INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20–0616/202x/00–0–000–000
DOI: 10.17957/IJAB/15.1619
http://www.fspublishers.org

Full Length Article

Cryopreservation of Embryogenic Callus of Date Palm (*Phoenix dactylifera*) cv. Magdoul through Encapsulation-Dehydration Technology

Ehab M.R. Metwali[1,2*†], Naif M.S. Kadasa[1†], Hemaid I.A. Soliman[3,4], Omar A. Almaghrabi[1] and Michael P. Fuller[5]
[1]*Department of Biology, College of Science, University of Jeddah, Jeddah, Saudi Arabia*
[2]*Genetic Branch, Department of Botany, Faculty of Agriculture, Suez Canal University, Ismailia, Egypt*
[3]*Plant Genetic Resources Department, Desert Research Center, El-Matariya, Cairo 11753, Egypt*
[4]*Tissue Culture and Biotechnology Labs., Maryout Research Station, Desert Research Center, Alexandria, Egypt*
[5]*School of Biological and Marine Sciences, Faculty of Science and Engineering, University of Plymouth, Plymouth, UK*
[*]Correspondence author: egharib@uj.edu.sa; ehabmetwali@hotmail.com
[†]Contributed equally to this work and are co-first authors
Received 16 April 2020; Accepted 22 August 2020; Published ____________

Corrected Proof, In Press

Abstract

To overcome the genetic deterioration and the extinction of date palm genotypes and species as result of environmental challenges, it has become necessary to develop techniques that allow the remaining genetic resources to be persevered under *in-vitro* conditions for long period without a substantial decline in the vitality, genetic stability and low survival. The development of an effective cryopreservation method for date palm (cv. Magdoul) *via* an encapsulation-dehydration method for long term conservation was researched in this study. Embryogenic callus, obtained from shoot tip culture, was used as explants and exposed to different concentration of sucrose (0.0, 0.5, 0.75, 1.0 and 1.5 *M*) combined with dehydration time (0–10 days) and different drying time (1–10 h). It was found that using sucrose at 0.75 *M* was more effective compared with using 1.0 and 1.5 M. Among the drying time tested, 4 h gave the best result for survival. The interaction treatment between sucrose, dehydration and moisture content (MC) was studies. After different periods of time in liquid nitrogen, the greatest values of survival (74.4%) and regrowth (71.25%) after 6 weeks of storage were obtained when 0.75 *M* sucrose for three days followed by 4 h dehydration period with a 39.50% MC was applied. The present results indicated that encapsulation-dehydration can be applied as a simple and effective protocol to a diverse range of cv. Magdoul genetic resources using embryogenic calli. © 2020 Friends Science Publishers

Keywords: Cryopreservation; Encapsulation; Germplasm; *Phoenix dactylifera* L.; Tissue culture

Introduction

The date palm (*Phoenix dactylifera* L.) is considered as one of the earliest cultivated fruit trees and is now cultivated across the world. Date palm has a high nutritional value and it is a staple food source especially for many inhabitants of the Arabian Gulf include Saudi Arabia. The quality of date palm varietal clones fruits varies and as a consequence price varies according to the variety. Many producing countries, including Saudi Arabia, are keen to keep distinct variety homogenetiy and avoid clonal deterioration and variety extinction (El-Juhany 2010). The number of palm trees around the world has reached about 150 million trees (Al-Khayri *et al.* 2018) but is subject an annual decline due to environmental conditions including desertification and salinization and biotic stress from insects and pathogens. Such factors are a major constraint on the conservation of date germplasm and consequently lead to a threat to locally maintained germplasm (Khan *et al*. 2012). Date palm is a dioecious fruit tree that is vegetatively propagated through offshoots, and its germplasm cannot be stored or handled easily using conventional means. Furthermore a deterioration in the productivity of date palm plants due to the use of traditional methods of propagation has been reported (Rajmohan 2011). It is inevitable that with the loss of date palm cultivars, the genetic diversity will decrease and threaten the future productivity.

In order to limit the loss of genetic diversity in many crops, plant breeders have resorted to preserving germplasm either in seed-banks or tissue culture clone banks as a source of diversity for use in plant breeding programes. *In vitro* methods to propagate the date palm have been developed which may also be employed to preserve germplasm (Taha *et al.* 2003). By combining the benefits of the vegetative propagation system with the capability of long-term storage, synthetic seeds have been created which have great potential in date palm agriculture (Bekheet *et al*. 2002). Conservation, diversity and ecological restoration are terms that have had

relevance in recent times (Duarte *et al.* 2018). For date palm *ex situ* conservation of genetic material under field conditions suffers from such several disadvantages including extensive labour cost andl the risk of losses due to pathological and environmental threats (Shatnawi 2013). Preserving germplasm as seeds through seed banks is normally the first resort for the *in situ* conservation for many plants (Bonner 1990; Towill 2005) but is nt very applicable to Date palm. Although conventional methods of storing seeds is an inexpensive method for moste plants for conserving germplasm, but in the case of palm there are several drawbacks to using this method. Firstly there is decline in vitality of seeds with increasing preservation period and most importantly seeds are very heterozygous and therefore, not suitable for maintaing true-to-type palm genotypes (Bekheet *et al.* 2007). As a result, those working in this field are beginning to use modern technology methods, such as cryopreservation of clonal material for the preservation the germplasm of palm trees and Soliman (2013) indicated that crystorage is probably the priniciple long-term *in vitro* conservation method to be used for such biological materials.

Cryopreservation of different plant explants has been used for storage of genetic material under low temperature (−196°C) for long periods (Panis and Lambardi 2006). The major advantages of this technique are: 1) it is safe, 2) cells retain their viability and vitality, 3) cryopreserved materials remain genetically stable, 4) metabolic process and biological deterioration are considerably slowed or even halted, 5) the process facilitates the exchange of germplasm between countries and regions and 6) it helps in the reduction or eradication of viruses from plant tissues (Kohmura *et al.* 1992; Feng *et al.* 2011). Cryopreservation of plant materials requires optimization of the such as: 1) size of explant, 2) concentration of cryoprotective amendments, 3) sample water content, 4) rate of freezing, 5) rate of thawing, 6) fitness of the explants, 7) materials, 8) culture conditions, 9) nature of liquid nitrogen solidifying agents, 10) dehydration processes and 11) cooling rate (Alansi *et al.* 2019). Therefore, establishment of a successful protocol for cryopresrvation can be a difficult and laborious process that requires the manipulation of all these factors to achieve success of the cryopresrvation technique. There are relatively few accounts of research in the literature on germplasm conservation of date palm somatic embryos by *in vitro* encapsulation/dehydration. The current work describes the results of a study implemented to develop an effective and simple protocol with maximum viability for long term *in vitro* conservation of date palm via the cryopreservation of somatic embryos.

Material and Methods

Plant material preparation and disinfection: Vegetative offshoots of mother trees of Saudi Arabian date palm (*Phoenix dactylifera* L.) cv. Magdoul was collected, washed with distilled water (DW) and shoot tip explants (8–10 cm in length) were exposed and excised using a scalpel. Shoot tips was surface sterilized using the following procedure: 1) Shoot tips were immersed in freshly prepared 50% Clorox (NaOCl at 5.25%) containing 2 drops of Tween-20 for 30 min followed by washing three times with sterilized distilled water (SDW), 2) then immersed in 0.2% $HgCl_2$ solution for 5 minutes and rinsed three times with SDW, 3) each shoot tip explant was then divided into 4 sections in preparation for culture on callus induction medium. All steps of the disinfection procedure were performed in a Laminar Air Flow "Hood" and aseptic procedures were applied according to Soliman *et al.* (2010)

Somatic embryogenesis induction: Shoot tip section explants, that were prepared as mentioned above, were cultured on callus induction medium (M1) including : 1) 4.4 mg L^{-1} MS (Murashige and Skoog 1962) 2) 170 mg L^{-1} Na H_2PO_4, 3) 125 mg L^{-1} myo-inositol, 4) 200 mg L^{-1} glutamine, 5) 100 mg L^{-1} ascorbic acid, 6) 100 mg L^{-1} citric acid, 7) 5.0 mg L^{-1} thiamine-HCl, 8) 1.0 mg L^{-1} nicotinic acid, 9) 1.0 mg L^{-1} pyridoxine-HCl, 10) 30 mg L^{-1} sucrose, and 11) 2.0 g L^{-1} gelrite, supplemented with the growth regulator hormones (GRHs) 10.0 mg L^{-1} 2,4-di-chloro-phenoxy-acetic acid (2,4-D), 8.0 mg L^{-1} 2–isopentenyle adenine (2iP) and 2.5 g L^{-1} activated charcoal according to (Aldhebiani *et al.* 2018) with some modification. The pH of the medium was adjusted to pH 5.6 before the addition of 2.5% phytagel and then sterilized by autoclaving for 15 minutes at 121°C. To stimulate induction and growth of callus, the cultures were incubated in total darkness in a growth room at 25 ± 2°C for 12 months.

To induce somatic embryogenesis, healthy callus (100–200 mg fresh weight) obtained from the previous stage was cultured on MS basal medium (MS2) containing the GRHs 3.0 mg L^{-1} Naphthalene Acetic Acid (NAA), 6.0 mg L^{-1} 2iP and 2.5 g L^{-1} activated charcoal. The culture jars were placed in a growth room for 8–10 weeks at 25 ± 2°C under cool white fluorescent lamps with an intensity 75 μmol m^{-2} s^{-1} and 16 h photoperiod. Sub-culturing of embryogenic callus formation was performed once every five weeks to generate sufficient embryogenic callus stocks for cryopreservation experiment.

Preparation of embryogenic callus for cryopreservation: Embryogenic callus was placed vertically on initiation medium (M3) consisted of MS medium containing the GRHs 2.0 mg L^{-1} NAA, 3.0 mg L^{-1} 2iP and 2.5 g L^{-1} activated charcoal, the pH of the medium was adjusted to 5.6 before the addition of 2.5% phytagel. Cultures were incubated for two weeks at 25°C and 16h photoperiod, then transferred to the same initiation medium (M3) but without any other supplement of GHRs and only containing 0.5 *M* sucrose for one week. Embryogenic callus was excised and used as explants for encapsulation.

Corrected Proof: In Press

Encapsulation and dehydration: Embryogenic callus was suspended in a calcium-free liquid MS medium with 3% (w/v) Na-alginate according to (Bose *et al.* 2017). Drops of the same alginate solution with contained embryogenic calli were dispensed as drops into 0.1 *M* calcium chloride. Encapsulated embryogenic calli were precultured in MS medium with different concentration of sucrose (0.0, 0.5, 0.75, 1.0 and 1.5 *M*) free from growth regulators and incubated at 25 ± 2°C under cool white fluorescent lamps with an intensity 75 μmol m^{-2} s^{-1} with a 16 h photoperiod for 24 hours following by incubation at 4°C in the dark for 24 h.

Dehydration of preculture encapsulated embryogenic callus: To measure the optimum drying time, the encapsulated embryogenic calli were placed on sterilized filter paper and exposed to continuous air in a laminar flow cabinet for periods ranging between 0.0 to 10 h at 25 ± 2°C. Samples were weighed every 2 h to obtain the dessication curves for each osmoticum treatment. Samples were also taken and tested for their liquid nitrogen tolerance where beads were placed in 2.0 mL sterile cryovials and directly rinsed into liquid nitrogen. Percentage of beads moisture content (MC%) were calculated using following equation: MC% = 100*[(Fresh weight - Dry weight (/ Fresh weight]. Also, Liquid nitrogen tolerance for each drying time at 2 h intervals was tested.

Freezing progress: Beads containing embryogenic calli were incubated in darkness at 4°C for one day and then transferred to liquid nitrogen (at -196°C) directly and storage for different periods (1, 2, 3, 4, 5 and 6 weeks).

Thawing and regrowth after cryopreservation: Beads following cryopreservation and non-cryopreserved controls, were transferred to fresh medium, as mentioned below, for assessment of recovery growth. Cryopreserved samples were taken after 1, 2, 3, 4, 5 and 6 weeks from cryopreservation to assess the effect of different preservation periods on vitality or survival of embryogenic callus. Cryopreserved vials containing beads with embryo calli were rapidly thawed at 37°C for 3 minutes using a water bath, then beads were removed and washed with liquid MS medium. Encapsulated non-cryopreserved (-LN) and encapsulated cryopreserved (+LN) beads were cultured on MS2 as described above for somatic embryogenesis germination and then transferred to MS medium (MS4) containing the GRHs 6.0 mg L^{-1} 2iP, 2.0 mg L^{-1} kinetin (Kin), 1.0 mg L^{-1} Indole Butyric Acid (IBA) and 2.5 g L^{-1} activated charcoal, and incubated in the dark at 25°C in the growth room for 3 days and then exposed to light with a 16 h photoperiod. Survival rates were recorded after 35 days and cultures were kept for observation of recovery growth and development of plantlets.

Statistical analysis

The experiments were designed in a completely randomized design (CRD). Analysis of variance (ANOVA) and the calculation of LSD or Duncan's Mulitple Range test (0.05) was undertaken using SAS, 2000 software programme, All values were reported as means ± standard error according to Snedecor and Cochran (1989).

Results

Effect of preculture treatments

Callus and somatic embryogenesis were successfully initiated on MS basal medium M1 (Fig. 1a) and M2 (Fig. 1b), supplemented with GRHs 10.0 mg L^{-1} 2,4-D, 8.0 mg L^{-1} 2iP ; 3.0 mg L^{-1} NAA , 6.0 mg L^{-1} 2iP , repectively. Then embryogenic callus was cultured on initiation medium (M3) consisting of MS medium along with GRHs 2.0 mg L^{-1} NAA and 3.0 mg L^{-1} 2iP, in order to improve the quality of the callus to obtain friable call a key step for encapsulated embryogenic callus.

The survival rate of encapsulated embryogenic callus after pre-growth varied depending on the sucrose content in the preculture medium and the duration of the pre-culture. The results indicated that high survival rates (98.2, 91.4 and 78.5%) were obtained after pre-culture of the embryogenic callus with 1.0 and 1.5 *M* sucrose at 1, 2 and 1 day, respectively. The highest survival rates (100%) of the embryogenic callus was observed when encapsulated embryogenic callI were pre-grown for 3 and 4 d in media with a sucrose concentration of 0.75 and 0.5 *M*, respectively (Table 1). The addition of 0.75, 1.0 and 1.5 M sucrose after 9, 7 and 4 days to the pre-culture medium inhibited the survival rates. However, low survival rate (12.7, 14.5 and 17.5%) was obtained at 0.75, 1.0 and 1.5 *M* sucrose after 8, 6 and 3 days, respectively. Based on our the results, the lowest concentration tested *i.e.*, 0.75 *M* sucrose for 3 days was considered to be optimal for pre-culture of encapsulated-dehydrated embryogenic calli of date palm cv. Magdoul.

Encapsulation/dehydration in sterile airflow

The modified encapsulation-dehydration method was studied to determine the optimial dehydration time. There was a significant impact of sucrose concentration and dehydration duration on the survival and regrowth of cryopreserved and non-cryopreserved embryogenic calli (Table 2). Minium survival (28.15%) and regrowth (10.08%) for encapsulated cryopreserved (+LN) calli occurred only when calli were suspended in 1.0 M sucrose for 3 days with 10 h dehydration time in a sterile airflow, where the beads attained 13.05% MC. In contrast, pre-treated with 0.5 and 0.75 *M* sucrose for 4 and 3 days followed by 0–4 and 0–2 h dehydration period recorded the greatest survival (100%) of encapsulated non-cryopreserved (-LN) embryogenic calli where the beads attained between 66.18–81.50% and 45.00–78.35% MC, respectively (Table

Proof. In Press

Table 1: Effect of preculture duration and sucrose concentration on survival percentage for encapsulated non-cryopreserved (-LN) embryogenic calli of date palm (*Phoenix dactylifera* L.) cv. Magdoul

Preculture duration (days)	Sucrose concentration (*M*)			
	0.5	0.75	1.0	1.5
0	100.0 ± 0.45^{a}	100.0 ± 0.00^{a}	100.0 ± 0.00^{a}	100.0 ± 0.00^{a}
1	100.0 ± 0.33^{a}	100.0 ± 0.00^{a}	98.24 ± 0.28^{b}	78.50 ± 0.00^{b}
2	100.0 ± 0.28^{a}	100.0 ± 0.00^{a}	91.42 ± 0.35^{c}	45.82 ± 0.00^{c}
3	100.0 ± 0.15^{a}	100.0 ± 0.00^{a}	82.50 ± 0.55^{d}	17.58 ± 0.00^{d}
4	100.0 ± 0.44^{a}	91.54 ± 0.14^{b}	65.25 ± 0.14^{e}	00.00 ± 0.00^{e}
5	96.45 ± 0.29^{b}	78.26 ± 0.23^{c}	38.72 ± 0.27^{f}	00.00 ± 0.00^{e}
6	92.82 ± 0.35^{c}	55.82 ± 0.45^{d}	14.51 ± 0.31^{g}	00.00 ± 0.00^{e}
7	88.51 ± 0.66^{d}	29.58 ± 0.56^{e}	00.00 ± 0.00^{h}	00.00 ± 0.00^{e}
8	72.48 ± 0.23^{e}	12.70 ± 0.18^{f}	00.00 ± 0.00^{h}	00.00 ± 0.00^{e}
9	69.52 ± 0.41^{f}	00.0 ± 0.00^{g}	00.00 ± 0.00^{h}	00.00 ± 0.00^{e}
10	65.25 ± 0.22^{g}	00.0 ± 0.00^{g}	00.00 ± 0.00^{h}	00.00 ± 0.00^{e}

Values are means ± standard error of three replicates. For each cultivar, bars with the same letters are not significantly different at $P \leq 0.05$ level

Table 2: Effect of sucrose concentration and dehydration duration on survival and regrowth percentages for encapsulated non-cryopreserved (-LN) and cryopreserved (+LN) embryogenic calli of date palm (*Phoenix dactylifera* L.) cv. Magdoul

Sucrose concentration (*M*)	Dehydration duration (h)	Survival%		Regrowth%		Moisture content (%)
		-LN	+LN	-LN	+LN	
0.5	0	100.0 ±0.45^{a}	15.35±0.22^{k}	100.0±0.43^{a}	13.25±0.36^{k}	78.50±0.25^{a}
	2	100.0 ±0.35^{a}	28.25±0.18^{j}	100.0±0.52^{a}	25.38±0.42^{i}	62.45±0.35^{c}
	4	100.0 ± 0.18^{a}	37.22 ± 0.26^{i}	97.35 ± 0.18^{b}	31.45 ± 0.21^{h}	56.18 ± 0.18^{d}
	6	94.35 ± 0.28^{c}	48.45 ± 0.33^{h}	92.25 ± 0.32^{c}	37.95 ± 0.34^{g}	49.25 ± 0.55^{e}
	8	91.40 ± 0.39^{e}	61.29 ± 0.19^{f}	88.75 ± 0.72^{d}	60.25 ± 0.46^{e}	45.55 ± 0.38^{f}
	10	84.29 ± 0.22^{h}	78.77 ±0.42^{b}	83.30 ± 0.22^{f}	71.85 ± 0.29^{c}	38.55 ± 0.25^{g}
0.75	0	100.0 ± 0.15^{a}	17.15 ± 0.51^{k}	100.0 ± 0.31^{a}	16.75 ± 0.37^{j}	69.35 ± 0.44^{b}
	2	100.0 ± 0.44^{a}	62.24 ± 0.28^{e}	97.18 ± 0.29^{b}	58.62 ± 0.20^{f}	45.05 ± 0.69^{e}
	4	97.37 ± 0.26^{b}	83.25 ± 0.35^{a}	96.85 ± 0.52^{b}	79.35 ± 0.25^{a}	39.50 ± 0.22^{g}
	6	90.18 ± 0.62^{e}	79.88 ± 0.26^{b}	89.50 ± 0.43^{d}	73.85 ± 0.15^{b}	37.25 ± 0.15^{g}
	8	86.25 ± 0.52^{f}	75.35 ± 0.42^{c}	85.37 ± 0.45^{e}	65.35 ± 0.30^{d}	33.75 ± 0.36^{h}
	10	71.45 ± 0.39^{j}	69.63 ± 0.55^{d}	69.50 ± 0.22^{h}	61.75 ± 0.48^{e}	31.50 ± 0.28^{h}
1.0	0	93.55 ± 0.41^{d}	22.92 ± 0.32^{j}	93.25 ± 0.65^{c}	17.15 ± 0.25^{j}	63.90 ± 0.65^{c}
	2	88.23 ± 0.33^{g}	49.38 ± 0.41^{h}	87.55 ± 0.39^{d}	38.95 ± 0.31^{g}	33.00 ± 0.37^{h}
	4	84.35 ± 0.25^{h}	55.62 ± 0.72^{g}	82.15 ± 0.52^{f}	52.28 ± 0.44^{f}	30.80 ± 0.42^{i}
	6	76.50 ± 0.14^{i}	32.95 ± 0.63^{i}	75.35 ± 0.77^{g}	28.50 ± 0.29^{i}	25.20 ± 0.19^{j}
	8	55.48 ± 0.26^{k}	19.44 ± 0.40^{k}	48.65 ± 0.15^{i}	00.00 ± 0.00^{l}	18.25 ± 0.24^{k}
	10	28.15 ± 0.35^{l}	00.00 ± 0.00^{l}	10.08 ± 0.32^{j}	00.00 ± 0.00^{l}	13.05 ± 0.32^{l}

Values are means ± standard error of three replicates. For each cultivar, bars with the same letters are not significantly different at $P \leq 0.05$ level
M Molar; **h** Hours; **LN** Liquid Nitrogen; **%** percentage

2 and Fig. 2). While, the highest regrowth percentage (100–97.18%) of encapsulated non-cryopreserved (-LN) embryogenic calli were obtained with 0.5 *M* for 4d and 0.75 *M* sucrose for 3d followed by 2 h dehydration period, where the beads attained 45.05–76.45% MC. Treating encapsulated cryopreserved embryogenic calli with LN gave significantly lower values compared with that of control non-cryopreserved treatment (-LN) (Table 2). The greatest survival (83.25%) and regrowth (79.35%) was obtained when encapsulated cryopreserved (+LN) embryogenic calli were pretreated with 0.75 *M* sucrose for 3 d followed by 4 h dehydration period with 39.50% bead MC.

Survival of desiccated encapsulated embryogenic calli decreased in line with decreasing bead MC after cryopreservation (Fig. 3). When embryogenic calli had a MC between 31.50 and 45.55% they retained high viability whilst a reduction of MC content of 18.25% or less led to embryogenic calli death. Our results also, suggested that, pre-culture encapsulated embryogenic calli in medium supplemented with 0.75 M sucrose for 3 d and dehydrated to 39.50% MC followed with exposure to LN was the good optimized treatment and led to excellent regrowth (Fig. 4).

Corrected Proof, In Press

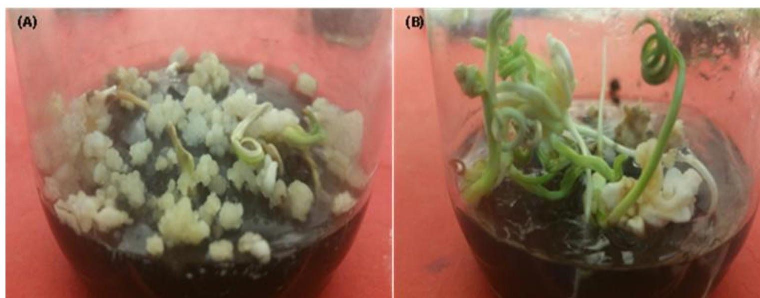

Fig. 1: Friable embryogenic callus induction, using MS3 containing 2.0 mg L^{-1} NAA, 3.0 mg L^{-1} 2iP and 2.5 g L^{-1} activated charcoalto use it as an explants source for encapsulation **(A).** Somatic embryogenesis formation and plantlet obtained on MS2 supplemented with 3.0 mg L^{-1}NAA, 6.0 mg L^{-1} 2iP and 2.5 g L^{-1} activated charcoal after 8-10 weeks **(B)**

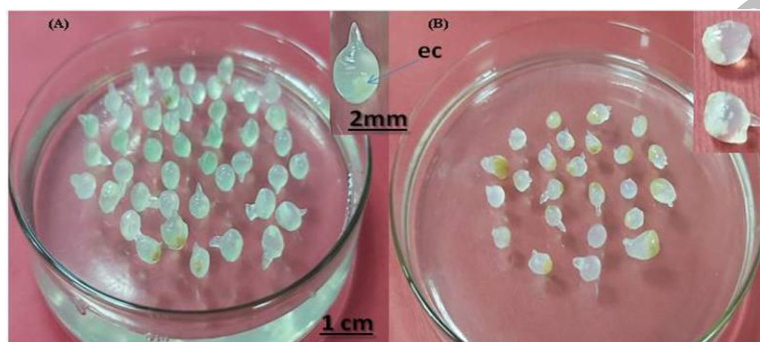

Fig. 2: Encapsulated embryogenic calli of date palm (*Phoenix dactylifera* L.) cv. Magdoul; Calcium alginate beads formed by encapsulation of explants using 3% sodium alginate. Encapsulated cryopreserved embryogenic calli were pretreated with 0.75 *M* sucrose for 3 d followed by (**A**) 4 h dehydration period and (**B**) 2 h dehydration period. ec = embryogenic callus

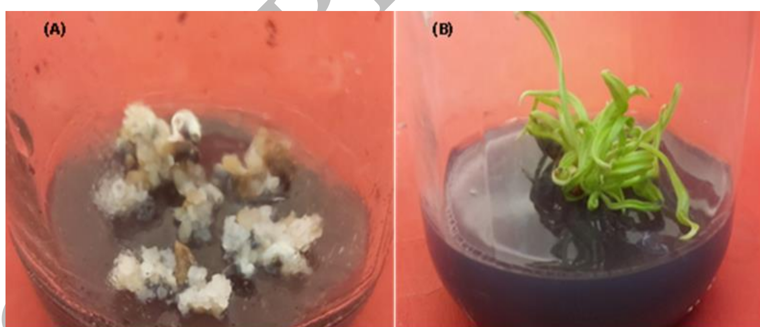

Fig. 3: Somatic embryogenesis formation recovery from embryogenic calli of date palm (*Phoenix dactylifera* L.) cv. Magdoul obtained on MS medium supplemented with 3.0 mg L^{-1} NAA, 6.0 mg L^{-1} 2iP and and 2.5 g L^{-1} activated charcoal after eight weeks (**A**). Plantlet development was obtained on 6.0 mg L^{-1} 2iP, 2.0 mg L^{-1} kin and 1.0 mg L^{-1} IBA and 2.5 g L^{-1} activated charcoal after 35 days (**B**)

Growth recovery after cryopreservation

The recovery and subsequent growth of cryopreserved embryogenic calli occurred directly without the process of callus multiplication. This allows us to assume that most of the cells of the callus region were largely undamaged during the process of cryopreservation in LN. The encapsulated cryopreserved embryogenic calli of each pre-treatment were re-cultured on MS2 medium containing 3.0 mg L^{-1} NAA, 6.0 mg L^{-1} 2iP and 2.5 g L^{-1} activated charcoal for the somatic embryogenesis development (Fig. 5) and then the somatic embryos were cultured on MS which contained 6.0 mg L^{-1} 2iP, 2.0 mg L^{-1} Kin and 1.0 mg L^{-1} IBA and 2.5 g L^{-1} activated charcoal for plantlet development (Fig. 5). The greatest survival percentage (76.35%) and regrowth (72.9%) of the encapsulated cryopreserved embryogenic calli was obtained following the combination of pre-culture in MS medium containing 0.75 *M* sucrose for 3 d with air dehydration for 4 hours after one week in LN storage. While, the survival percentage (74.4%) and regrowth (71.25%) of encapsulated cryopreserved embryogenic calli were obtained with MS medium plus 0.75 *M*

Corrected Proof Press

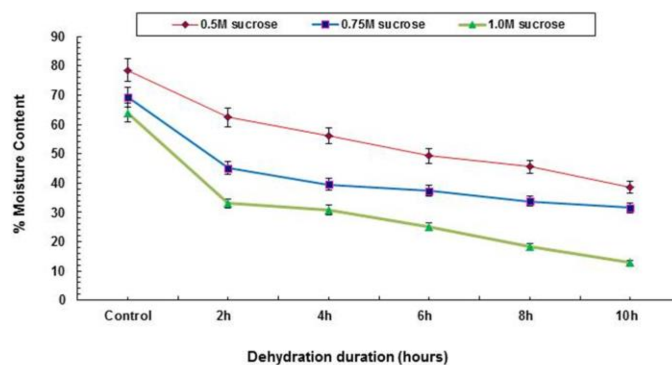

Fig. 4: Effect of dehydration duration on moisture content after preculture treatments of cryopreserved (+LN) embryogenic calli of date palm (*Phoenix dactylifera* L.) cv. Magdoul. Vertical bars indicate ± SE for means

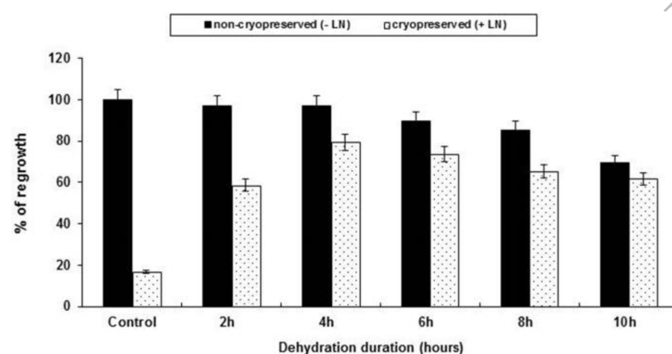

Fig. 5: Percentage of regrowth of encapsulated non-cryoprserved (-LN) and cryopreserved (+LN) embryogenic calli of date palm (*Phoenix dactylifera* L.) cv. Magdoul with 0.75 *M* sucrose for 3 d, following encapsulation in alginate beads, air drying for 0 to 10 h and exposure in liquid nitrogen. Vertical bars indicate ± SE for means

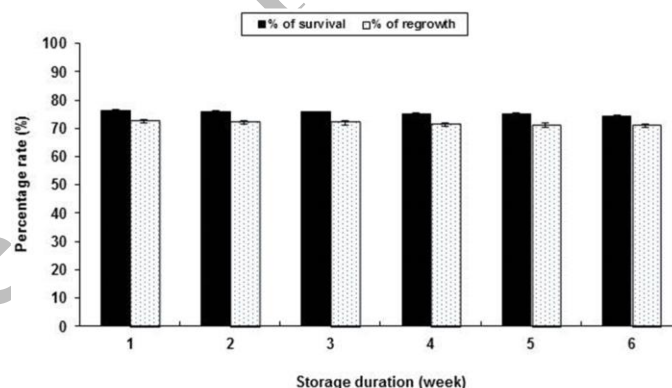

Fig. 6: The effect of storage duration on percentage of survival and regrowth of cryopreserved (+LN) embryogenic calli of date palm (*Phoenix dactylifera* L.) cv. Madgoul. Vertical bars indicate ± SE for means

sucrose for 3 d followed by dehydration for 4 h after 6 weeks in LN storage (Fig. 6).

Discussion

Encapsulation dehydration technology is a widely used technique for the preservation of genotypes *in situ* (Rihan *et al.* 2017). This is due to several advantages which include low cost, genetic stability for longer time, high ability to survive and high cell viability (Farag *et al.* 2012), however, it requires more handling of alginate beads and some species do not tolerate the high sucrose concentrations employed (Kaviani 2011).

In order to establish an effective encapsulation dehydration protocol for date palm (cv. Magdoul), the production of friable callus was achieved by culturing

Corrected Proof Press

somatic embryogenic callus on initiation medium (M3) consisting of MS medium containing the GRHs 2.0 mg L^{-1} NAA, 3.0 mg L^{-1} 2iP and 2.5 g L^{-1} activated charcoal (Fig. 1). Obtaining friable callus has been preferred in several studies where it was concluded that a high success rate in most plant biotechnology protocols such as transformation and *in situ* conservation was achieved by using friable callus (Utsumi *et al.* 2017).

In the current study drops of alginate solution containing embryogenic callus was dispensed as drops into 0.1 M calcium chloride where polymerization was completed and solid beads formed around embryogenic callus. This protocol was in agreement with a previous study (Bose *et al.* 2017) where they used different concentrations of Na-alginate with concentrations ranging from (1–4%) and found that a concentration of 3% was most successful in producing uniform beads with regular shape and good coverage over the callus. Concentrations lower than 3% failed to create a solid cover, whereas, 4% Na-alginate produced a very hard cover which was subsequently difficult to rupture. Several other studies have also successfully used the combination of 3% Na-alginate and 0.1 *M* $CaCl_2$ for the encapsulation of cryopreserved embryogenic calli in several plant species (Saha *et al.* 2015; Haque and Ghosh 2016; Kaya *et al.* 2020). Sodium alginate was also applied for encapsulated cryopreserved embryogenic calli in order to prevent the building of ice inside the cells during the cryopreservation process (Patra and Gupta 2020).

The key to success in the process of cryopreservation using the encapsulation/dehydration technique depends largely on avoiding the formation of ice crystals inside the cells to prevent ice injury to the cells which will negatively affect survival and regrowth. Ice formation during cryopreservation can mechanically damage the biological structure of cells resulting in ice injury (Yang *et al.* 2019). Factors that can affect the formation of ice crystals and thus the success of cryopreservation can be applied during the encapsulation dehydration process and have been found to include: 1) osmoticums such assucrose concentration, 2) duration of hydration and 3) moisture content (Soliman 2013). The success in achieving better treatment of sucrose and duration of dehydration will help in the induction of cytoplasmic vitrification (Wilkinson *et al*. 1998). Different concentrations of sucrose as an osmoticum and different periods of dehydration were applied in the current investigation (Table 1) to study the effect of pre-culture duration and sucrose concentration on survival percentage for encapsulated non-cryopreserved (-LN) embryogenic calli**.** The results indicated that the lowest concentration tested *i.e*., 0.75 *M* sucrose for 3 days was optimal for pre-culture of encapsulated-dehydrated embryogenic calli. These results agree with those reported by Farag *et al.* (2012), who noted that there is an inverse relationship between increased sucrose concentration and hydration time with the rate of regrowth and survival. Increasing sucrose to high concentrations in the pre-culture stage led to sugar accumulation, a decrease in the water content and induced cytoplasmic vitrification that helped to prevent ice crystalization in apricot (*Prunus armeniaca* L.) (Soliman 2013). Previous studies (Al-Ababneh *et al.* 2003; Moges *et al*. 2004; Baghdadi *et al*. 2010; Kaya *et al.* 2020) stated that the highest percentages of survival and regrowth were achieved when shoot tips were pre-cultured for two or three days with sucrose ranging from 0.1 to 0.5 *M*. Several other studies (Crowe *et al.* 1987; Kendall *et al.* 1993) suggest that maintaining a higher concentration of sucrose inside the cells may stimulate the expression of some genes responsible for preventing the degradation of sugars and thus maintain the integrity of cell membranes and cell walls as well as maintaining the stability of proteins under frozen conditions. Accumulation of sugar in plant tissues protects cells against these cryo-injuries and helps to restore vitality during freezing and allows successful storage at cryogenic temperatures (Yang *et al*. 2019).

A degree of dehydration and cellular dehydration tolerance is required for successful cryopreservation because the cells must reduce their freezable water in order to avoid ice crystal formation when exposed to liquid nitrogen (Benson 1999). The results presented here showed that a decrease in the MC of the bead (dehydration) led to a decrease in survival and regrowth. Maximum tolerance of non-cryopreserved beads reached a maximum when beads were exposed to 10 h dehydration whilst cryopreserved embryogenic calli had the highest rate of survival (83.25 ± 0.35^{a}) and regrowth (79.35 ± 0.25^{a}) under treatment with 0.75 *M* sucrose and 4 h dehydration with MC of 39.50 ± 0.22 Table (2). These results agree with the earlier work of Wang *et al*. (2000) who found that after 10 h of dehydration under laminar-air flow, the viability of dehydrated cells of grape was 42% at a MC of 16.2%, while 6 h of dehydration recorded highest viability (78%) with 20.6% MC. Hirai and Sakai (1999) pointed out the effect of the dehydration process on the ability of the plant tissues to resist freezing during cryopreservation and also showed that the dehydration duration not only increased the freezing tolerance rate but also contributed to improving the regrowth rate of date palm. Our results also support that of earlier work by Gupta and Reed (2006), where they were noted that it is important to optimize the dehydration period, where increasing the dehydration period led to damage of the encapsulated shoot tip, whilst insufficient dehydration was associated with ice nucleation and thereby freezing damage (Villouta *et al.* 2020). With regard to MC previous studies stated that decreasing bead water content and reduction of the MC of encapsulated explants to a minimal level is a necessary step for successful cryopreservation and it leads to an increase in survival rates. However, it was found that the shrivelling of the cell and its exposure to the condition of hypertension arise from a lack of water content, which resulted in changes in the plasma membrane (Palanyandy *et al.* 2020). Correlation have been found

Corrected Proof, In Press

between dehydration time, low moisture contents, the formation of the glassy state (vitrification) and survival after cryopreservation (Martinez and Revilla 1998).

Cryopreserved vials containing beads with embryogenic calli were rapidly thawed at 37°C for 3 minutes using a water bath, then beads were removed and washed with liquid MS medium to test growth recovery. This is in a good agreement with previous finding on garlic plants (Kim *et al.* 2004), where they observed that rapid rewarming resulted in more regrowth than slower warming. In our study, cryopreserved embryogenic calli of date palm regrew and successfully developed into plantlets (Fig. 3) with intermediary callus to reduce somaclonal variation. Takagi (2000) and Fernandes *et al.* (2008) also observed that producing plants directly without intermediary callus minimizes the risk of somaclonal variation. Our results found that the highest value for growth recovery, whether by calculating the survival rate or the regrowth was 76.35 and 72.9% respectively after storage in LN for the first week, but there was an non-significant decrease in those values by increasing the storage period in LN (Fig. 6). This result may suggest that thawed cryopreserved embryogenic calli had high viability and exhibited long-term survival. Previous studies support this contention, as both Nogueira *et al.* (2013) and Vujović *et al.* (2011) indicated a significant decrease in survival rates to reach maximum rates of 20 and 37% with increased storage time in LN. The growth recovery ratio that was recorded in our current research is considered one of the best survival rates that has been reached with date palm plants and this may be due to the optimised protocol . Recovery and regrowth in other studies of date palm cv. Sagai were above 30% for all the protocols used such as dehydrated-encapsulation, vitrification, and vitrification-encapsulation (Alansi *et al.* 2019) . It was reported by (Panis *et al.* 2005; Chen *et al.* 2011) the average survival rate also varies between varieties even when the same cryopreservation treatment conditions were applied in the experiment.

Conclusion

In conclusion, this study defines the first efficient, simple protocol for cryopreservation of date palm (cv. Magdoul) germplasm by encapsulation-dehydration. Encapsulation plays an important role in the success of cryopreservation, it is nessary to induce a high level of dehydration tolerance for preservation. It has been shown that encapsulation and dehydration increased viability of cryopreserved date palm calli. Also, encapsulation-dehydration improved the survival of these plants after exposure to LN. Cryopreservation is now a viable long-term storage technique for date palm germplasm. This leads to the preservation of good varieties in the Kingdom of Saudi Arabia for long periods and the reproduction of them in the laboratory at any time of the year which will facilitate the clonal production of elite genotypes for future agricultural use.

Acknowledgements

This work was funded by the *University of Jeddah*, Saudi Arabia, under grant No. (UJ-11-18- ICP). The authors, therefore, acknowledge with thanks the University technical and financial support.

Author Contributions

EMRM, NMSK and HIAS planned the experiments, OAA and MPF interpreted the results, EMRM and HIAS made the write up and NMSK statistically analyzed the data and made illustrations, MPF made the language editing.

Refernces

Al-Ababneh SS, RA Shibli, NS Karam, MA Shatnawi (2003). Cryopreservation of bitter almond (*Amygdalus communis* L.) shoot tips by encapsulation-dehydration and vitrification. *Adv Hortic Sci* 17:15–20

Alansi S, F Al-Qurainy, M Nadeem, S Khan, M Tarroum, A Alshameri, ARZ Gaafar (2019). Cryopreservation: A tool to conserve date palm in Saudi Arabia. *Saud J Biol Sci 26*:1896–1902

Aldhebiani AY, EM Metwali, HI Soliman, SM Howladar (2018). Response of different date palm cultivars to salinity and osmotic stresses using tissue culture technique. *Intl J Agric Biol* 20:1581–1590

Al-Khayri JM, PM Naik, SM Jain, DV Johnson (2018). Advances in date palm (*Phoenix dactylifera* L.) Breeding. *In: Advances in Plant Breeding Strategies: Fruits*, pp:727–771. Springer

Baghdadi SH, RA Shibli, MQ Syouf, MA Shatnawai, A Arabiat, IM Makhadmeh (2010). Cryopreservation by encapsulation vitrification encapsulation vitrification of embryogenic callus of wild Crocus (*Crocus hyemalis* and *Crocus moabiticus*). *Jor J Agric Sci* 6:436–443

Bekheet SA, HS Taha, MM Saker, ME Solliman (2007). Application of cryopreservation technique for in vitro grown date palm (*Phoenix dactylifera* L.) cultures. *J Appl Sci Res* 3:859–866

Bekheet SA, HS Taha, MM Saker, HA Moursy (2002). A synthetic seed system of date palm through somatic embryos encapsulation. *Ann Agric Sci Ain Shams Univ Cairo* 47:325–337

Benson EE (1999). *Plant Conservation Biotechnology*, pp:83–95. Benson E. (Ed.). Taylor & Francis, London

Bonner FT (1990). Storage of seeds: Potential and limitations for germplasm conservation. *For Ecol Manage 35*:35–43

Bose S, J Karmakar, DP Fulzele, U Basu, TK Bandyopadhyay (2017). In vitro shoots from root explant, their encapsulation, storage, plant recovery and genetic fidelity assessment of Limonium hybrid ‘Misty Blue’: A florist plant. *Plant Cell Tiss Org Cult* 129:313–324

Chen XL, JH Li, X Xin, ZE Zhang, PP Xin, XX Plu (2011). Cryopreservation of in vitro-grown apical meristems of Lilium by droplet-vitrification. *S Afr J Bot* 77:397–403

Crowe JH, LM Crowe, JF Carpenter, CA Wistrom (1987). Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochem J* 242:1–10

Duarte E, R González, R Rubenich, P Rocha (2018). Vegetative propagation method for ex situ conservation of *Sida ramoniana* (Malvaceae): An endemic species with medicinal potential in danger of extinction. *Intl J Agric Biol* 20:2779–2784

El-Juhany LI (2010). Degradation of date palm trees and date production in Arab countries: Causes and potential rehabilitation. *Aus J Basic Appl Sci 4*:3998–4010

Farag KM, NMN Nagy, HI Soliman, MEE Ahmed (2012). Preservation of rare apricot and walnut germplasms grown in Saint Catherine Valleys by encapsulation dehydration technique. *Aus J Basic Appl Sci* 6:626–635

Corrected Proof In Press

Feng C, Z Yin, Y Ma, Z Zhang, L Chen, B Wang, B Li, Y Huang, Q Wang (2011). Cryopreservation of sweet potato (*Ipomoea batatas*) and its pathogen eradication by cryotherapy. *Biotechnol Adv* 29:84–93

Fernandes P, E Rodriguez, G Pinto, I Roldán-Ruiz, MD Loose, C Santos (2008). Cryopreservation of *Quercus suber* somatic embryos by encapsulation-dehydration and evaluation of genetic stability. *Tree Physiol* 28:1841–1850

Gupta S, BM Reed (2006). Cryopreservation of shoot tips of blackberry and raspberry by encapsulation-dehydration and vitrification. *Cryo Lett* 27:29–42

Haque SM, B Ghosh (2016). High-frequency somatic embryogenesis and artificial seeds for mass production of true-to-type plants in Ledebouria revoluta: An important cardioprotective plant. *Plant Cell Tiss Org Cult* 127:71–83

Hirai D, A Sakai (1999). Cryopreservation of *in vitro-grown* axillary shoot-tip meristems of mint (*Mentha spicata* L.) by encapsulation vitrification. *Plant Cell Rep* 19:150–155

Kaviani B (2011). Conservation of plant genetic resources by cryopreservation. *Aus J Crop Sci* 5:778–800

Kaya E, S Galatali, S Guldag, O Celik (2020). A New Perspective on Cryotherapy: Pathogen Elimination Using Plant Shoot Apical Meristem via Cryogenic Techniques. *In: Plant Stem Cells*, pp:137–148. Humana, New York, USA

Kendall EJ, KK Kartha, JA Qureshi, P Chermak (1993). Cryopreservation of immature spring wheat zygotic embryos using an abscisic acid pretreatment. *Plant Cell Rep* 12:89–94

Khan S, F Al-Qurainy, M Nadeem (2012). Biotechnological approaches for conservation and improvement of rare and endangered plants of Saudi Arabia. *Saud J Biol Sci* 19:1–11

Kim HH, JB Kim, HJ Baek, EG Cho, YA Chae, F Engelmann (2004). Evolution of DMSO concentration in garlic shoot tips during a vitrification procedure. *Cryo Lett* 25:91–100

Kohmura H, A Sakai, S Chokyu, T Yakuwa (1992). Cryopreservation of in vitro-cultured multiple bud clusters of asparagus (*Asparagus officinalis* L. cv Hiroshimagreen (2n= 30) by the techniques of vitrification. *Plant Cell Rep* 11:433–437

Martinez D, MA Revilla (1998) Cold acclimation and thermal transitions in the cryopreservation of hop shoot tips. *Cryo Lett* 19:313–342

Moges AD, RA Shibli, NS Karam (2004). Cryopreservation of African violet (*Saintpaulia ionantha* Wendl.) shoot tips. *In Vitro Cell Dev Biol Plant* 40:389–395

Murashige T, F Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plantarum* 15:473–497

Nogueira GF, M Pasqual, JE Scherwinski-Pereira (2013). Survival of sugarcane shoot tips after cryopreservation by droplet-vitrification. *Pesq Agrop Bras* 48:1524–1527

Palanyandy SR, S Gantait, S Subramaniam, UR Sinniah (2020). Cryopreservation of oil palm (*Elaeis guineensis* Jacq.) polyembryoids via encapsulation–desiccation. *3 Biotech* 10:1–9

Panis B, M Lambardi (2006). Status of cryopreservation technologies in plants (crops and forest trees). *The role of Biotechnology in Exploring and Protecting Agricultural Genetic Resources*, pp:61–78. FAO, Rome, Italy

Panis B, B Piette, R Swennen (2005). Droplet vitrification of apical meristems: A cryopreservation protocol applicable to all Musaceae. *Plant Sci* 168:45–55

Patra T, MK Gupta (2020). Evaluation of sodium alginate for encapsulation-vitrification of testicular Leydig cells. *Intl J Biol Macromol* 150:128–137

Rajmohan K (2011). Date palm tissue culture: A pathway to rural development. *In*: *Date palm biotechnology*, pp:29-45. Springer, Dordrecht, The Netherlands

Rihan HZ, F Kareem, ME El-Mahrouk, MP Fuller (2017). Artificial seeds (principle, aspects and applications). *Agronomy* 7; Article 71

Saha S, C Sengupta, P Ghosh (2015). Encapsulation, short-term storage, conservation and molecular analysis to assess genetic stability in alginate-encapsulate microshoots of *Ocimum kilimandscharicum* Guerke. *Plant Cell Tiss Org Cult* 120:519–530

Shatnawi MA (2013). Multiplication and Cryopreservation of Yarrow (*Achillea millefolium* L., Asteraceae). *J Agric Sci Technol* 15:163–173

Snedecor GW, WG Cochran (1989). *Statistical Methods*, 7th edn. The Iowa State University, Ames. Iowa, USA

Soliman HI (2013). Cryopreservation of in vitro-grown shoot tips of apricot (*Prunus armeniaca* L.) using encapsulation-dehydration. *Afr J Biotechnol* 12:1419–1430

Soliman HI, M Gabr, NA Abdallah (2010). Efficient transformation and regeneration of fig (*Ficus carica* L.) via somatic embryogenesis. *GM Crops* 1:40–51

Taha HS, SA Bekheet, MK El-Bahr (2003). Alternative approach for micropropagation of the date palm cv. Zaghlool. *Arab J Biotechnol* 6:103–112

Takagi H (2000). Recent developments in cryopreservation of shoot apices of tropical species. Cryopreservation of tropical plant germplasm. *In: Current Research Progress and Application,* pp:178–193. Engelmann F, H Takagi (Eds.). Japan International Research Center for Agricultural Sciences, Tsukuba, Japan/International Plant Genetic Resources Institute, Rome, Italy

Towill LE (2005). Germplasm conservation. *In: Plant Development and Biotechnology*, pp:277–284. Trigiano R, D Gray (Eds.). CRC Press, New York, USA

Utsumi Y, C Utsumi, M Tanaka, VT Ha, A Matsui, S Takahashi, M Seki (2017). Formation of friable embryogenic callus in cassava is enhanced under conditions of reduced nitrate, potassium and phosphate. *PLoS One* 12; Article e0180736

Villouta C, BA Workmaster, J Bolivar-Medina, S Sinclair, A Atucha (2020). Freezing stress survival mechanisms in *Vaccinium macrocarpon* Ait. terminal buds. *Tree Physiol* 40:841–855

Vujović T, I Sylvestre, D Ružić, F Engelmann (2011). Droplet-vitrification of apical shoot tips of *Rubus fruticosus* L. and *Prunus cerasifera* Ehrh. *Sci Hortic* 130:222–228

Wang Q, E Tanne, A Arav, R Gafny (2000). Cryopreservation of *in vitro*-grown shoot tips of grapevine by encapsulation-dehydration. *Plant Cell Tiss Org Cult* 63:41–46

Wilkinson T, A Wetten, MF Fay (1998). Cryopreservation of *Cosmos atrosanguineus* shoot tips by a modified encapsulation/dehydration method. *Cryo Lett* 19:293–302

Yang J, L Gao, M Liu, X Sui, Y Zhu, C Wen, L Zhang (2019). Advanced Biotechnology for Cell Cryopreservation, pp:1–15. Trans Tianjin University, Beijing, China