Cryopreservation of date palm meristematic cells

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ABSTRACT

Date palm (*Phoenix dactylifera* L.) is the most important perennial plant in sub-Saharan and hot regions. Genetic erosion is among the serious problems which date palm is facing. This study aimed to produce and cryopreserve meristematic cell aggregates having the capacity to generate adventitious buds or somatic embryos. A biochemical investigation was carried out to explain the utility of the sucrose preculture and the cold hardening phases in a cryopreservation protocol. MS medium supplemented with 70 g/L sucrose was effective to get meristematic cells having the capacity to generate buds or somatic embryos from in vitro tissue culture. Compared to the standard vitrification protocol, the encapsulation vitrification and particularly the ultra-rapid droplet freezing techniques proved their high efficiency for the cryopreservation of the date palm meristematic cells. Thus, the highest survival rates using these techniques were 26.7, 53.3 and 66.7 % respectively. The multiplication rates, measured after a cultivation period of 3 months, of control and cryopreserved plant material were 3 and 2 successively. Sucrose preculture and cold hardening which both could induce activation of genes coding for resistance towards osmotic stress, as observed in total soluble proteins profiles and proline content measurements, increased considerably post thaw recovery rates after vitrification.. We showed that cryopreservation does not affect the morphogenetic capacities of this plant material. Indeed, multiple bud or embryogenic suspension cultures were established. Morphological studies showed the genetic stability of clonal material following cryopreservation.

Key words: date palm, genetic resources, cryobiology, tissue culture, caulogenesis, embryogenesis, SDS PAGE, proline

INTRODUCTION

Socio-economically, one of the most important perennial plant in sub-Saharan and hot regions is date palm (*Phoenix dactylifera* L.). This is why extensive efforts have been undertaken by the scientific community to overcome constraints hampering the extension of date palm plantations (El Hadrami and El Hadrami 2009). Biotechnological tools are effective to propagate, improve and preserve plant genetic resources (Pati et al. 2006; Parveez et al. 2000; Engelmann 2004; Panis 2008). In case of date palm, biotechnologies have already been fully employed for large scale propagation (Fki et al. 2003; Fki et al. 2010). Nevertheless, biotechnological approaches for date palm improvement and preservation still need more investigations. This study aims to produce and cryopreserve meristematic cells having the capacity to generate true-to-type in vitro date palms. A biochemical study was carried out to explain the benefits of the sucrose preculture and the cold hardening phases in a cryopreservation protocol.

MATERIALS AND METHODS

Meristematic cell aggregates were initiated from date palm in vitro chlorophyll-free leaves using MS medium supplemented with 70 g/L sucrose (Murashige and Skoog 1962). Embryogenic suspensions were established from calli (0.5 g) and maintained on a rotary shaker at 100 rpm. For shoot multiplication, RITA bioreactors (Alvard et al. 1993) for the temporary immersion of cultures in liquid medium were used.

The RITA vessel 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 ml of MS medium supplemented with 70 g/L 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 28 °C under a 16h photoperiod (photon flux: 30 µEm-2 s-1).

Prior to cryopreservation, explants (< 3 mm) bearing meristematic cell aggregates were cultured on MS medium enriched with 180 g l-1 sucrose or incubated at 4 °C for 2, 5 and 10 days. For cryopreservation, the standard vitrification, the encapsulation vitrification and the droplet vitrification protocols have been applied (Panis et al. 2005). Three different cryopreservation protocols were assessed in the present study, namely: standard (tube) vitrification, droplet-vitrification and encapsulation-vitrification. Three replicates of ten samples were used for each experiment.

In the first two protocols (standard vitrification and droplet-vitrification), explants were transferred into 15 cm3 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 % (w/v) sodium alginate dissolved in MS medium, with 7 % (w/v) sucrose and no CaCl2; then they were sucked up with a micropipette and gently dropped into 75 mM CaCl2, 2 H2O 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 PVS2 solution (Sakai et al. 1990). This solution consisted of 30% (3.26 M) glycerol, 15% (2.42 M) ethylene glycol (EG) and 15% (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 for 15, 30, 60 or 120 min at 0 °C.

Explants were transferred into 2 ml cryotubes containing 0.5 ml 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 ml cryotubes filled with liquid nitrogen then closed.

For encapsulated explants, alginate beads were transferred into 2 ml cryotubes filled with 0.5 ml PVS2 solution then plunged in liquid nitrogen (encapsulation-vitrification protocol).

After one hour of LN storage, strips of aluminium foil were transferred to recovery solution (RS) containing 1.2 M sucrose dissolved in MS medium for 15 min at room temperature (25 °C). Cryotubes containing the meristems or alginate beads were thawed in a water bath at 40 °C for 2 min 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/L sucrose and then incubated in the dark. After 2 days, tissues were transferred onto MS medium containing 50 g/L sucrose and 0.1 mg/L 2,4-D. Survival rates were estimated using growth measurement at 4-6 weeks after thawing.

For histological examinations, explants were fixed in Svaloff Navashine solution (chromic acid 0.5 %, glacial acetic acid 5%, formaldehyde 15% and ethanol 5 %), then gradually dehydrated using ethanol solutions (50 to 100%) and finally embedded in paraffin. Serial sections (10 µm) were cut with a rotary microtome and stained with acetohematoxylin (Sass 1958).

For protein extraction, samples (0.5 g FW) were ground in liquid nitrogen then homogenized in 1 cm3 of maleate/Tris buffer 50 mM (pH 8.3) containing 2 % SDS, 0.5 mM EDTA, 2 mM PMSF, 1 mM DTT and 2 mM β-mercaptoethanol. Homogenates were centrifuged at 13,000 g for 15 min at 4 °C. Total soluble protein content of the supernatant was estimated according to Bradford (1976). For SDS-PAGE protein electrophoresis, samples (10 µg per lane) were loaded onto 12 % SDS gels and stained with Coomassie Brilliant Blue-R250 (Stone and Gifford 1997).

Proline content was estimated according to Bates et al. (1973) on 1 gFW of leaf tissue using 6 ml 3% sulfosalicylic acid. Two cm3 of the extract were placed for 1 h in boiling water with 2 cm3 ninhydrin and 2 cm3 glacial acetic acid and then cold toluene (4 ml) was added. Extracts were then filtered through a Whatman paper filter. Proline content was estimated spectrophotometrically at 520 nm and calculated as µmol/g against standard L-proline (Sigma-Germany P-0380).

Statistical analyses of data were performed using one-way ANOVA and Duncan's test. *P* values < 0.05 were considered as statistically significant. Statistical analysis was computed using SPSS 13 software. Experiments were replicated three times. Data expressed in percentage were transformed by arcsin transformation and then analyzed. Arcsin transformation (y' = arcsin y ½, y = original percentage/100) was undertaken in order to stabilize the variance of data.

RESULTS

Murashige and Skoog medium supplemented with 70 g/L sucrose was effective to generate date palm meristematic cells from in vitro tissue culture. Hypertrophied

chlorophyll-free leaves showed a high morphogentic capacity as they produced number of meristematic cell aggregates after only 3 months (Fig. 1).

Compared to the standard vitrification protocol, the encapsulation vitrification and particularly the ultra-rapid droplet freezing techniques proved their high efficiency for the cryopreservation of the date palm meristematic cells. Thus, the highest regeneration rates using these techniques were 26.7, 53.3 and 66.7 %, respectively (Table 1). Sucrose preculture and cold hardening both improved considerably post thaw recovery rates after vitrification. Both treatments were found to increase proline contents (Table 2) and to change the expression of 15 and 18 kDa proteins (Fig. 2). Besides, a newly expressed 21 kDa protein was detected only after cold hardening (Fig. 2). We also showed that cryopreservation does not affect the morphogenetic capacities of this plant material. Indeed, cryopreserved meristematic cells could produce proembryos or adventitious buds. Furthermore, multiple bud cultures and embryogenic suspension cultures were established employing temporary immersion system (TIS) and agitated liquid media, respectively. With respect to the effect of the cryogenic treatments on the genetic integrity, no morphological differences were observed between plants regenerated from non-cryopreserved controls and cryopreserved meristematic cells. All the plants showed a similar growth rate in the greenhouse (0.5 ± 0.2 cm in length per month), leaf colour and morphology. These observations are encouraging as regards genetic stability of cryopreserved material.

DISCUSSION

In this paper, we showed that cryopreservation of meristematic cells is a promising tool to establish date palm cryobanks. *In vitro* generated chlorophyll-free leaves were found to be a choice material to get meristematic cells. Enhancing sucrose concentration in the medium was sufficient for cell dedifferentiation. Our previous studies showed that PGRs such as 2,4-D were essential for cells dedifferentiation within primary explants tissue and that the culture period required to observe neoformations was much longer, especially when low concentrations of 2,4-D were used (Drira, 1983; 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. Fki et al. (2011b) confirmed that high level of PGRs was the cause of somaclonal variation in date palm.

We proved the benefits of the sucrose preculture and the cold hardening on post-thaw regeneration. Both treatments seem to be effective to activate genes coding for resistance towards severe osmotic stress and ultra-low temperature. Basic knowledge about cryoprotection is improving fast: indeed, the determination of physical and biochemical changes associated with tolerance to cryopreservation is a very interesting approach to optimize cryopreservation protocols (Kaviani 2011). We carried out such a biochemical study in order to assess the effect of sucrose preculture and cold hardening on the total soluble protein profiles and proline content of explants. Helliot et al. (2003) monitored ultra-structural changes occurring during the cryopreservation of banana apical meristems. Moreover, differential scanning calorimetry (DSC) was used to discover the principal thermal events connected with plant cryopreservation procedures (Nadarajan et al. 2008, Sisunandar et al. 2010). 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 modulate the expression of genes which are essential for the acquisition of freezing tolerance. On the other hand, Zhu et al. (2006) demonstrated that sucrose pretreatment induced changes in sugar, sterol and fatty acid composition in banana meristems.

Many reports showed the efficiency of the vitrification technique and its two derived protocols, encapsulation-vitrification and droplet-vitrification (see Sakai and Engelmann 2007, for a review). In this study, we concluded that droplet vitrification remains the best way for date palm germplasm cryobanking.

Acknowledgment

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Tables

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

Regeneration (%)												
PVS2 exposure at 0 °C (min)	-LN -PC -CH	-LN +PC	-LN +CH	+LN v -PC -CH	+LN v +PC	+LN v +CH	+LN ev -PC -CH	+LN ev +PC	+LN ev +CH	+LN dv -PC -CH	+LN dv +PC	+LN dv +CH
0	96.7a	93.3a	96.7a	0a	0a	0 a	0a	0a	0a	0a	0a	0a
15	93.3a	96.7a	93.3a	6.7b	16.7c	6.7b	0a	0a	0a	13.3c	46.7d	36.7d
30	93.3a	96.7a	93.3a	13.3c	26.7d	23.3d	13.3b	23.3b	16.7b	36.7e	66.7e	53.3e
60	93.3a	93.3a	93.3a	6.7b	6.7b	6.7b	26.7cd	53.3d	46.7cd	16.7d	26.7c	23.3c
120	96.7a	93.3a	93.3a	0a	0a	0a	23.3c	33.3c	43.3c	6.7b	13.3b	6.7b

Table 2: Effect of sucrose (180 g/L) and cold (4 °C) treatments on proline content in date palm leaf tissue bearing meristematic cells. Experiments were replicated three times. Data followed by the same letter within the same column are not significantly different according to Duncan's test ($P < 0.05$)

Duration of the treatment (days)	Proline content (µg proline per g FW)	
	Sucrose (180 g/L) Treatment	Cold (4 °C) Treatment
0	99,3 a	99 a
2	370 b	376 b
5	373,3 b	365 b
10	368,3 b	378,3 b

Figures

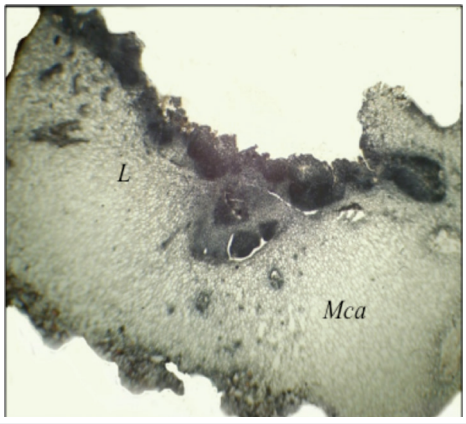

Fig. 1. Cross section showing meristematic cell aggregates within *in vitro* date palm chlorophyll-free leaf. *Mca* meristematic cell agregate, *L* hypertrophied chlorophyll-free leaf. *Scale bar* 1 mm

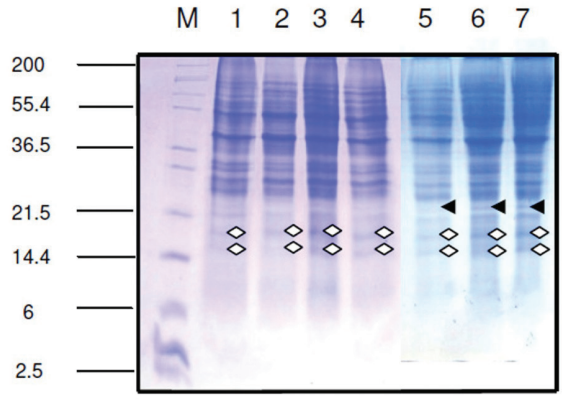

Fig. 2. Effects of sucrose preculture and cold hardening on the total soluble proteins profiles of the highly proliferating meristems. M: marker; Lane 1: control; Lane 2: 2days 180 g/L sucrose; Lane 3: 5 days 180 g/L sucrose; Lane 4: 10 days 180 g/L sucrose; Lane 5: 2 days 4°C; Lane 6: 5 days 4°C; Lane 7: 10 days 4 °C