

Cryopreservation of citrus seed via dehydration followed by immersion in liquid nitrogen

Ergun KAYA^{1*}, Fernanda Vidigal Duarte SOUZA², Emel YILMAZ GÖKDOĞAN¹, Muammer CEYLAN¹, Maria M. JENDEREK³

¹Department of Molecular Biology and Genetics, Faculty of Science, Muğla Sıtkı Koçman University, Kötekli, Muğla, Turkey

²Embrapa Cassava & Fruits, Cruz das Almas, Bahia, Brazil

³National Laboratory for Genetic Resources Preservation, USDA-ARS, Fort Collins, Colorado, USA

Received: 28.03.2016 • Accepted/Published Online: 01.10.2016 • Final Version: 20.02.2017

Abstract: An important method for plant germplasm conservation is offered by a biotechnology-based approach of cryopreservation. Cryopreservation refers to the storage of plant material at ultralow temperatures in liquid nitrogen. A procedure for cryopreservation of polyembryonic seeds was improved for selected citrus cultivars from Turkey. Seed dehydration was performed at different exposure times, in sterile conditions of a laminar flow-hood. The tested cultivars showed the highest tolerance to low temperature storage when the seeds were first dehydrated to a moisture content of 21.8% for *Poncirus trifoliata* Raf. × *Citrus sinensis* Osb. and to 17.6% for *Citrus limonia* Osbeck. The postcryopreservation germinability ranged from 73.3% (*Poncirus trifoliata* Raf. × *C. sinensis* Osb. and *Fortunella margarita* (Lour.) Swingle) to 93.3% (*C. jambhiri* Lush.). Dehydration was beneficial for germination of seeds from all of the tested citrus species after the liquid nitrogen exposure. Seedlings derived from cryopreserved seeds had well-formed shoots and roots and were easily acclimated to greenhouse conditions.

Key words: Polyembryonic seed, viability and germination, dehydration time, moisture content

1. Introduction

Conventionally, citrus germplasm is conserved in orchards and greenhouses, where it is subjected to losses due to pests, diseases, and climatic hazards (Duran-Vila, 1995). Germplasm storage at ultralow temperatures has proven to be an economic and reliable method for long-term storage of genetic resources of several plant species. In recent years, many studies have been conducted on cryopreservation of citrus genetic resources. As a result, effective freezing protocols have been developed for different types of plant tissues and organs such as shoot tips (Wang and Deng, 2004; Kaya et al., 2013), embryonic axes (Cho et al., 2001, 2002a, 2002b), seeds (Cho et al., 2002c; Lambardi et al., 2004), somatic embryos (Marin and Duran-Vila, 1988), ovules (Gonzales-Arno et al., 2003), nucellar cells (Sakai et al., 1991), and embryogenic calli (Olivares-Fuster et al., 2000).

According to desiccation responses, seeds have been divided into two main categories: desiccation-tolerant (orthodox) and desiccation-sensitive (recalcitrant). A third category has been known as suborthodox, which are relatively desiccation-tolerant seeds, but they cannot resist desiccation down to water contents as low as those tolerated by orthodox seeds and they are freezing-sensitive. The suborthodox seeds are represented by many

tropical and subtropical species (oil palm, coffee, and citrus species). Thus, the only effective technique for long-term germplasm conservation of polyembryonic seeds that are grouped among suborthodox and recalcitrant seed species is cryopreservation (Roberts, 1973; Ellis et al., 1990; Hor et al., 2005).

Cryopreservation of polyembryonic citrus seed allows long-term preservation of seeds that contain both nucellar and zygotic embryos. While zygotic embryos are the product of controlled or open pollination and are desired in conventional breeding, nucellar embryos originate from maternal cells and seedlings derived from such embryos have the same genotype as the female plant. Seedlings originating from nucellar embryos are of great value in citrus rootstock production (Koltunow, 1993, 1995; Ruiz et al., 2000).

Polyembryonic seeds might be a valuable material for long-term conservation of citrus germplasm. Citrus seeds are considered primarily suborthodox and good germination can be maintained after controlled drying (King et al., 1981; Bonner, 1990). This gives a good premise for their long-term storage after dehydration and direct plunging in liquid nitrogen. The traditional seed preservation system in seed banks at above-zero

* Correspondence: ergunkaya@mu.edu.tr

temperatures (5 °C) is not reliable, because low above-zero temperatures slow down the loss of viability but fail to provide long-term preservation (Kholina and Voronkova, 2012). Seed moisture content is considered the most crucial factor for maintaining viability after exposure to liquid nitrogen (-196 °C) (Stanwood, 1985). Seed moisture should be decreased to a content that prevents intracellular ice crystal formations during ultrarapid freezing (occurring by the direct immersion of seeds in liquid nitrogen), thus conserving the integrity of seed tissues (Benson, 1999).

The aim of the present research was to evaluate the time of desiccation and moisture content in seed that would maintain the highest germination after cryopreservation for selected Turkish citrus cultivars that are under risks of orchard conditions (pests, diseases, and climatic hazards) at the West Mediterranean Agricultural Research Institute.

2. Materials and methods

2.1. Plant Materials

Seeds of six different cultivars of citrus species (*Citrus limon* (L.) Burm.f. 'Kara Limon'; *C. limonia* Osbeck 'Volkamer Limon'; *C. jambhiri* Lush. 'Kaba Limon'; *C. aurantium* L. 'Yerli Turunc'; *Poncirus trifoliata* Raf. × *C. sinensis* Osb. 'Troyer'; *Fortunella margarita* (Lour.) Swingle 'Kamkat') were provided by the West Mediterranean Agricultural Research Institute, Antalya, Turkey. The study was carried out with 28 accessions (each accession belonged to one different rootstock), with three accessions for the Kamkat cultivar and 25 accessions belonging to the other five cultivars from the collection orchards (five accessions were used for each cultivar). At least 70 seeds were used for moisture content (MC) determination, cryopreservation, and control treatments of each accession and each treatment was repeated at least three times (totally at least 210 seeds for each accession).

2.2. Surface sterilization of *Citrus* spp. cultivars seeds

The seeds were surface sterilized using a modified procedure developed for *Thymus* spp. seeds (Ozudogru et al., 2011). In short, the seeds were soaked in 70% ethanol for 4 to 5 min, then exposed to 10% H₂O₂ for 4 to 5 min and subsequently disinfected twice in 20% commercial bleach (Domestos, Turkey, 30% NaClO) for 10 min and rinsed in sterile distilled H₂O (at least three times) after the exposure to bleach.

2.3. Culture conditions and seed germination assay

Surface-sterilized seeds were germinated in vitro in petri dishes (100 × 15 mm) on semisolid (1.5 g L⁻¹ Phytigel (Sigma-Aldrich, Turkey), 3.5 g L⁻¹ agar (Sigma-Aldrich)) MS medium (Murashige and Skoog, 1962; Sigma-Aldrich, pH 5.8) supplemented with 0.1 μM gibberellic acid (GA) (Phytotech Laboratories, filter-sterilized) and 20 g L⁻¹ sucrose (Phytotech Laboratories). The seeds were kept at 27 ± 2 °C with a 16-h photoperiod, under white cool

fluorescent light of 50 μmol⁻¹ m⁻² s⁻¹, at a laboratory in Gebze, Turkey.

Germination was evaluated 6 weeks after the seeds were recovered from liquid nitrogen (for cryopreserved seeds) and dehydration treatment (control group seeds). Seeds that produced at least one morphologically normal seedling were considered germinated. Percent germination and the average number of seedlings per seed were calculated using at least 30–45 seeds per treatment, and each experiment was repeated at least three times for each accession. Levels of seed germination and viability (seeds that did not germinate but were alive) were based on a 2,3,5-triphenyltetrazolium (TZ) test; samples were incubated overnight with 1% TZ solution (pH 7.4) at 30 °C in the dark and observed for the appearance of red staining (Van Waes and Debergh, 1986).

2.4. Determination of the moisture content (MC)

In a preliminary trial, MC was determined for ten different seeds (these seeds were used only for determination of MC for each accession) by using Pixton's (1966) formula: moisture content (%) = (AC - BD) / AC × 100 (where A is the original weight of the portion of sample, B is the weight after drying, C is the initial weight of ground sample, and D is the final weight of dried ground sample). The seeds were placed on top of open petri dishes under the air current of a laminar flow hood. The MC (%) of the ten seeds was established every 3 h until a MC below 10% was reached. During the dehydration process, the temperature in the laboratory was 32 to 35 °C and relative humidity was 16%–23%. The seed type and the initial moisture content for the six species tested were different; hence, the dehydration times in the Table are also different.

2.5. Cryopreservation by dehydration technique

After the preliminary dehydration trial, seeds were exposed directly to liquid nitrogen. The seeds were placed in 1.5-mL cryovials (NALGENE, Turkey), with five seeds per cryovial, and were directly plunged into liquid nitrogen. After at least 24 h of storage at -196 °C, the seeds were rewarmed in the cryovials under laminar flow hood at room temperature for 15 min. After 15 min, seeds were transferred to the germination medium, under the conditions stated above.

2.6. Statistical analysis

Statistical analysis of the nonparametric data (frequencies) was carried out either by the chi-square test to make comparisons between two percentage values or the test for homogeneity ratios. Significant differences of treatments were selected using a nonparametric statistical test, the post hoc multiple comparisons test, to compare multiple percentage values (Marascuilo and McSweeney, 1977). Discrete data were subjected to ANOVA to compare means followed by the least significant difference test at P ≤ 0.05. Data of all accessions for each cultivar were evaluated together.

2.7. Acclimatization of cryopreserved seedlings

Rooted seedlings were acclimatized under greenhouse conditions. The seedlings were planted into 250-mL plastic pots under high relative humidity (65% or more). The humidity level, kept by using transparent plastic pots with a hole over the seedlings, was checked daily and was gradually decreased. As a planting substrate (Plantistanbul Tropical, Turkey) nitrogen-rich peat with high nutrient retention and water-holding capacity, free of weed seeds and pathogens, was used. Plants were irrigated daily (50 mL of sterilized tap water) for the first 3 days, at 2-day intervals between the 4th and 7th days, and at 3-day intervals for the rest of the acclimatization period.

3. Results and discussion

3.1. Dehydration effect on citrus seed viability and germination

For all accessions, the initial seed germination rate was 100%. Although seeds of all cultivars were collected from the same area and date, and kept in the same conditions (temperature, moisture), the initial moisture content in seeds with different shapes and sizes of the cultivars varied, ranging from a minimum of 25.1% (*Fortunella margarita* (Lour.) Swingle ‘Kamkat’) to a maximum of 56.3% (*P. trifoliata* Raf. × *C. sinensis* Osb. ‘Troyer’). The MC in the seeds desiccated over time also varied between the species but was similar for *C. jambhiri* and *C. limon* (Figure 1); the difference between the initial MC in seeds of the two species

was only 1.4%. According to Bäumler et al. (2006), the volume and weight of the seed, the expansion coefficient, the equivalent diameter, and the sphericity increase lineally with the increase in the seed MC, as was also observed in this study. After dehydration in a sterile air flow, the seed viability and germination ranged from 76.6% viability and 70% germination for *C. limon* (L.) Burm.f. to 100% viability and germination for *C. limonia* Osbeck, *C. jambhiri* Lush., *C. aurantium* L., and *F. margarita* (Lour.) Swingle. With the exceptions of *P. trifoliata*, *C. jambhiri*, and *C. limon*, the effect of MC on the viability and germination was not statistically significant for the control seeds, i.e. those without exposure to liquid nitrogen (Table).

Our study results are similar to those obtained by Hor et al. (2005) for seeds of three different *Citrus* species, Dussert et al. (2001) for seeds of five species of *Coffea*, and Michalak et al. (2015a, 2015b) for mazzard cherry (*Prunus avium* L.) and wild apple (*Malus sylvestris*) seeds. They observed that there was a decline in seed survival after liquid nitrogen exposure with dehydrating seeds to water content below the specific high-moisture freezing limit (MC around or under 20%). On the basis of our results and previous studies we could conclude that oil-rich seeds better tolerate the temperature of liquid nitrogen at higher MCs and for long-term storage they should be cryopreserved at their specific high-moisture freezing limit. On the other hand, Michalak et al. (2013) investigated the sensitivity of *Corylus avellana* seeds to extreme desiccation and cryopreservation in

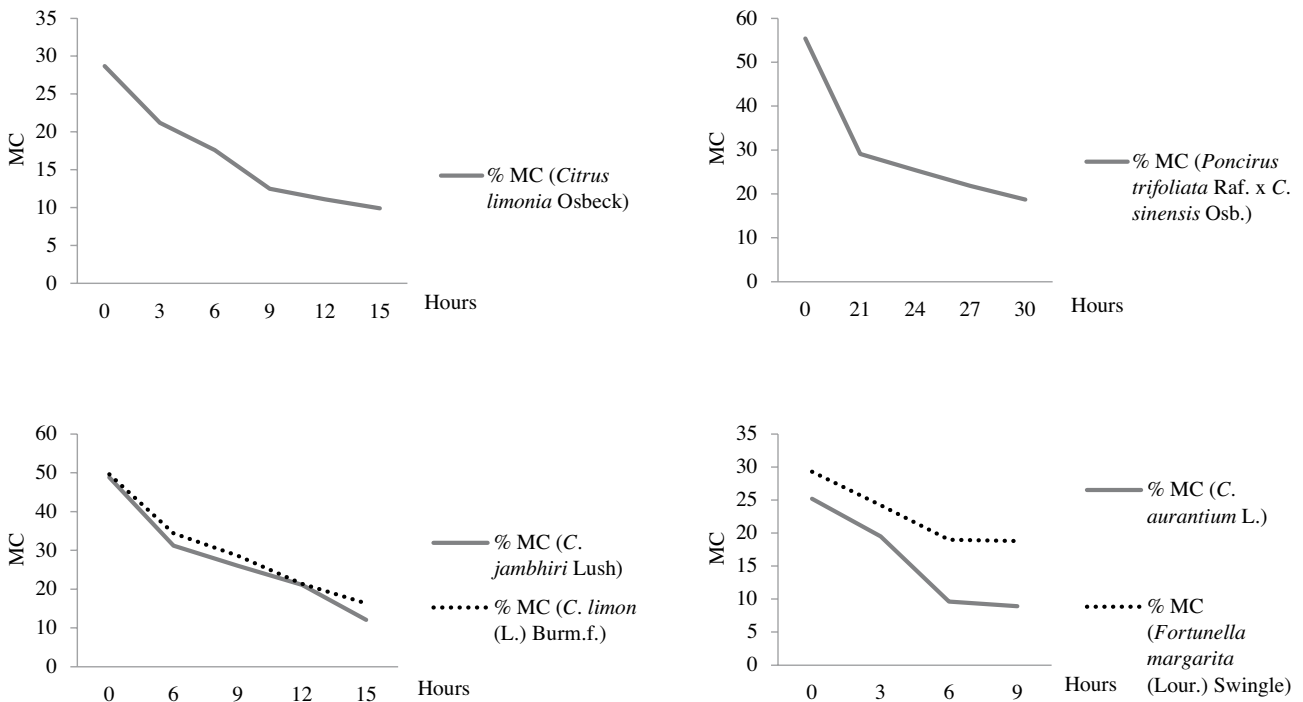


Figure 1. Seed moisture content of six citrus species after 9, 15, and 30 h of dehydration under laminar air flow conditions. Ten seeds of each accession were used for moisture content determination (these seeds were used only for determination of MC) and results of all accessions for each cultivar were evaluated together.

Table. Germination and viability of seeds of 28 accessions belonging to six different cultivars after dehydration (control) and liquid nitrogen treatment (all accession results of each cultivar were calculated as an average value).

Species	Cultivar	Group	Initial		Dehydration		Control		Liquid nitrogen		
			Moisture content (%)	Viability and germination (% ± SE ^a)	Time (h)**	Moisture content (%)	Total viability*** (% ± SE)	Germination (% ± SE)	Total viability (% ± SE)	Germination (% ± SE)	
<i>Citrus limonia</i> Osbeck	Volkamer limon	Lemon			6	17.6	100 ± 00 ^{a**}	100 ± 00 ^a	83.3 ± 0.4 ^b	83.3 ± 0.4 ^b	
				28.8	100 ± 00 ^a	9	12.5	100 ± 00 ^a	100 ± 00 ^a	83.3 ± 0.4 ^b	73.3 ± 0.4 ^c
				56.3	100 ± 00 ^a	12	11.1	100 ± 00 ^a	100 ± 00 ^a	56.7 ± 1.1 ^d	56.7 ± 1.1 ^d
<i>Poncirus trifoliata</i> Raf. × <i>C. sinensis</i> Osb.	Troyer	Trifoliolate			24	25.4	86.7 ± 0.8 ^b	86.7 ± 0.8 ^b	60 ± 0.7 ^d	60 ± 0.7 ^d	
				56.3	100 ± 00 ^a	27	21.8	100 ± 00 ^a	93.3 ± 0.8 ^{ab}	73.3 ± 0.4 ^c	73.3 ± 0.4 ^c
				49.7	100 ± 00 ^a	30	18.7	96.7 ± 0.4 ^a	93.3 ± 0.8 ^{ab}	66.7 ± 0.4 ^{cd}	60 ± 1.1 ^d
<i>C. jambhiri</i> Lush.	Kaba limon	Lemon			9	26	100 ± 00 ^a	100 ± 00 ^a	83.3 ± 0.9 ^c	80 ± 0.6 ^c	
				49.7	100 ± 00 ^a	12	21.1	100 ± 00 ^a	100 ± 00 ^a	93.3 ± 0.7 ^b	93.3 ± 0.7 ^b
				51.1	100 ± 00 ^a	15	12.1	100 ± 00 ^a	93.3 ± 0.3 ^b	73.3 ± 0.9 ^d	60 ± 0.0 ^c
<i>C. limon</i> (L.) Burm.f.	Kara limon	Lemon			9	28.6	100 ± 00 ^a	100 ± 00 ^a	80 ± 0.9 ^{bc}	76.7 ± 0.8 ^c	
				51.1	100 ± 00 ^a	12	21.4	83.3 ± 0.8 ^b	83.3 ± 0.8 ^b	86.7 ± 0.3 ^b	83.3 ± 0.6 ^b
				29.5	100 ± 00 ^a	15	16.3	76.7 ± 0.3 ^c	70 ± 0.5 ^d	66.7 ± 0.8 ^d	66.7 ± 0.8 ^d
<i>C. aurantium</i> L.	Yerli turunc	Sour orange			3	19.5	100 ± 00 ^a	100 ± 00 ^a	90 ± 0.4 ^b	86.7 ± 0.2 ^b	
				29.5	100 ± 00 ^a	6	9.6	100 ± 00 ^a	100 ± 00 ^a	76.7 ± 0.2 ^c	76.7 ± 0.2 ^c
				25.1	100 ± 00 ^a	9	8.9	100 ± 00 ^a	100 ± 00 ^a	76.7 ± 0.7 ^c	76.7 ± 0.7 ^c
<i>Fortunella margarita</i> (Lour.) Swingle	Kamkat	Kumquat			3	24.2	100 ± 00 ^a	100 ± 00 ^a	43.3 ± 0.3 ^c	43.3 ± 0.3 ^c	
				25.1	100 ± 00 ^a	6	19.0	100 ± 00 ^a	100 ± 00 ^a	73.3 ± 0.6 ^b	73.3 ± 0.6 ^b
				18.8	100 ± 00 ^a	9	18.8	100 ± 00 ^a	100 ± 00 ^a	60 ± 0.0 ^c	53.3 ± 0.2 ^d

^aSE values (%) were statistically analyzed by a nonparametric test, the post hoc multiple comparisons test (Marascuilo and McSweeney, 1977).
^{**}The seeds of the six species have different types and different initial moisture contents; hence, dehydration times were different to achieve ≤20% MC for each species.
^{***}Statistical analysis performed by ANOVA, followed by LSD test at P ≤ 0.05.
^{****}Total viabilities evaluated by TZ method.

liquid nitrogen ($-196\text{ }^{\circ}\text{C}$). Their results demonstrated the feasibility of long-term cryopreservation of European hazelnut seeds. As the seeds of this species have been classified in several different categories (orthodox, suborthodox, and recalcitrant), based on their response to desiccation and low temperature, the assignment of the seeds of hazelnut to a specific category was provided and discussed. The present data indicate that citrus seeds should be classified as suborthodox (intermediate) rather

than orthodox, due to the fact that these seeds are not resistant to drying.

3.2. Cryopreservation of citrus seeds

Maximum tolerance to the cryopreservation procedure, in terms of viability and germination, was observed for seeds of *C. jambhiri* Lush., in which 93.3% of seeds germinated after 12 h of dehydration to 21.1% MC, followed by direct immersion in liquid nitrogen, thawing, and plating on germination medium (Figure 2A). Conversely, the



Figure 2. Germinated and acclimatized seedlings derived from cryopreserved citrus seeds: A) seedlings of *Citrus jambhiri* Lush. germinated after 12 h of dehydration and B) *Fortunella margarita* (Lour.) Swingle germinated after 3 h of dehydration, followed by direct immersion in liquid nitrogen, thawing, and plating on germination medium. C–H) Plantlets derived from seeds 4 or 6 weeks after cryopreservation: C- *Citrus limonia* Osbeck, D- *Poncirus trifoliata* Raf. × *C. sinensis* Osb., E- *C. jambhiri* Lush., F- *C. limon* (L.) Burm.f., G- *C. aurantium* L., H- *Fortunella margarita* (Lour.) Swingle. I) Acclimatized plantlets of *Citrus limonia* Osbeck in greenhouse conditions (bars: 1 cm).

minimum tolerance was observed for seeds of *F. margarita* (Lour.) Swingle, in which only 43.3% of seeds germinated after 3 h of dehydration to a MC of 24.2%, followed by direct immersion in liquid nitrogen, thawing, and plating on germination medium (Figure 2B). However, this species showed 73.3% germination after the seeds were dehydrated for 6 h to a MC of 19.0%. In the other tested species, the best germination was obtained at 17.6% MC in *C. limonia* Osbeck (83.3%), at 21.8% MC in *P. trifoliata* Raf. × *C. sinensis* Osb. (73.3%), at 12.0% MC for *C. limon* (L.) Burm.f. (83.3%), and at 19.5% MC for *C. aurantium* L. (86.7%). Reducing the MC below 20% benefited postcryopreservation seed germination of *C. limonia* Osbeck, *C. aurantium* L., and *F. margarita* (Lour.) Swingle, while *P. trifoliata* Raf. × *C. sinensis* Osb., *C. jambhiri* Lush., and *C. limon* (L.) Burm.f. seeds performed similarly when the MC was in the range of 21.1%–21.8% (73.3%–93.3% germination; Table).

Marzalina and Krishnapillay (1999) reported that the MC of citrus seeds should be reduced to 20% or less in order to achieve high germination after storage in liquid nitrogen. In the present study, seeds of all six cultivars benefited from dehydration in terms of germination after cryopreservation. However, their responses to increasing levels of dehydration varied. As confirmation, Mumford and Grout (1979), Normah and Serimala (1995), and Lambardi et al. (2007) reported high tolerance of *C. limon*, *C. halimii*, and *C. volkameriana* seeds to dehydration below 10% MC, and, in the present study, *C. aurantium* L. showed similar results (9.6% and 8.9% MC, 76.7% germination); however, seeds with a MC of 19.5% germinated at significantly higher rates (86.7%). For all tested cultivars, 4 to 6 weeks after germination, the seedlings originating from cryopreserved seeds developed healthy shoots and long, well-formed roots (Figures 2C–2H). Subsequent plantlet acclimatization to in vivo conditions was easily

obtained by means of a gradual reduction of the relative humidity (Figure 2I).

Seeds with high lipid contents can provide high germination rates after liquid nitrogen treatment from seeds with high MC (Hor et al., 2005). The same results for oil-rich seeds considered as intermediate were obtained from citrus (Graiver et al., 2011) and coffee (Dussert et al., 2001) species for liquid nitrogen at higher MCs. The capability of oily seeds to survive at low temperatures is strictly connected to avoidance of intracellular ice formation (Chien and Chen, 2008). In this study results are similar to the results of Graiver et al. (2011) for citrus species (*C. reticulata*, *C. sinensis*, and *C. paradisi*). They observed that seed MC under the high-moisture freezing limit caused a decrease in seed survival after liquid nitrogen exposure. We may deduce from previous studies and our results that seeds with high oil contents resist low temperature better and for long-term storage they should be cryopreserved at their high-moisture freezing limit.

The dehydration process before liquid nitrogen treatment provides a satisfactory method by which nonorthodox oily citrus seeds might resist cryopreservation damage. The results of this study demonstrated a simple and rapid technique that might be used to predict the tolerance of citrus seeds to liquid nitrogen treatment. All six citrus species benefited from dehydration before storage in liquid nitrogen in terms of germination after cryopreservation. Seedlings derived from cryopreserved seeds had well-formed roots and shoots and acclimated easily to in vivo conditions.

Acknowledgments

This study was supported by the Muğla Sıtkı Koçman University Scientific Research Projects Coordination Unit (Muğla, Turkey, MSKÜ-BAP 16/021). The Republic of Turkey Ministry of Food, Agriculture, and Livestock is acknowledged for providing the plant material.

References

- Bäumler E, Cuniberti A, Nolasco SM, Riccobene IC (2006). Moisture dependent physical and compression properties of safflower seed. *J Food Eng* 72: 134-140.
- Benson EE (1999). Cryopreservation. In: Benson EE, editor. *Plant Conservation Biotechnology*. London, UK: Taylor & Francis, pp. 83-96.
- Bonner FT (1990). Storage of seeds: potential and limitation for germplasm conservation. *Forest Ecol Manag* 35: 35-43.
- Cho EG, Hor YL, Kim HH, Rao VR, Engelmann F (2001). Cryopreservation of *Citrus madurensis* zygotic embryonic axes by vitrification: importance of pregrowth and preculture conditions. *Cryo-Lett* 22: 391-396.
- Cho EG, Hor YL, Kim HH, Rao VR, Engelmann F (2002a). Cryopreservation of *Citrus madurensis* embryonic axes by vitrification: importance of loading and treatment with vitrification solution. *Cryo-Lett* 23: 317-324.
- Cho EG, Hor YL, Kim HH, Rao VR, Engelmann F (2002b). Cryopreservation of *Citrus madurensis* embryonic axes by encapsulation-dehydration. *Cryo-Lett* 23: 325-332.
- Cho EG, Hor YL, Kim HH, Rao VR, Engelmann F (2002c). Cryopreservation of *Citrus aurantifolia* seeds and embryonic axes using a desiccation protocol. *Cryo-Lett* 23: 309-316.
- Duran-Vila N (1995). Cryoconservation of germplasm of *Citrus*. In: Bajaj YPS, editor. *Biotechnology in Agriculture and Forestry* 32. Cryopreservation of Plant Germplasm. Berlin, Germany: Springer-Verlag, pp. 70-85.

- Dussert S, Chabrillange N, Rocquelin G, Engelmann F, Lopez M, Hamon S (2001). Tolerance of coffee (*Coffea* sp.) seeds to ultra-low temperature exposure in relation to calorimetric properties of tissue water, lipid composition, and cooling procedure. *Physiol Plantarum* 112: 495-504.
- Ellis RH, Hong TD, Roberts EH (1990). An intermediate category of seed storage behaviour? I. Coffee. *J Exp Bot* 41: 1167-1174.
- Gonzales-Arno MT, Ortega JC, Navarro L, Duran-Vila N (2003). Cryopreservation of ovules and somatic embryos of Citrus using the encapsulation-dehydration technique. *Cryo-Lett* 24: 85-94.
- Graiver N, Califano A, Zaritzky N (2011). Partial dehydration and cryopreservation of Citrus seeds. *J Sci Food Agr* 91: 2544-2550.
- Hamilton KN, Ashmore SE, Pritchard HW (2009). Thermal analysis and cryopreservation of seeds of Australian wild Citrus species (Rutaceae): *Citrus australasica*, *C. inodora* and *C. garrawayi*. *Cryo-Lett* 30: 268-279.
- Hor YL, Kim Y, Ugap JA, Charbrillange N, Sinniah UR, Engelmann F, Dussert S (2005). Optimal hydration status for cryopreservation of intermediate oily seeds: citrus as a case study. *Ann Bot* 95: 1153-1161.
- Kaya E, Alves A, Rodrigues L, Jenderek M, Hernandez-Ellis M, Ozudogru A, Ellis D (2013). Cryopreservation of *Eucalyptus* genetic resources. *Cryo-Lett* 34: 608-618.
- Kholina AB, Voronkova NM (2012). Seed cryopreservation of some medicinal legumes. *Journal of Botany* 2012: 186891.
- King MW, Soetisna U, Roberts EH (1981). The dry storage of citrus seeds. *Ann Bot* 48: 865-872.
- Koltunow AM (1993). Apomixes: embryo sac and embryos formed without meiosis or fertilization in ovules. *Plant Cell* 5: 1425-1437.
- Koltunow AM, Soltys K, Nito N, McClure S (1995). Anther, ovule, seed and nucellar embryo development in *Citrus sinensis* L. cv Valencia. *Can J Bot* 73: 1567-1582.
- Lambardi M, De Carlo A, Biricolli S, Puglia AM, Lombardo G, Siragusam DE, Pasaquale F (2004). Zygotic and nucellar embryo survival following dehydration and cryopreservation of citrus intact seeds. *Cryo-Lett* 25: 81-90.
- Lambardi M, Halmagyi A, Benelli C, De Carlo Vettori C (2007). Seed cryopreservation for conservation of ancient Citrus germplasm. *Adv Hort Sci* 21: 198-202.
- Marasculo LA, McSweeney M (1977). Post-hoc multiple comparisons in sample preparations for test of homogeneity. In: McSweeney M, Marasculo LA, editors. *Non-Parametric and Distribution-Free Methods for the Social Sciences*. Pacific Grove, CA, USA: Brooks/Cole Publications, pp. 141-147.
- Marin ML, Duran-Vila N (1988). Survival of somatic embryos and recovery of plants of sweet orange [*Citrus sinensis* (L.) Osb.] after immersion in liquid nitrogen. *Plant Cell Tiss Org Cult* 14: 51-57.
- Marzalina M, Krishnapillay B (1999). Recalcitrant seed biotechnology application to rain forest conservation. In: Benson EE, editor. *Plant Conservation Biotechnology*. London, UK: Taylor & Francis, pp. 265-276.
- Michalak M, Plitta BP, Chmielarz P (2013). Desiccation sensitivity and successful cryopreservation of oil seeds of European hazelnut (*Corylus avellana*). *Ann Appl Biol* 163: 351-358.
- Michalak M, Plitta-Michalak BP, Chmielarz P (2015a). A new insight in desiccation tolerance and cryopreservation of mazzard cherry (*Prunus avium* L.) seeds. *Open Life Science* 10: 354-364.
- Michalak M, Plitta-Michalak BP, Chmielarz P (2015b). Desiccation tolerance and cryopreservation of wild apple (*Malus sylvestris*) seeds. *Seed Sci Technol* 43: 1-12.
- Mumford PM, Grout BWW (1979). Desiccation and low temperature (-196°C) tolerance of *Citrus limon* seed. *Seed Sci Technol* 7: 407-410.
- Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473-497.
- Normah MN, Serimala SD (1995). Cryopreservation of seeds and embryonic axes of several Citrus species. In: Ellis RH, Black M, Murdoch AJ, Hong TD, editors. *Basic and Applied Aspects of Seed Biology*. Dordrecht, the Netherlands: Kluwer, pp. 817-823.
- Olivares-Fuster O, Asins MJ, Duran-Vila N, Navarro L (2000). Cryopreservation of callus, a source of protoplasts for Citrus improvement. *J Hortic Sci Biotech* 75: 635-640.
- Ozudogru EA, Kaya E, Kirdok E, Issever-Ozturk S (2011). In vitro propagation from young and mature explants of thyme (*Thymus vulgaris* and *T. longicaulis*) resulting in genetically stable shoots. *In Vitro Cell Dev Biol-Plant* 47: 309-320.
- Pixton SW (1966). Moisture content—its significance and measurement in stored products. *Stored Prod Res* 3: 35-47.
- Roberts EH (1973). Predicting the storage life of seeds. *Seed Sci Technol* 1: 499-514.
- Ruiz C, Breto MP, Asins MJ (2000). A quick methodology to identify sexual seedlings in citrus breeding programs using SSR markers. *Euphytica* 112: 89-94.
- Sakai A, Kobayashi S, Oiyama I (1991). Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb.) by a simple freezing method. *Plant Sci* 74: 243-248.
- Stanwood PC (1985). Cryopreservation of seed germplasm for genetic conservation. In: Kartha KK, editor. *Cryopreservation of Plant Cells and Organs*. Boca Raton, FL, USA: CRC Press, pp 199-226.
- Van Waes JM, Debergh PC (1986). Adaptation of the tetrazolium method for testing the seed viability, and scanning electron microscopy study of some Western European orchids. *Physiol Plantarum* 66: 435-442.
- Wang ZC, Deng XX (2004). Cryopreservation of shoot tips of Citrus using vitrification: effect of reduced form of glutathione. *Cryo-Lett* 25: 43-50.