

Cryopreservation and Acclimatization of *Lycopersicon esculentum* (Mill.) Genotypes

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Abstract

Romanian tomato (*Lycopersicon esculentum* Mill.) cultivars have been cryopreserved by encapsulation-dehydration and successfully acclimatized to *ex vitro* growth conditions. Shoot tips were excised from *in vitro* grown plants then precultured for 24 h in various sucrose concentrations, dehydrated up to 6 h in laminar air flow prior to direct immersion in liquid nitrogen (−196 °C) for 24 h. Different parameters have been studied: the effects of osmoprotection and desiccation duration on the regrowth of cryopreserved shoot tips, the effects of various IBA concentrations on rooting and the *ex vitro* acclimatization of plants recovered from liquid nitrogen. The highest frequency of regrowth (72% cv. ‘Pontica’) was obtained when encapsulated explants were precultured in 0.5 M sucrose and the moisture content (fresh weight basis) of alginate beads was 23%. The highest rooting rates (58% to 77%) for all cultivars were observed for shoots grown on MS medium supplemented with 1.0 mg/l IBA. The rooted plants could be easily acclimatized *ex vitro* with up to 100% survival.

Keywords: alginate beads, cryopreservation, encapsulation-dehydration, tomato

Introduction

Lycopersicon esculentum (Mill.) is one of the most extensively studied species not only for their importance as crop species but also as model system for genetic, molecular or physiological analyses (Gubiš *et al.*, 2004). Various aspects of *in vitro* grown tomato cultures have been studied including micropropagation, selection of cell lines resistant to biotic / abiotic stress and production of haploids (Bhatia *et al.*, 2004) or cryopreservation (Zevallos *et al.*, 2013). Breeding efforts have raised the need for the conservation of valuable tomato germplasm (Grout and Crisp, 1995). Advances in the characterization of its genetic diversity led to *ex situ* plant conservation (Bauchet and Causse, 2012). Although tomato seeds show orthodox storage behaviour and can be maintained at −20 °C (Ellis and Roberts, 1981), loss of viability over time is inevitable due to biochemical activity, which is not completely stopped at this temperature (Roberts and Ellis, 1984; Stanwood, 1985). For the long-term conservation of genetic resources the availability or development of reliable and cost effective strategies and the subsequent regeneration of plants following storage are basic requirements (Kaviani, 2010, 2011). Cryopreservation techniques optimized for differentiated plant material contributed significantly to the conservation of various plant species (Lambardi *et al.*, 2000; Sakai and Engelmann, 2007; Ozudogru *et al.*, 2011). Methods involving alginate encapsulation followed by desiccation have been applied to

numerous plant species (González-Arnáo and Engelmann, 2006; Harding *et al.*, 2009; Engelmann, 2011; Cruz-Cruz *et al.*, 2013) since their development by Fabre and Dereuddre (1990).

Cryopreservation protocols can be used as such or adjusted for the purpose of cryotherapy which is emerging as a powerful technique for virus elimination and was successfully used to eradicate severe pathogens in different plant species (Wang *et al.*, 2009). Therefore, the establishment of an efficient cryopreservation protocol could be a prerequisite for cryotherapy and virus eradication in tomato. The aim of our work was (1) to establish a protocol for cryopreservation of five elite Romanian tomato cultivars by encapsulation-dehydration and (2) to acclimatize plants regenerated from low temperature storage to *ex vitro* growth conditions.

Materials and methods

Plant material and growth conditions

For initiation of *in vitro* cultures, tomato (*Lycopersicon esculentum* Mill., cvs. ‘Capriciu’, ‘Darsirius’, ‘Kristin’, ‘Pontica’ and ‘Siriana’) seeds were surface sterilized with 75% Clorox for 15 min and rinsed three times with sterile distilled water. The seeds were then germinated on half-strength (1/2) Murashige and Skoog (1962) (MS) medium supplemented with 30 g/l sucrose and solidified with 7.6 g/l agar. The pH of the medium was adjusted to 5.7 before autoclaving. The cultures were maintained in the dark for four days and then transferred to 16 h light/8 h dark photoperiod (36 μmol/m²/s photosynthetic

active radiation) at 24 ± 1 °C. Shoot tips (2-3 mm in length) consisted of the apical meristem covered by 2-3 leaf primordia were excised from 1 month old *in vitro* plants under a stereo microscope in sterile conditions and were further used in cryopreservation studies.

Encapsulation and osmoprotection

For encapsulation of shoot tips two solutions were prepared, a 3% (w/v) Na - alginate (low viscosity 250 centipoise - cps) solution in Ca^{2+} - free liquid MS medium containing 30 g/l sucrose and a 100 mM CaCl_2 solution in MS medium. The pH of the encapsulation matrix was adjusted to 5.7 after the addition of sodium alginate. Both solutions were sterilized by autoclaving. For encapsulation shoot tips were transferred to the Na-alginate solution and then to the sterile CaCl_2 solution and beads (approximately 4-5 mm in diameter) were formed instantaneously (Fig. 1a). After 30 min of polymerization the CaCl_2 solution was drained off and the beads were washed using liquid MS medium without sucrose (pH 5.7). Further the beads were osmoprotected in liquid MS medium containing sucrose in various concentrations (0.25, 0.5, 0.75 and 1.0 M) for 24 h on a rotary shaker (98 rpm) at 24 ± 1 °C and 16 h light/8 h dark photoperiod.

Desiccation, cryopreservation and regrowth

Osmoprotected beads were blot dried using sterile filter paper, transferred to open Petri dishes (10 cm diameter) and desiccated in sterile laminar air-flow up to 6 h. During air desiccation the environmental conditions of the room were monitored for temperature (24 ± 1 °C) and relative humidity (RH $65 \pm 4\%$). At 1-h intervals desiccated beads were placed in 1.8 ml cryovials (5 beads/cryovial) and immersed into liquid nitrogen contained in a 25 l Dewar flask for 24 h. Rewarming was performed in water bath at 38 °C for 2 min and then shoot tips were transferred in Petri dishes on semisolid (5.5 g/l agar) MS medium for regrowth under the same light and temperature conditions as mentioned above.

Moisture content of the alginate beads

To determine the optimum desiccation time for high regrowth percentages following storage in liquid nitrogen the amount of water loss in the beads was monitored. For dry weight determination, ten beads for three replications were weighed and dried at 60 °C until constant weight was attained. The percentage of moisture content was expressed on fresh weight basis using the formula: $\text{MC} (\%) = [(\text{fresh weight} - \text{dry weight}) / \text{fresh weight}] \times 100$.

Rooting and acclimatization

For rooting, one node shoot segments (2-2.5 cm in length) from shoots regenerated following cryopreservation were transferred to MS medium supplemented with 1.5 mg/l N^6 -benzyladenine (BA) and various concentrations (0, 0.5, 1.0, 1.5 mg/l) of indole-3-butyric acid (IBA). For each auxin concentration 10 nodal explants were used. After six weeks roots were formed and plants were removed from *in vitro* growth conditions, washed (to remove agar) and planted in sterile perlite in 150 ml plastic cups stored in glass boxes (50 x 50 x 30 cm, length/width/height) covered with glass lids to ensure high humidity in the initial stages of the acclimatization period. First day under *ex vitro* growth conditions the lids were

removed for 1 hour, followed by one additional hour every day and after 10 days the plants were transferred to soil in pots and exposed to normal ambient light with daytime illumination at 22 ± 1 °C.

Assessment of recovery and statistical analyses

Survival and regrowth following cryopreservation was assessed 15 and respectively 30 days after rewarming and transfer to regeneration medium. For each treatment of the cryopreservation protocol three replicates of 10-12 explants were used and the experiments were performed twice. Encapsulated explants treated with sucrose and desiccated but not exposed to liquid nitrogen were used as controls. The frequency of survival (shoot tips which developed green tissue) and regrowth (shoot growth and development, expansion of leaves from the original shoot tip) were calculated according to the formulas:

$$\text{Survival} (\%) = (\sum \text{surviving shoot tips} / \sum \text{number of total shoot tips}) * 100$$

$$\text{Regrowth} (\%) = (\sum \text{regrowing shoot tips} / \sum \text{number of total shoot tips}) * 100$$

Data for rooting of shoots recovered from liquid nitrogen storage were recorded six weeks after transfer of node segments to rooting medium and was expressed as percentage of nodal explants forming roots from the total number of inoculated nodal segments. Acclimatization was expressed as the percentage of plants which survived and showed growth under *ex vitro* conditions from the total total number of plants transferred to pots.

The results were expressed as the mean \pm standard deviation (SD). Data expressed in percentages were subjected to arcsine transformation before statistical analysis and were analyzed by ANOVA using the Tukey's test for data comparison.

Results and discussion

Effects of osmoprotection on survival and regrowth of shoot tips

In preliminary experiments the regrowth ability of non-encapsulated and encapsulated tomato shoot tips was assessed. The non-encapsulated shoot tips started to grow after 3-4 days in culture and within 10 days the shoot formation was 100%. Encapsulation of explants did not significantly affect regeneration although shoot growth was slower and shoot formation ranged between 96% (cvs. 'Capriciu' and 'Siriana') and 100% (cvs. 'Pontica', 'Kristin' and 'Darsirius'). In our current study a significant decrease in survival and regrowth of non-cryopreserved shoot tips was observed with increasing concentration of sucrose (Tab. 1). Although, results obtained for survival can offer information about the efficiency of osmoprotection in early stages of recovery for establishment of a cryopreservation protocol essential is the recovery of shoot tips, therefore, only this parameter will be further considered. It was shown that preculture of shoot tips in sucrose plays an important role in the acquisition of resistance to desiccation and liquid nitrogen exposure (Suzuki *et al.*, 2006; Barraco *et al.*, 2011) increasing the efficiency of cryopreservation in many species (Padrò *et al.*, 2012; Kim *et al.*, 2012; Feng *et al.*, 2013; Le Bras *et al.*, 2014).

Tab. 1. Effect of preculture with different sucrose concentrations on survival and regrowth of non-cryopreserved tomato shoot tips

| Cultivar | Sucrose (M) | | | | |
|-------------|--------------------|-------------|-------------|-------------|--------------|
| | 0 | 0.25 | 0.5 | 0.75 | 1.0 |
| | Survival (% ± SD)* | | | | |
| 'Capriciu' | 100 ± 0.0a | 93.3 ± 5.7a | 89.6 ± 0.6a | 75.5 ± 7.6a | 61.8 ± 7.3a |
| 'Darsirius' | 100 ± 0.0a | 89.6 ± 0.6b | 86.7 ± 5.7b | 68.8 ± 1.9c | 51.8 ± 3.2d |
| 'Kristin' | 96.6 ± 5.7b | 89.6 ± 0.6b | 82.9 ± 5.1c | 68.8 ± 1.9c | 55.5 ± 9.6c |
| 'Pontica' | 100 ± 0.0a | 92.9 ± 6.1a | 83.3 ± 5.7c | 71.4 ± 5.7b | 58.8 ± 8.3b |
| 'Siriana' | 96.6 ± 5.7b | 86.2 ± 6.4a | 81.4 ± 1.9d | 53.7 ± 3.2d | 42.9 ± 2.5e |
| | Regrowth (% ± SD)* | | | | |
| 'Capriciu' | 96.6 ± 5.7b | 90.0 ± 0.0a | 85.9 ± 7.0b | 72.5 ± 4.4b | 48.1 ± 3.2de |
| 'Darsirius' | 100 ± 0.0a | 89.0 ± 0.6b | 80.0 ± 0.0d | 53.3 ± 5.7d | 48.1 ± 3.2de |
| 'Kristin' | 93.3 ± 5.7c | 86.0 ± 5.4c | 82.0 ± 6.5c | 54.8 ± 8.9d | 40.7 ± 6.4f |
| 'Pontica' | 93.3 ± 5.7c | 85.9 ± 5.1c | 83.3 ± 5.7c | 67.7 ± 1.9c | 55.5 ± 9.6c |
| 'Siriana' | 96.6 ± 5.7b | 82.9 ± 5.1c | 79.0 ± 1.2d | 40.7 ± 6.4e | 39.2 ± 5.5f |

*Data represent mean values ± SD. Values followed by the same letter within a column are not significantly different ($P \leq 0.05$).

Tab. 2. Effect of various concentrations of IBA on rooting of shoots regenerated from encapsulated and cryopreserved shoot tips after six weeks of culture

| Cultivar | IBA (mg/l) | Rooting rate (% ± SD)* | Number of roots (Nr ± SD)* | Length of roots (cm ± SD)* |
|-------------|------------|--------------------------|----------------------------|----------------------------|
| 'Capriciu' | 0 | 16.6 ± 1.0 ^f | 1.3 ± 0.5 ^d | 1.4 ± 0.4 ^e |
| | 0.5 | 58.3 ± 1.7 ^c | 7.6 ± 2.0 ^a | 3.0 ± 0.8 ^b |
| | 1.0 | 77.7 ± 1.5 ^a | 4.3 ± 2.5 ^c | 4.7 ± 0.8 ^b |
| | 1.5 | 38.8 ± 2.3 ^d | 3.0 ± 1.7 ^{cd} | 2.3 ± 0.7 ^c |
| 'Darsirius' | 0 | 19.4 ± 2.3 ^e | 1.6 ± 0.5 ^d | 1.7 ± 0.4 ^e |
| | 0.5 | 47.2 ± 1.5 ^d | 5.6 ± 0.5 ^{bc} | 2.0 ± 0.5 ^c |
| | 1.0 | 69.4 ± 2.5 ^a | 5.3 ± 1.1 ^c | 5.5 ± 1.0 ^a |
| | 1.5 | 61.1 ± 2.8 ^b | 2.6 ± 0.5 ^d | 2.5 ± 1.0 ^{bc} |
| 'Kristin' | 0 | 25.0 ± 1.7 ^e | 2.6 ± 0.5 ^d | 2.3 ± 0.7 ^c |
| | 0.5 | 41.6 ± 1.7 ^d | 7.0 ± 2.6 ^{ab} | 3.1 ± 1.0 ^b |
| | 1.0 | 63.8 ± 3.2 ^b | 6.6 ± 1.1 ^b | 7.4 ± 1.1 ^a |
| | 1.5 | 55.5 ± 3.2 ^c | 4.3 ± 1.5 ^c | 2.8 ± 0.2 ^b |
| 'Pontica' | 0 | 22.2 ± 1.1 ^e | 1.3 ± 0.5 ^d | 1.2 ± 0.3 ^e |
| | 0.5 | 33.3 ± 1.7 ^c | 6.3 ± 1.1 ^b | 3.2 ± 1.6 ^b |
| | 1.0 | 66.6 ± 2.0 ^{ab} | 5.6 ± 2.0 ^{bc} | 6.0 ± 1.3 ^a |
| | 1.5 | 47.2 ± 1.5 ^d | 4.6 ± 1.5 ^c | 3.1 ± 0.5 ^b |
| 'Siriana' | 0 | 27.7 ± 1.1 ^e | 2.6 ± 0.5 ^d | 1.2 ± 0.6 ^e |
| | 0.5 | 36.1 ± 1.5 ^d | 8.0 ± 1.0 ^a | 2.8 ± 1.0 ^b |
| | 1.0 | 58.3 ± 2.6 ^{bc} | 6.3 ± 0.5 ^b | 6.9 ± 1.2 ^a |
| | 1.5 | 50.0 ± 1.7 ^c | 4.3 ± 2.5 ^c | 3.3 ± 0.6 ^b |

Shoots regenerated from encapsulated, dehydrated and cryopreserved shoot tips were transferred to MS medium supplemented with IBA in various concentrations. Each value is a mean of three replicates ± SD. Values followed by the same letter within a column are not significantly different ($P < 0.05$).

Tolerance of encapsulated shoot tips to desiccation and cryopreservation

The highest regrowth in all cultivars was obtained for shoot tips treated either with 0.5 M or 0.75 M sucrose in combination with 3 or 4 h desiccation time (Fig. 2a-e). Non-dehydrated cryopreserved shoot tips did not survive regardless of genotype (Fig. 2a-e). The extent of sucrose treatment varied from 24 h (González-Arnáez *et al.*, 1993) to several days (Reed *et al.*, 2005). The initial water content of the osmoprotected beads was up to 84%, declined to 25-35% within the first 3 h of air drying and achieved 13-19% following 6 h of desiccation with consequences on shoot regrowth following liquid nitrogen storage in all cultivars. Our findings showed that the moisture content of the alginate beads is critical to ensure regrowth of cryopreserved tomato shoot tips (Fig. 2a-e). Combining osmoprotection of the beads in sucrose with their desiccation, a reduction of the water content was achieved to a level which allowed shoot regrowth following cryopreservation. To ensure high recovery rates after liquid nitrogen storage the moisture content of the beads had to be between 21-26% with a maximum regrowth (72% cv. 'Pontica') at 23% moisture content of the alginate beads (Fig. 2d). Although, the alginate bead moisture content that ensures high regeneration after cryopreservation is approximately 20%

(Engelmann *et al.*, 2008), high regrowth was obtained at lower or higher values of beads moisture content (Padrò *et al.*, 2012). The decline in regrowth of cryopreserved shoot tips with increasing desiccation is may be due to the osmotic shock at higher sucrose concentrations leading to decreased viability of encapsulated shoot tips after liquid nitrogen exposure (Rabba'a *et al.*, 2012). Following osmotic dehydration an extensive evaporative desiccation is necessary to achieve moisture content compatible with cryopreservation (Dumet *et al.*, 2002). Due to desiccation of explants most or all freezable water is removed from cells and vitrification of internal solutes takes place during rapid exposure to liquid nitrogen avoiding lethal intracellular ice crystallization (Engelmann, 2000). Sucrose treatment and the moisture content of alginate beads are key factors for successful application of encapsulation-dehydration (Gonzalez-Benito *et al.*, 1998). Cryopreserved shoot tips developed directly shoots and no callus formation was observed (Fig. 1b, d). Cryopreservation of tomato has been reported for wild tomato seeds (60% regrowth) (Zevallos *et al.*, 2013, 2014), shoot meristems (52% regeneration) (Grout *et al.*, 1978), and pollen (Karipidis and Douma, 2011).

Rooting and ex vitro acclimatization

Rooting occurred also on medium without IBA but was significantly reduced in all cultivars compared to the medium with auxin (Tab. 2). The highest rooting rates for all cultivars were obtained for shoots grown on MS medium supplemented with 1.0 mg/l IBA, whereas the highest number of roots was noted on medium with 0.5 mg/l IBA with significant differences among cultivars (Tab. 2). The acclimatization percentages for plants derived from cryopreserved shoot tips were high, ranging between 90 and 100% (cvs. 'Kristin' and 'Capriciu') with no significant differences between cultivars. The recovered plants showed growth in length, developed new leaves, were morphologically similar to the control plants and no visible malformations were observed in any of the tested cultivars. Rooting of *in vitro* grown shoots prior their transfer to soil in pots was a prerequisite for successful *ex vitro* acclimatization (Fig. 1e, f).



Fig. 1. Cryopreservation of tomato shoot tips: (a) alginate coated shoot tips; (b) regrowth of shoot tip (4 weeks after liquid nitrogen treatment), cst - cryopreserved shoot tip, rs - regrowing shoot; (c, d) shoots 6 weeks after liquid nitrogen treatment ('Darsirius' and 'Capriciu'), circle - dead shoot tip; (e) rooted shoots ('Kristin'); (f) acclimatized tomato plant ('Kristin'); Bar 0.5 cm

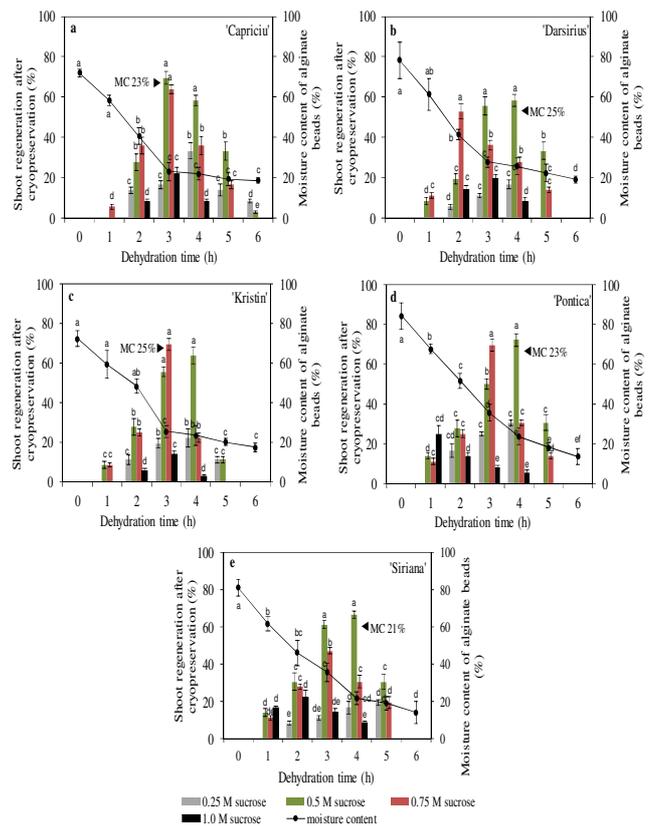


Fig. 2. Regrowth of encapsulated cryopreserved tomato shoot tips as a function of beads moisture content. Encapsulated shoot tips were osmoprotected in sucrose (0.25 M, 0.5 M, 0.75 and 1.0 M), desiccated in laminar air flow up to 6 h followed by immersion in liquid nitrogen. (a) 'Capriciu'; (b) 'Darsirius'; (c) 'Kristin'; (d) 'Pontica'; (e) 'Siriana'; MC = moisture content. Arrows indicate the percent (%) MC of alginate beads corresponding to the highest regeneration rate of each cultivar. Results are expressed as means of three replicates \pm SD. Different letters indicates significant differences ($P \leq 0.05$)

The conditions for acclimatization of tissue cultured plants have been reviewed (Pospíšilová *et al.*, 1999; Chandra *et al.*, 2010). Important issues during acclimatization include, the exposure of plants to light (which enables them to establish photosynthesis), appropriate temperatures without major air fluctuations (Lesar *et al.*, 2012) or the control of relative humidity (Jang *et al.*, 2011). Plant acclimatization following *in vitro* culture can be improved by application of abscisic acid to the last subculture (Pospíšilová *et al.*, 2009), using an alternate substrate (Deb and Imchen, 2010).

Conclusions

A simple and efficient protocol for shoot tips cryopreservation of five *Lycopersicon* cultivars using encapsulation-dehydration was developed in the present study. Shoots recovered from liquid nitrogen storage were rooted and successfully acclimatized to *ex vitro* growth conditions. Further cryopreservation techniques must be evaluated to increase regrowth percentages.

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References

- Barraco G, Sylvestre I, Engelmann F (2011). Comparing encapsulation-dehydration and droplet-vitrification for cryopreservation of sugarcane (*Saccharum* spp.) shoot tips. *Sci Hortic* 130:320-324.
- Bauchet G, Causse M (2012). Genetic diversity in tomato (*Solanum lycopersicum*) and its wild relatives, p. 133-162. In: Caliskan M (Ed.). *Genetic Diversity in Plants*, InTech.
- Bhatia P, Ashwath N, Senaratna T, Midmore D (2004). Tissue culture studies of tomato (*Lycopersicon esculentum*). *Plant Cell Tiss Org Cult* 78:1-21.
- Chandra S, Bandopadhyay R, Kumar V, Chandra R (2010). Acclimatization of tissue cultured plantlets: from laboratory to land. *Biotech Lett* 32:1199-1250.
- Cruz-Cruz CA, González-Arnao MT, Engelmann F (2013). *Biotechnology and Conservation of Plant Biodiversity*. Resources:73-95.
- Deb CR, Imchen T (2010). An efficient *in vitro* hardening of tissue culture raised plants. *Biotechnology* 9:79-83.
- Dumet D, Grapin A, Bailly C, Dorion N (2002). Revisiting crucial steps of an encapsulation/desiccation based cryopreservation process: importance of thawing method in the case of *Pelargonium* meristems. *Plant Sci* 163:1121-1127.
- Ellis RH, Roberts EH (1981). The quantification of ageing and survival in orthodox seeds. *Seed Sci Tech* 9:373-409.
- Engelmann F (2000). Importance of cryopreservation for the conservation of plant genetic resources, p. 8-20. In: Engelmann F, Takagi H (Eds.). *Cryopreservation of Tropical Plant Germplasm - Current Research Progress and Applications*, JIRCAS, Tsukuba/IPGRI, Rome.
- Engelmann F (2011). Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cell Dev-Pl* 47:5-16.
- Engelmann F, Gonzalez-Arnao MT, Wu Y, Escobar R (2008). The development of encapsulation-dehydration, p. 59-68. In: Reed BM (Ed.). *Plant Cryopreservation: A Practical Guide*, Springer.
- Fabre J, Dereuddre J (1990). Encapsulation-dehydration: a new approach to cryopreservation of *Solanum* shoot tips. *Cryo-Lett* 11:413-426.
- Feng CH, Cui ZH, Li BQ, Chen L, Ma YL, Zhao YH, Wang QC (2013). Duration of sucrose preculture is critical for shoot regrowth of *in vitro*-grown apple shoot-tips cryopreserved by encapsulation-dehydration. *Plant Cell Tiss Org Cult* 112:369-378.
- González-Arnao MT, Engelmann F, Huet C, Urrea C (1993). Cryopreservation of encapsulated apices of sugarcane: effect of freezing procedure and histology. *Cryo-Lett* 14:303-308.
- González-Arnao MT, Engelmann F (2006). Cryopreservation of plant germplasm using encapsulation-dehydration technique: review and case study on sugarcane. *Cryo-Lett* 27:155-168.
- Gonzalez-Benito ME, Nuñez-Moreno Y, Martin C (1998). A protocol to cryopreserved nodal explants of *Antirrhinum microphyllum* by encapsulation-dehydration. *Cryo-Lett* 19:225-230.
- Grout BW, Crisp PC (1995). Cryopreservation of germplasm of tomato, p. 371-380. In: Bajaj YPS (Ed.). *Biotechnology in Agriculture and Forestry* 32, *Cryopreservation of Plant Germplasm I*, Springer.
- Grout BW, Westcott RJ, Henshaw GG (1978). Survival of shoot meristems of tomato seedlings frozen in liquid nitrogen. *Cryobiology* 15:478-483.
- Gubiš J, Lajchová Z, Faragó J, Jureková Z (2004). Effect of growth regulators on shoot induction and plant regeneration in tomato (*Lycopersicon esculentum* Mill.). *Biol Bratislava* 59:405-408.
- Harding K, Johnston JW, Benson EE (2009). Exploring the physiological basis of cryopreservation success and failure in clonally propagated *in vitro* crop plant germplasm. *Agric and Food Sci* 18:103-116.
- Jang Y, Goto E, Ishigami Y, Mun B, Chun C (2011). Effects of light intensity and relative humidity on photosynthesis, growth and graft-take of grafted cucumber seedlings during healing and acclimatization. *Hort Environ Biotechnol* 52:331-338.
- Karipidis CH, Douma D (2011). Tomato pollen storage at freeze and cryogenic temperature – effects on viability and fecundity. *Acta Hort* 908:257-263.
- Kaviani B (2010). Cryopreservation by encapsulation-dehydration for long-term storage of some important germplasm: seed of lily (*Lilium ledebourii* (Baker) Bioss.), embryogenic axe of persian lilac (*Melia azedarach* L.) and tea (*Camelia sinensis* L.). *Plant Omics J* 3:177-182.
- Kaviani B (2011). Conservation of plant genetic resources by cryopreservation. *Australian J of Crop Sci* 5:778-800.
- Kim HH, Popova E, Shin DJ, Yi JY, Kim CH, Lee JL, Yoon MK, Engelmann F (2012). Cryobanking of Korean *Allium* germplasm collections: results from a 10 year experience. *Cryo-Lett* 33:45-57.
- Lambardi M, Fabbri A, Caccavale A (2000). Cryopreservation of white poplar (*Populus alba* L.) by vitrification of *in vitro* grown shoot tips. *Plant Cell Rep* 19:213-218.
- Le Bras C, Le Besnerais L, Hamama L, Grapin A (2014). Cryopreservation of ex-vitro-grown *Rosa chinensis* 'Old Blush' buds using droplet-vitrification and encapsulation-dehydration. *Plant Cell Tiss Org Cult* 116:235-242.
- Lesar H, Hlebec B, Čeranič N, Kastelec D, Luthar Z (2012). Acclimatization of terrestrial orchid *Bletilla striata* Rehb.f. (*Orchidaceae*) propagated under *in vitro* conditions. *Acta Agric Slovenica* 99:69-75.

- Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473-497.
- Ozudogru EA, Kirdok E, Kaya E, Capuana M, Benelli C, Engelmann F (2011). Cryopreservation of redwood (*Sequoia sempervirens*) in vitro buds using vitrification-based techniques. *Cryo-Lett* 32(2):99-110.
- Padrò MDA, Frattarelli A, Sgueglia A, Condello E, Damiano C, Caboni E (2012). Cryopreservation of white mulberry (*Morus alba* L.) by encapsulation-dehydration and vitrification. *Plant Cell Tiss Org Cult* 108:167-172.
- Pospišilová J, Tichá I, Kladaček P, Haisel D, Plzánková S (1999). Acclimatization of micropropagated plants to *ex vitro* conditions. *Biol Plant* 42:481-497.
- Pospišilová J, Synková H, Haisel D, Batková-Spoustová P (2009). Improvement of *ex vitro* transfer of tobacco plantlets by addition of abscisic acid to the last subculture. *Biol Plant* 53:617-624.
- Rabba'a MM, Shibli RA, Shatnawi MA (2012). Cryopreservation of *Teucrium polium* L. shoot-tips by vitrification and encapsulation-dehydration. *Plant Cell Tiss Org Cult* 110:371-382.
- Reed BM, Schumacher L, Dumet D, Benson E (2005). Evaluation of a modified encapsulation-dehydration procedure incorporating high sucrose pretreatments for the cryopreservation of *Ribes* germplasm. *In Vitro Cell Dev-Pl* 41:431-436.
- Roberts EH, Ellis RH (1984). The implications of orthodox seeds during storage for genetic resources conservation, p. 18-35. In: Holden JHW, Williams JT (Eds.). *Crop Genetic Resources and Evaluation*, George Allen & Unwin Ltd.
- Sakai A, Engelmann F (2007). Vitrification, encapsulation-vitrification and droplet-vitrification: a review. *Cryo-Lett* 28:151-172.
- Stanwood PC (1985). Cryopreservation of seed germplasm for genetic conservation, p. 199-226. In: Kartha KK (Ed.). *Cryopreservation of Plant Cells and Organs*. CRC Press, Boca Raton.
- Suzuki M, Ishikawa M, Okuda, H, Noda K, Kishimoto T, Nakamura T, Ogiwara I, Shimura I, Akihama T (2006). Physiological changes in gentian axillary buds during two-step preculture with sucrose that conferred high levels of tolerance to desiccation and cryopreservation. *Ann Bot* 97:1073-1081.
- Zevallos B, Cejas I, Valle B, Yabor Y, Aragón C, Engelmann F, Martínez ME, Lorenzo JC (2013). Short-term liquid nitrogen storage of wild tomato (*Solanum lycopersicum* Mill.) seeds modifies the levels of phenolics in 7 day-old seedlings. *Sci Hortic* 160:264-267.
- Zevallos B, Cejas I, Engelmann F, Carputo D, Aversano R, Scarano MT, Yanes E, Martínez-Montero M, Lorenzo JC (2014). Phenotypic and molecular characterization of plants regenerated from non-cryopreserved and cryopreserved wild *Solanum Lycopersicum* Mill. seeds. *Cryo-Lett* 35:216-225.
- Wang QC, Panis B, Engelmann F, Lambardi M, Valkonen JPT (2009). Cryotherapy of shoot tips: a technique for pathogen eradication to produce healthy planting materials and prepare healthy plant genetic resources for cryopreservation. *Ann App Biol* 154:351-363.