expression and phenotype. Al-Mazroui *et al.* (2007) confirmed that high amount of PGRs was the cause of somaclonal variation in date palm.

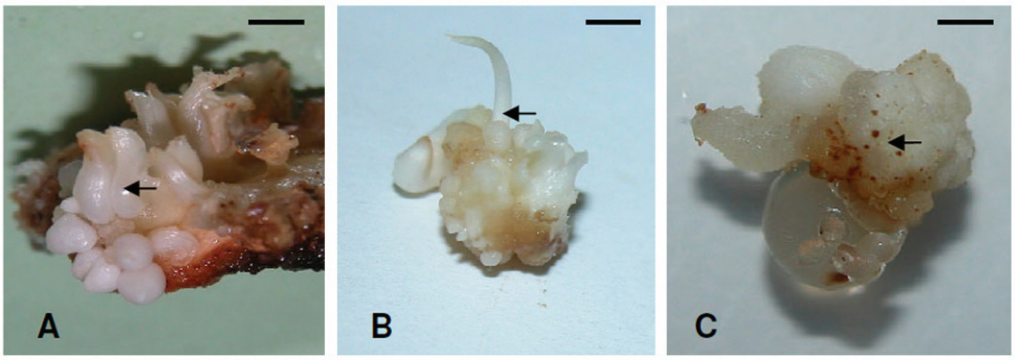

Fig. 1. *A* - Bud development from adventitious meristems initiated in date palm cv. Khenizi *in vitro* cultivated leaf explants (bud is shown by an *arrow*, *bar* 2 mm. *B, C* - Regeneration from cryopreserved date palm cv. Khenizi adventitious vegetative meristems at 4 months after thawing.: *B* - using the droplet vitrification protocol (bud is shown by an arrow, *bar* 2 mm); *C* - using the encapsulation-vitrification protocol, (callus is shown by an *arrow*, *bar* 1 mm).

Table 1. Regeneration [%] of caulogenic meristems after 0 to 120 min exposure to PVS2 solution at 0 °C followed or not by cryopreservation**.** Ten samples were used in each of the three replicates. Data within a column with the same letters are not significantly different according to Duncan's test after arcsin transformation ($P < 0.05$). +LN - cryopreserved meristems, -LN - non cryopreserved meristems, PC - sucrose (180 g dm^{-3}) preculture for 2 d, CH - cold hardening (2 d at 4 °C), v - standard vitrification, ev - encapsulation-vitrification, dv - droplet-vitrification.

PVS2 [min]	-LN -PC,-CH	-LN +PC	-LN +CH	+LN -PC,-CH v	+LN +PC v	+LN +CH v	+LN -PC,-CH ev	+LN +PC ev	+LN +CH ev	+LN -PC,-CH dv	+LN +PC dv	+LN +CH dv
0	93.3bc	86.7c	86.7c	0a	0a	0 a	0a	0a	0a	0a	0a	0a
15	93.3bc	86.7c	86.7c	3.3c	13.3c	6.7c	0a	0a	0a	13.3c	46.7d	36.7d
30	86.7b	86.7c	73.3b	13.3c	26.7d	23.3d	13.3b	23.3b	16.7b	36.7e	60.0e	56.7e
60	86.7b	73.3b	73.3b	6.7b	6.7b	3.3b	26.7cd	40.0cd	36.7d	16.7d	26.7c	23.3c
120	66.7a	63.3a	63.3a	0a	0a	0a	23.3c	33.3c	26.7c	6.7b	13.3b	6.7b

Compared to the standard vitrification protocol and the encapsulation-vitrification, particularly the ultra-rapid droplet freezing techniques was found to be very efficient for the cryopreservation of date palm proli-ferating meristems (Table 1). We measured the post-cryopreservation growth of explants which is an essential parameter to evaluate the morphogenetic capacity of cryopreserved plant material. The highest regeneration rates obtained with the standard vitrification, the encapsulation vitrification, and the droplet vitrification protocols reached 26.7, 40.0, and 60.0 %, respectively. Only small explants (2 mm) were able to resist cryogenic treatments. Larger explants (> 3 mm) were found to die after thawing. The importance of explant size for cryopreservation is well known. Indeed, Baek *et al.* (2003) confirmed that smaller garlic shoot apices (1.5 or 3.0 mm in diameter) displayed a higher regeneration capacity than larger ones (4.5 mm). Besides, we found that alginate was not toxic to date palm meristems and was efficient at protecting them against cryo-damages, thus confirming previous results from Daikh and Demarly (1987).

Using the standard vitrification and droplet-vitrification protocols, the optimal PVS 2 treatment was 30 min (Fig. 1*B*). When the encapsulation-vitrification protocol was used, the optimal duration of treatment increased to 60 min (Fig. 1*C*). This result can be explained by the fact that enclosed tissues need longer treatment with PVS 2 to be sufficiently dehydrated. Both sucrose preculture and cold hardening improved considerably post-thaw recovery rates (Table 1). Sucrose preculture has been used in several plant cryopreservation protocols (Ganino *et al.* 2012). Sucrose treatments increase the osmolarity of intracellular solutes, thus reducing the detrimental effect of PVS 2 and the formation of intracellular ice upon subsequent immersion of the explants in liquid nitrogen (Reinhoud *et al.* 2000).

Table 2. Effect of duration of sucrose (180 g dm^{-3}) and cold (4 °C) treatments on proline content [µg g^{-1}(f.m.)] in date palm caulogenic cultures. Experiments were replicated three times. Data followed by the same letter within the same column are not significantly different according to Duncan's test ($P < 0.05$).

Time [d]	Sucrose	Cold
0	105.3 a	96.0 a
2	390.0 b	386.0 b
5	383.3 b	395.0 b
10	378.3 b	398.3 b

Cold acclimation was found to improve post-cryopreservation recovery in several plant species such as apple (Kushnarenko *et al.* 2009) and rubus (Reed 1990). The determination of biochemical and physical changes associated with tolerance to cryopreservation is a very interesting approach to optimize cryopreservation protocols (Kaviani 2011). We studied the effect of sucrose preculture and cold hardening on proline content of explants. Both sucrose and cold acclimation treatments were found to increase proline contents in date palm tissues (Table 2). According to Pociecha *et al*. (2009), the specific accumulation of this amino acid improves resistance to both severe osmotic stress and low temperature. The impact of sucrose preculture on protein metabolism in banana meristems was studied by Carpentier *et al*. (2010) through 2-D gel electrophoresis. These authors demonstrated that preculture was able to change the expression of genes which are essential for the acquisition of freezing tolerance.

Using LS, the regeneration of explants after freezing and thawing was promoted since post-thaw regeneration rates were never higher than 15 % without LS treatment. We also observed that in our case RS treatments were not essential so they can be removed from our cryopreservation protocols.

Cryopreserved explants produced buds and organogenic callus could also be observed (Fig. 1*B,C*). After cryopreservation, plant material grew very slowly during the first five months. After this period, multiple bud cultures were successfully re-established in *RITA* bioreactors. Advantages of temporary immersion bioreactors have been underlined by Afreen (2006). Such systems provide a simple way of using liquid medium while controlling the gaseous environment of *in vitro* cultures. For date palm, similar results were obtained in our group using cv. Barhee (Fki *et al.* 2011*a*).

Whole plants have been regenerated and no phenotypic abnormalities could be observed, thus suggesting that genetic integrity might not be severely affected by the cryopreservation process under study. Sisunandar *et al*. (2010) recently explored genetic and epigenetic status of cryopreserved coconut plants and did not find any significant changes. More often, simple molecular markers are not accurate enough to detect subtle somaclonal variations (Jaligot *et al.* 2011). We are therefore focusing on the phenotypic study of in-field behaviour of palms regenerated from cryopreserved plant material in order to confirm their fidelity to selected mother palms.

Our findings show that cryopreservation is a promising tool for the successful establishment of a stable and available stock of highly proliferating tissues. In such a bank, adequate plant material is readily available for the rapid regeneration and distribution.

Many reports showed the efficiency of the vitrification technique and its two derived protocols, encapsulation-vitrification and droplet-vitrification (Sakai and Engelmann 2007). Our results demonstrated that droplet-vitrification was the most efficient strategy for the cryobanking of date palm tissues.

Genotypic effects on success rates in tissue culture as well as in germplasm cryopreservation are already well documented (Panis *et al.* 2005). Our approach, which was designed for date palm cv. Khenizi, of large scale propagation should be optimized for other elite date palm cultivars and it paves the way for the improvement of cryopreservation techniques for other *Arecaceae* species.

The successful cryopreservation of proliferating tissues can be of great interest for the development of large scale micropropagation strategies. Indeed, its availability as a safe back-up of cryopreserved germplasm enables the sequential rejuvenation of cultures under production, thus circumventing unwanted drifts linked to long term proliferation such as hormone habituation, loss of regeneration capacity, or somaclonal variation.

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