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REVIEW

# Chrysanthemum low-temperature storage and cryopreservation: a review

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**Abstract** Chrysanthemum (*Dendranthema* × *grandiflora* (Ramat.) Kitamura) is an ornamental plant that responds well to in vitro growth conditions. This receptivity makes it a particularly attractive target for low-temperature storage and cryopreservation studies. This review examines in detail the protocols thus far used to achieve the short- to long-term low-temperature and cryostorage of important chrysanthemum germplasm. Occasionally, medicinal chrysanthemum species have also been cryostored, and these studies are also examined in detail. Since chrysanthemum is sensitive to both osmotic stress and chemical toxicity of vitrification solutions, a generalized protocol for the cryopreservation of chrysanthemum apical or axillary shoot tips is proposed: excision of apical or axillary shoot tips after 4 or 7 weeks, respectively, from final subculture; progressive preculture with 10 % sucrose for 31 h, 17.5 % sucrose for 17 h, then 25 % sucrose for 7 h; osmoprotection with 17.5 % glycerol + 17.5 % sucrose for 40 min; cryoprotection with PVS3 (50 % glycerol + 50 % sucrose) vitrification solution for 60 min (axillary) or 90 min (apical); cooling and warming using aluminium foil strips; unloading with 30 % sucrose for 40 min. When smaller axillary shoot tips are used, cryoprotection of samples with 37.5 % glycerol + 15 % DMSO + 15 % ethylene glycol + 22.5 % sucrose at 0 °C for about 60 min can be applied.

**Keywords** Cold storage · Cryostorage · Encapsulation · In vitro storage · Liquid nitrogen · Plant growth regulators

**Abbreviations**

| | |
|---|---|
| EG | Ethylene glycol |
| LN | Liquid nitrogen |
| LNC | Liquid nitrogen control (dehydrated control) |
| LS | Loading solution |
| LTS | Low-temperature storage |
| PVS2 | Plant vitrification solution 2 |
| PVS3 | Plant vitrification solution 3 |
| SIM | Shoot induction medium |
| Synseed | Synthetic seed |
| VS | Vitrification solution |

*In memoriam*: We dedicate this review to the legend of Prof. Akira Sakai, who passed away on October 5, 2013. Prof. Sakai advanced our knowledge on cryopreservation for plant tissues, including of chrysanthemum.

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## The importance of chrysanthemum and its response in vitro

The preservation of ornamental chrysanthemum (*Dendranthema grandiflorum* Ramat. Kitamura, syn. *Chrysanthemum morifolium* Ramat.; Shinoyama et al. 2006), would benefit from techniques to preserve tissues over time since the plant is an annual and is primarily propagated by

vegetative means. A sub-set of biotechnology, low-temperature storage (LTS), cryopreservation and synthetic seed (synseed) technology use in vitro culture to establish the short-, mid- and long-term storage of useful germplasm. However, since in vitro culture of chrysanthemum is very well explored, and with dozens of protocols published for individual genotypes (Teixeira da Silva 2003a, 2004a; Teixeira da Silva et al. 2013), exploring LTS, cryopreservation—the focus of this review—and synseed technology (Sharma et al. 2013) is somewhat facilitated.

## Low-temperature storage studies in chrysanthemum

LTS has applications in plant improvement, production and conservation programmes (Ashmore 1997). Decreasing the storage temperature (usually at 4–5 °C) reduces the growth rate of cultures, thus allowing maintaining them without subcultures for extended durations. As a slow growth approach, LTS often includes alterations to medium (e.g., lower osmotic strength through the application of osmotic agents) and culture conditions (reducing temperature, light intensity and photoperiod). However, the deterioration of stored materials can also be influenced by their morphology and physiological state (Ozudogru et al. 2010). New varieties can thus be conserved, ready for multiplication, while in vivo tests are being performed. In commercial laboratories, storing at low temperature varieties, which are not requested for a given period allows optimizing production management. Finally, LTS allows maintaining large numbers of varieties/species in germplasm conservation programmes.

Bannier and Steponkus (1972) conducted the first LTS study in 'Giant #4 Indianapolis White' chrysanthemum, in which callus cultures could be stored at −3.5 °C for 28 days when placed in MS medium containing 10 % (w/v) sucrose. LTS is already well established for chrysanthemum (Fukai et al. 1988). It is a relatively simple and easy technique since chrysanthemum roots easily in vitro. Rooted plantlets of 'Lineker' and 'Shuhou-no-Chikara' cultivars could withstand cold temperatures (4–10 °C) for several months (up to 1 year) with near-perfect regeneration when returned to control conditions (Teixeira da Silva unpublished data). This claim is supported by findings by Roxas et al. (1995) who found that shoots with one newly expanded leaf could be preserved on plant growth regulator-free Murashige and Skoog (MS; 1962) medium for up to 24 months at 5 °C. Budiarto (2009) found that shoot survival and plantlet viability decreased linearly from 2 to 12 months of LTS at 2 °C. Trifunović et al. (2007) found that storage of in vitro plantlets of varieties 'Reagan Sunny' and 'White Spider' for 2, 4 or 6 months at 4 °C did not significantly affect shoot or root formation, although 4 or 6 months storage significantly negatively impacted plantlet height (Fig. 1).

Liu and Gao (2010) investigated the effect of temperature, growth retardants, osmotic pressure and culture medium on in vitro conservation of *Chrysanthemum cinerariifolium* (Trev.) Vis. Conservation of germplasm as test tube plantlets (age and developmental stage undefined) increased when temperature was lowered from 25 to 4 °C, when sucrose concentration was reduced from 3 to 2 % and when 2 mg/l abscisic acid (ABA) were added to the medium. Under these optimized conditions, plantlets could be conserved up to 6 months at 4 °C (Table 1).

## Synthetic seed technology in chrysanthemum

Synthetic seeds (synseeds) refer to artificially encapsulated somatic embryos, shoot buds or any other meristematic tissues that can be used as functional mimic seed for sowing, possesses the ability to convert into a plant under in vitro or ex vitro conditions, and can be stored (Sharma et al. 2013). Synseed technology has always been envisaged for both the short-term and long-term storage of plant

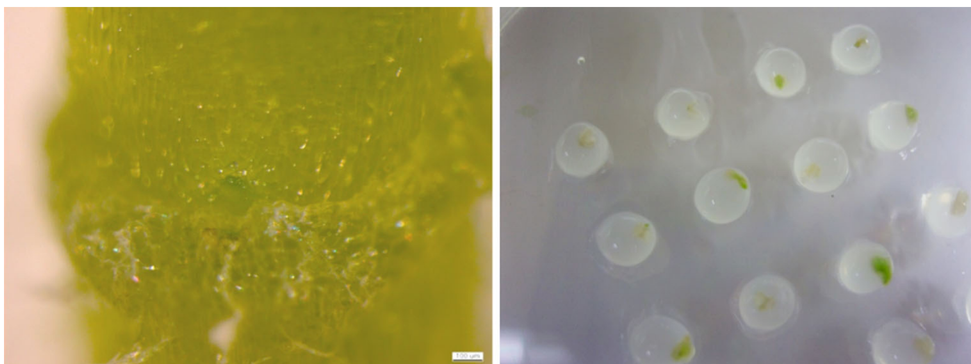

**Fig. 1** Seven-week subcultured axillary shoot tip after culture of 4-day-old nodal segment containing a single axillary bud (*left*) and encapsulated 4-week-old subcultured apical shoots of chrysanthemum 'Peak' (*right*). Unpublished photos (Haenghoon Kim)

**Table 1** Summary of chrysanthemum cryopreservation protocols

| Species and/or cultivar(s) | Explant cryopreserved | Optimized protocol (cryopreservation and subsequent regeneration) | References |
|---|---|---|---|
| Shuhounotikara then applied to 2 cultivars (Kenrokukougiku, Parliament), 12 species and 2 interspecific hybrids; Apricot Marble (1994) | Shoot tips (size and age of mother material NR) | To test for toxicity to DMSO, leaf-induced callus was tested in the presence of 5, 10, 15 or 20 % DMSO in MS + 1.0 mg/l BA + 2.0 mg/l NAA, 4 % sucrose and 0.8 % agar under continuous light at 19 μmol $m^{-2}$ $s^{-1}$ at 25 °C for 60 days. After establishing that 5 % DMSO was least toxic to callus, shoot tips (ca. 0.5–0.7 mm) were precultured on SIM + 5 % DMSO for 2–14 days. Pre-conditioned shoot tips were submerged in a cryoprotectant solution (10 % DMSO + 3.0 % sucrose) at a rate of 0.2 °C/min from 0 to −40 °C (i.e., CRF), then immersed and stored in LN for 15–30 min. Straws were rapidly thawed in a water bath at 25–30 °C. Shoot tips were rinsed with sterilized water and replated on SIM. Shoot formation was assessed after 60 days. In the 1991 study, Shuhounotikara thawed shoot tips were transferred to 3 % Hyponex + 2 % sucrose + 0.8 % agar for 60 days. 3-cm terminal shoots were transferred directly to Metromix in a greenhouse | Fukai (1990); Fukai and Oe (1990); Fukai et al. (1991, 1994) |
| *Chrysanthemum cinerariaefolium* (high pyrethrin-producing line) | Capitulum-derived callus | Callus 5 mm in diameter was exposed to sucrose at a high concentration (18 g/l) of sucrose for 30 days to provide a cryoprotective effect that led to callus with the best regrowth and viability. 250 mg of sucrose-treated callus was transferred to chilled liquid CIM (see Table 2) containing 5 % DMSO, added gradually over 30 min and left for 1 h. This callus was then frozen at a rate of 1 °C/min from 0 to −20 °C, stored for 324 h at this temperature, then immersed and stored in LN for 30 min. Cryotubes were thawed at 40 °C for 5 min. When DMSO was used, cryopreserved callus was then exposed to ½MS containing CIM with 30 g/l sucrose less every 30 min. Filter-sterilized callus was grown in liquid ½MS CIM for 1 day, then transferred to solid ½MS CIM. TTC tests for viability and quantitative analysis of pyrethrin content by HPLC were conducted | Hitmi et al. (1997, 1999b, 2000b) |
| *Chrysanthemum cinerariaefolium* (high pyrethrin-producing line) | Apical buds with 1–3 young leaves and a base (stem) 2 ± 0.3 mm long derived from 1-month-old in vitro commercial cultures | To establish the optimal conditions for pre-freezing tolerance, shoot tips were exposed to two treatments: (1) air-drying for 1–4 h after treatment, on ice, in 5, 7.5 or 10 % DMSO for 1, 3, 5 or 7 h; (2) preculture of apices to solid medium containing 0.45, 0.55 or 0.65 M sucrose (for an unspecifid amount of time) then transferred gradually over 30 min to a liquid solution with the same sucrose concentration + 7.5 % DMSO. Cryotubes containing 8 apices per tube were plunged directly into LN for at least 1 h. Cooling of cryotubes and transfer of explants to regeneration medium followed the same protocol as used for callus in the 1997 and 1997a studies | Hitmi et al. (1999a, 2000a) |
| Shuhounochikara | Shoot tips | Shoot tips derived from the treatments explained in Table 2 were first precultured in 0.3 M sucrose for 16 h (abstract) or 0.3 M sucrose at 10 °C in the dark for 3 days. Three protocols were established: (1) new ED; (2) V; (3) standard ED. In protocol 1, shoot tips were then placed in liquid calcium-free MS medium + 2 % (w/v) Na-alginate + LS (2 M glycerol + 0.4 M sucrose) then encapsulated and osmoprotected with 2 M glycerol + 0.4 M sucrose for 1 h. The mixture, including shoot tips, were dispensed into 0.1 M $CaCl_2$ + LS for 1 h at 25 °C. 20–30 osmoprotected and encapsulated beads were plated in one Petri dish with ~50 g silica gel, made air-tight with Parafilm®, dehydrated for 3 h at 0 °C then plunged directly into LN at 10 shoot tips/1.8 ml cryotube and maintained in LN for at least 1 h. Cryotubes were rapidly rewarmed at 35 °C for 3 min to prevent crystallization. Cryopreserved shoot tips were placed onto filter paper in Petri dishes for 1 day, then transferred to SIM, although it is not clear if the Ca-alginate bead was also cultured, or removed prior to re-culture. In protocol 2, precultured shoot tips were osmoprotected with LS for 20 min at 25 °C then dehydrated with PVS2 solution for 20 min at 25 °C. Explant density in cryotubes and all steps up to cryopreservation were the same as protocol 1. After rewarming by vigorous shaking at 40 °C for 1 min, PVS2 solution was drained off and replaced with MS + 1.2 M sucrose for 20 min and shoot tips were transferred directly to SIM. In protocol 3, precultured shoot tips were encapsulated in 2 % Na-alginate beads containing 0.4 M sucrose. Encapsulated shoot tips were treated in liquid MS medium containing 0.8 M sucrose for 16 h at 25 °C and then subjected to silica gel desiccation for 9 h. LN treatment, rewarming and reculture were as for protocol 1 | Sakai et al. (2000) |

**Table 1** continued

| Species and/or cultivar(s) | Explant cryopreserved | Optimized protocol (cryopreservation and subsequent regeneration) | References |
| --- | --- | --- | --- |
| Escort, Snowdon, Elegance Weiss, White Cindy, Trumpf Rot, Branilo, Branglow, Astro, Heidi Weiss | Shoot tips from 4-week-old mother plants (3–4 mm long, 1–2 mm thick with two leaf primordia) | Three cryopreservation techniques (CRF, ED, URF) were applied to Escort while V was applied to all nine cultivars. In the CRF protocol, shoot tips were cultured on SIM for 24 h followed by a second 24-h incubation period on SIM containing 0.3, 0.5 or 0.75 M sucrose. Shoot tips were cryoprotected in SIM + 10 % DMSO for 2 h at RT. Shoot tips were incubated with 1 ml of this latter medium in 2-ml cryovials, which were placed in a programmable freezer and cooled down at rates of −0.25, −0.5 and −1 °C/min to −40 °C then transferred into LN. Cryovials were rewarmed by incubating in a 40 °C water bath and re-cultured on SIM. In the ED protocol, as for the CRF protocol, there was a 24-h soaking in SIM medium. Shoot tips were immobilized by immersion in $Ca^{2+}$-free medium + 3 % alginic acid. This solution + shoot tips were added, drop by drop to medium (SIM?) containing 0.1 M $CaCl_2$ with gentle stirring for 30 min. Medium was poured off and alginic beads were washed three times with SIM. Beads were incubated for 4 h on SIM containing 0.3, 0.5 or 0.75 M sucrose. Beads were then dried off on dry filter paper and placed under a vertical laminal air-flow bench for 7 h. Every hour, beads were placed in a cryovial and plundeg into LN. Beads were thawed by adding them to SIM at RT. In the V protocol, shoot tips were cultured on SIM for 24 h followed by a second 24-h incubation period on SIM + 0.5 M sucrose. A third incubation period involved exposure to 20, 60 or 100 % of PVS2 for 5, 10, 15, 20, 25 or 30 min. Shoot tips were then added to 4-μl droplets (one shoot tip per droplet?) of PVS2 solution on small sheets (2 × 0.8 mm) of thin aluminum foil and immersed directly in LN in 2-ml cryovials. Shoot tips were rewarmed when aluminium foil was submerged in 15 ml SIM at RT. In the URF protocol, shoot tips were cultured on SIM for 24 h then cryoprotected in SIM + 7.5 % DMSO for 2 h. Shoot tips were then plunged one by one into cryovials containing LN using a hypothermic needle. Cryovials containing shoot tips were rewarmed in basal MS medium containing 50 g/l sucrose at 35 °C for 30 min. Shoot tips were then covered with a liquid drop of low-melting agarose (1 %) inside Petri dishes. After the agarose solidified, liquid SIM (volume NR) was added and left for 4 weeks in translucent plastic boxes. In all four protocols, shoot growth was assessed from shoot tips at 4 weeks or from callus at 8 weeks | Halmagyi et al. (2004) |
| Pasodoble (17 lines) | Shoot tips derived from adventitious buds from nodal segments | In the 2005 paper, V and ED were tested while in the 2011 paper, only ED was tested. For ED (2005 and 2011 papers), nodal segments were precultured on SIM for 3 weeks. 2-mm long shoot tips from adventitious shoots were cultured on semi-solid MS (agar conc. NR) + 0/3 M sucrose at 5 °C in darkness for 3 days. Pretreated shoot tips were immersed in a 3 % alginate solution prepared with MS liquid medium (without $CaCl_2$) + 0.4 M sucrose. Drops of this solution, each containing a shoot tip, were dispensed in liquid MS medium containing 100 mM $CaCl_2$ + 0.4 M sucrose. Alginic beads were maintained in this solution for ~30 min then cultured in liquid MS medium containing 0.8 M sucrose, at 130 rpm, for 16–18 h, removed, dabbed on filter paper and placed in a jar containing silica gel (50 g/5–15 beads), for 5 h. Beads were then added to cryovials (number of bead/cryovial NR) and rapidly immersed in LN for at least 1 day. Cryovials were rewarmed in a 35 °C water bath for 3 min and alginic beads were then cultured in 6-cm Petri dishes on SIM for 30 days. For the V protocol, pre-treated shoot tips (see ED protocol) were immersed in LS (MS liquid medium +2 M glycerol + 0.4 M sucrose), for 20 min at RT (0.7 ml for 4–16 apices, originating from the same shoot, in one 2-ml cryovial). Loading solution was removed from the cryovial and rapidly replaced by filter-sterilized PVS2 and left at RT for 20 min. Cryovials were rapidly immersed in LN for at least 1 day, rewarmed in a 40 °C water bath for 1 min then shaken vigorously. PVS2 solution was removed and 1.5 ml of MS liquid medium + 1.2 M sucrose was added for 20 min. Shoo tips were cultured in 6-cm Petri dishes on SIM then sub-cultured to fresh SIM after 24 h. After 30 days developed shoots were induced to form plantlets on plantlet maintenance medium | Martín and González-Benito (2005); Martín et al. (2011) |

**Table 1** continued

| Species and/or cultivar(s) | Explant cryopreserved | Optimized protocol (cryopreservation and subsequent regeneration) | References |
|---|---|---|---|
| *Chrysanthemum morifolium* 'Peak' then 'Baekma' | Apical shoots (1.5–2.0 mm long) or axillary shoot tips (1.2–1.5 mm long) from 3 to 4-day-old nodal segments | A DV protocol. Optimal stage for explant isolation was different, namely 4 weeks for apical shoots and 7 weeks for axillary shoot tips. Apical or axillary shoot tips were precultured step-wise in liquid sucrose-enriched medium (0.3, 0.5 and 0.7 M for 31, 17 and 7 h, respectively). Precultured explants were treated for 40 min with C4-35 % LS made up of (w/v) 17.5 % glycerol + 17.5 % sucrose, then dehydrated with PVS3 vitrification solution (w/v, 50 % glycerol + 50 % sucrose) for 60 min (axillary shoot tips) or 90 min (apical shoots). Explants were cryopreserved by direct immersion in LN in minute drops of PVS3 attached to aluminium foil strips. The optimal age of donor plants was 4–5.5 weeks for apical shoots and 7 weeks for axillary shoot tips, resulting in 81.9 and 84.9 % post-cryopreservation regeneration, respectively. Plants regenerated from cryopreserved samples showed no phenotypic abnormalities and similar profiles of relative DNA content were recorded for control and cryopreserved plants. A single-branch type variety, 'Baekma' produced 90 % post-cryopreservation regeneration with direct and rapid shoot development | Kim et al. (2009a, b); Lee et al. (2011a, b) |
| *Dendranthema morifolium* + 5 other species (*Ajanica pacificum*, *D. grandiflorum* 'Jinba', *D. indicum* 'Lishui yeju', *Dahlia pinnata* Cav.) | Shoot tips (1–2 mm) from 4-week old plantlets | A Vitrification (V) procedure. Shoot tips were precultured with 0.4 M sucrose for 2–3 days in the dark at 4 °C, osmoprotected with 2 M glycerol + 0.4 M sucrose for 20 min, cryoprotected with PVS2 for 15 min on ice, cooled and warmed using cryovials, then unloaded with 1.2 M sucrose for 20 min. With this protocol post-cryopreservation survival rate of 85.7 % was obtained. When procedures were compared, V produced highest survival (85.7 %), over EV (50.0 %) or encapsulation-programmed cooling (0 %). Shoots that survived developed into plantlets via direct regeneration or indirectly via multiple shoot formation through a callus phase. The optimized V protocol was applied to five other chrysanthemum species (*Ajanica pacificum*, *D. grandiflorum* 'Jinba', *D. indicum* 'Lishui yeju', *Dahlia pinnata* Cav.), with a survival rate of 6–84 % | Liu et al. (2009) |
| Indianapolis | Shoot tips (0.5–1.0 mm long) from transgenic and non-transgenic 4-month-old plantlets, 15 days after their last sub-culture on SIM | A V procedure. Two trehalose-accumulating transgenic lines (35S-8 "TL1", 35S-19 "TL2") were compared with non-transformed WT ("control"). The highest post-cryopreservation regeneration of 48–67 % for transgenic lines and 33–36 % for WT were obtained in preculture with 0.3 M sucrose for 4 days, osmoprotection with 2 M glycerol + 0.4 M sucrose for 20–30 min, cryoprotection with PVS2 or PVS3 for 40 min at RT. The transgenic lines, especially 35S-19 (TL2) produced 23–42 % regeneration after cryoprotection (-LN) with PVS2 or PVS3 for 40 min, since they were damaged due to the toxicity. However, their post-cryopreservation (+LN) regeneration was unusually high, i.e., 48–67 % with PVS2 and 52–54 % with PVS3. While control (WT) apices cryoprotected (-LN) with PVS2 or PVS3 for 40 min produced 86 and 72 % regeneration, respectively. After cryopreservation (+LN) regeneration decreased to 33 and 36 %, respectively. In an analysis of genetic stability using eight RAPD markers, a total of 101 monomorphic loci were amplified from 20 samples per primer. There was no variation in RAPD banding profiles from cryopreserved and non-cryopreserved plantlets. More importantly, genetic engineering can be used to acquire tolerance to the negative impacts of cryopreservation | Osorio-Saenz et al. (2011) |
| Dalmatian chrysanthemum (*Tanacetum cinerariifolium*) line '28v-75' + 6 other lines | Shoot tips were dissected from cold-hardened shoot cultures at 5 °C for 20–40 days | An EV procedure using aluminum cryo-plates. After preculture with 0.5 M sucrose medium at 5 °C for 2 days, shoot tips were placed on aluminum cryo-plates and embedded in an alginate gel. After loading with LS (2 M glycerol + 1.4 M sucrose) for 30 min, LS was replaced with PVS 7 M (30 % glycerol + 19.5 % ethylene glycol + 0.6 M sucrose) and dehydrated for 40 min. This procedure showed post-cryopreservation regrowth of 77 % and was further applied to six additional lines (19v-KO4, 26v-84, 28v-173, 29v-289, 36v-34, 41v-2) with a regrowth range of 65–90 %. The authors developed an aluminum cryo-plate technique which had 10 wells making the process more user-friendly and decrease the cytotoxicity by using less concentrated and less toxic VS (PVS 7 M) and encapsulation of shoot tips | Yamamoto et al. (2011) |

**Table 1** continued

| Species and/or cultivar(s) | Explant cryopreserved | Optimized protocol (cryopreservation and subsequent regeneration) | References |
|---|---|---|---|
| Lady Orange, Lady Salmon mutants | 2-week-old shoot tips (size NR) | An ED protocol. After preculture with MS medium (and modified vitamins) supplemented with 0.06 M sucrose for 7 days, then transferred to the same medium with 10 μM ABA for another 7 days. After encapsulation in 3 % Na-alginate (hardened with 0.1 M $CaCl_2$ for 45 min), beads were rinsed 3× with SDW, then exposed to a sucrose gradient: 0.3 M (48 h), 0.5 M (48 h), 0.7 M (48 h), 0.9 M (24 h) at 130 rpm. After a 3-h desiccation until 40 % fresh weight was achieved, encapsulated shoot tips were dipped in LN for 1 h, then thawed at 38 °C for 1 h. SIM was MS medium (+modified vitamins), 0.09 M sucrose and 0.25 mg/l kinetin. | Zalewska and Kulus (2013) |
| *Chrysanthemum morifolium* Japanese Red + 5 other genotypes (Fall Color, Xizi Qiuzhuang, Roma Red, Jinba, Hangju) | Shoot tips (2.0 mm) containing 5–6 leaf primordia were excised from elongated buds | A DV procedure. After preculture with MS medium supplemented with 0.5 M sucrose for 1 day, shoot tips were loaded with 0.4 M sucrose + 2 M glycerol for 20 min at RT. Shoots were dehydrated with PVS2 for 30 min at 0 °C and then transferred onto droplets of aluminium foil strips, prior to a direct immersion in LN for 1 h. Thawed shoot tips were unloaded with 1.2 M sucrose for 20 min and postcultured on MS medium supplemented with 0.05 mg/l $GA_3$ in the dark for 3 days and then transferred to standard culture conditions. The DV procedure resulted in 83 and 43 % shoot regrowth rates for *C. morifolium* 'Japanese Red' and 'Xizi Qiuzhuang', respectively with an average rate of 68 % across the six genotypes tested. Simple sequence repeats and flow cytometry analysis as well as histological analysis showed no significant differences between the two highest regenerating genotypes | Wang et al. (2014) |

ABA, abscisic acid; BA, $N^6$-benzyladenine (also represents BAP or 6-benzylaminopurine if so reported in the literature originally, according to Teixeira da Silva 2012); $CaCl_2$, calcium chloride; CRF, controlled rate freezing; DMSO, dimethyl sulphoxide; DV, droplet-vitrification; ED, encapsulation–dehydration; EV, encapsulation–vitrification; encapsulation-programmed cooling, programmed slow cooling of encapsulated samples; HPLC, high performance liquid chromatography; LN, liquid nitrogen; LS, loading dolution; MS, Murashige and Skoog (1962); NR, not reported; PPPR, post-publication peer review meant to identify problems, difficulties, inconsistencies or problematic protocols or data; PVS2, 30 % (w/v) glycerol + 15 % (w/v) ethylene glycol + 15 % (w/v) DMSO in liquid MS medium supplemented with 0.4 M sucrose (Sakai et al. 1990); PVS3, 50 % glycerol + 50 % sucrose (Nishizawa et al. 1993); RAPD, random amplified polymorphic DNA; RT, room temperature; SDW, sterile distilled water; SIM, shoot induction (and development) medium (see Table 2 for details); TTC, triphenyltetrazolium chloride; URF, ultra-rapid freezing; V, vitrification; VS, vitrification solution; WT, wild-type

germplasm, and being able to store partially dehydrated synseeds for several weeks or months at reduced temperature (around +5 °C) would allow for the optimization of the management of laboratories that produce ornamentals such as chrysanthemum on a large scale. Cryopreserving encapsulated shoot tips or somatic embryos using encapsulation–dehydration or encapsulation–vitrification techniques would allow for the long-term conservation of important germplasm. Since the establishment of synseed technology, synseeds have been efficiently used for propagation, exchange and short- to medium-term storage of germplasm of numerous species (Sharma et al. 2013). In case of chrysanthemum, there is only one report of the application of synseed technology (Pinker and Abdel-Rahman 2005). These authors encapsulated nodal segments of six ornamental chrysanthemum genotypes with the objective of using them for non-sterile sowing. Depending on the experimental conditions, up to 100 % of the encapsulated nodal segments formed roots and shoots following sowing in pots containing vermiculite.

## Cryopreservation technology for preservation of chrysanthemum germplasm

Cryopreservation (liquid nitrogen, −196 °C) is currently the only technique ensuring the safe and cost-effective long-term storage of vegetatively propagated and non-orthodox seed species, of rare and endangered species and of biotechnology products (Engelmann 2004, 2011). Today, cryopreservation protocols have been developed for several hundred plant species including food crops, forest trees, fruit trees and ornamental plants from temperate and tropical origin (Reed 2008). The number of cryopreserved plant germplasm collections is still limited, but it is nevertheless progressing steadily, due to the increasing activity

**Table 2** Summary of chrysanthemum regeneration protocols prior to or following cryopreservation

| Species and/or cultivar(s) | Explant used (type, size, origin) | Basal medium | PGR (type and concentration, mg/l or molar)* | Other medium additives* | Other culture conditions | Effect on tissue culture, development, etc. (regeneration type)** | Productivity, somaclonal variation and abnormalities | Acclimatization (% survival) | References |
|---|---|---|---|---|---|---|---|---|---|
| Shuhounotikara (Shuhounochikara) then applied to 2 cultivars (Kenrokukougiku, Parliament), 12 species and 2 interspecific hybrids; Apricot Marble (1994) | Shoot tips (size and age of mother material NR) | MS | 0.1 mg/l BA + 1.0 mg/l NAA + 0.8 % agar (SIM) | 2.0 % sucrose pH NR | 25 °C; continuous light (16-h PP for Apricot Marble); 19 µmol $m^{-2}$ $s^{-1}$ | DMSO at 10 % or more negatively impacted callus growth. DMSO at 5 % in SIM decreased shoot regeneration rate. Cryopreservation of Apricot Marble shoot tips showed a 70 % reversion from wild type apricot colour to pink, suggesting regeneration from epidermal tissue | For Shuhounotikara, 87 % of shoot tips survived; 47 % of shoot tips regenerated shoots. Shoot tips tended to form callus, and result in deformed shoots. For all other cultivars and species, shoot regeneration varied widely from 9.4 to 100 %. | Rooting and acclimatization not performed in 1990's studies. In 1991 study, no apparent differences in flowering characteristics between cryopreserved and control Shuhounotikara plants. In 1994 study, 73.7 % of flowers of cryopreserved shoot tips regenerated on 1.0 mg/l BA reverted to pink colour (61.7 % of shoot tips on 0.1 mg/l BA) | Fukai (1990); Fukai and Oe (1990); Fukai et al. (1991) |
| *Chysanthemum cinerariaefolium* (high pyrethrin-producing line) | Flower head used to generate callus | MS (CIM) (4–5 weeks) then ½MS | No PGRs in CIM; In ½MS, 4 mg/l NOAA + 0.4 mg/l BA | 3.0 % sucrose; pH 5.8 | 1.0 % agar; 23 °C; 16-h PP; 60 µmol $m^{-2}$ $s^{-1}$ | Exposure of callus to 18 g/l sucrose for 30 days resulted in 57 ± 9.8 % viability and 72.3 ± 14.2 % regrowth. When 5 % DMSO was added to sucrose-containing medium for the same period, viability was 90.8 ± 4.8 % and regrowth was 96.2 ± 15.2 % | In most cases of pyrethrins, treatment with sucrose + DMSO resulted in higher secondary metabolite production than untreated cryopreserved callus. One notable exception was cinerin II + jasmonin II, whose production was stimulated significantly more when only sucrose was used | Rooting and acclimatization not performed | Hitmi et al. (1997, 1999b, 2000b) |

**Table 2** continued

| Species and/or cultivar(s) | Explant used (type, size, origin) | Basal medium | PGR (type and concentration, mg/l or molar)* | Other medium additives* | Other culture conditions | Effect on tissue culture, development, etc. (regeneration type)** | Productivity, somaclonal variation and abnormalities | Acclimatization (% survival) | References |
|---|---|---|---|---|---|---|---|---|---|
| *Chrysanthemum cinerariaefolium* (high pyrethrin-producing line) | Apical buds with 1–3 young leaves and a base (stem) 2 ± 0.3 mm long derived from 1-month-old in vitro commercial cultures | MS | 11 µm NAA + 4.5 µm BA | 83 mM sucrose | Agar conc. NR; 23 °C; 16-h PP; 60 µmol $m^{-2}$ $s^{-1}$ | 3 h of exposure to 7.5 % DMSO resulted in highest survival (~50 %). Desiccation for 2 h on a laminar flow bench after treatment with 7.5 % DMSO resulted in 50 % survival. Pre-culture for 3 days in 0.55 M sucrose resulted in highest survival (~61 %) | Sucrose + DMSO pre-treatment resulted in higher total pyrethrins but due to incorrect statistical analyses, difficult to assess whether differences with the control were significant | 100 % rooting and acclimatization claimed (but unclear from what treatments) | Hitmi et al. (1999a, 2000a) |
| Shuhounochikara | Shoot tips (1–1.5 mm long) with 2 enlarged leaves from 3-week-old shoots derived from cold-treated axillary bud culture | MS | 0.2 mg/l BA | 2.5 % sucrose; pH 5.8 (stock plants) 3–5 mm long node on 0.1 M sucrose for 3 weeks (SIM) | 0.8 % agar; temp. NR; 16-h PP; 50 µmol $m^{-2}$ $s^{-1}$ (stock plants) Agar conc. NR; 10 °C; 8-h PP; 10 µmol $m^{-2}$ $s^{-1}$ (SIM) | The new ED protocol or vitrification resulted in 85 % shoot formation versus 20 % in the standard ED protocol | Somaclonal variation and abnormalities NR | Rooting and acclimatization not performed | Sakai et al. (2000) |

**Table 2** continued

| Species and/or cultivar(s) | Explant used (type, size, origin) | Basal medium | PGR (type and concentration, mg/l or molar)* | Other medium additives* | Other culture conditions | Effect on tissue culture, development, etc. (regeneration type)** | Productivity, somaclonal variation and abnormalities | Acclimatization (% survival) | References |
|---|---|---|---|---|---|---|---|---|---|
| Escort, Snowdon, Elegance Weiss, White Cindy, Trumpf Rot, Branilo, Branglow, Astro, Heidi Weiss | Shoot tips from 4-week-old mother plants (3–4 mm long, 1–2 mm thick with two leaf primordia) | MS | No PGRs (stock plants) 1 mg/l BA + 0.1 mg/l NAA (SIM) | 3.0 % sucrose; pH 5.7 (stock plants and SIM) | Magenta boxes (stock plants) or 30-mm agar plates (SIM); 0.8 % agar; 24 °C; 16-h PP; 2,000 lm $m^{-2}$ (stock plants and SIM?) | For CRF, −0.25 °C/min at any concentration of sucrose resulted in highest shoot % regeneration (35–45 %); in the ED protocol, 4–5 h of pre-incubation at any concentration of sucrose resulted in highest shoot % regeneration (31–45 %); in the V protocol, highest shoot % regeneration (80 %) resulted with 5 min of 100 % PVS2 or 15 min of 60 % PVS2; in the URF protocol, wide variation in shoot % regeneration (1.4–28.0 %) between the 9 cultivars | Somaclonal variation and abnormalities NR | Rooting and acclimatization not performed | Halmagyi et al. (2004) |
| Pasodoble | Shoot tips (stock plants), nodal segments then shoot tips derived from adventitious buds from nodal segments | MS | 0.1 mg/l BA + 0.01 mg/l NAA (plantlet maintenance); 0.1 mg/l BA (SIM) | Carbon source and pH NR (plantlet maintenance and SIM) | 25 °C; 50 µmol $m^{-2}$ $s^{-1}$ (plantlet maintenance) or 10 °C; 10 µmol $m^{-2}$ $s^{-1}$ (SIM); culture vessel, agar concentration, PP NR (plantlet maintenance and SIM) | 64 % of shoot tips regrew following ED, but only 43 % from V | RAPD and RFLP indicated somaclonal variation, but several results were suspect (see criticisms in text) | Rooting and acclimatization not performed | Martín and González-Benito (2005); Martín et al. (2011) |

**Table 2** continued

| Species and/or cultivar(s) | Explant used (type, size, origin) | Basal medium | PGR (type and concentration, mg/l or molar)* | Other medium additives* | Other culture conditions | Effect on tissue culture, development, etc. (regeneration type)** | Productivity, somaclonal variation and abnormalities | Acclimatization (% survival) | References |
|---|---|---|---|---|---|---|---|---|---|
| Optimized for Peak, then applied to Baekma | Axillary shoot tips (1.2–1.5 mm long) from 3–4-day-old nodal segments | MS | 0.15 mg/l IAA + 0.2 mg/l Zea (plantlet maintenance) + 0.05 mg/l $GA_3$ (SIM) | 0.1 M sucrose (plantlet maintenance and SIM) | 2.0 % phytagel; 24 °C; 16-h PP; 50 µmol $m^{-2}$ $s^{-1}$ (plantlet maintenance) 2.0 % phytagel; 10 °C; PP and PPFD NR (SIM) | The droplet-vitrification protocol resulted in 81.9 and 84.9 % regeneration with apical and axillary shoot tips, respectively. >90 % regeneration was observed with var. 'Baekma' | No phenotypic abnormalities and similar profiles of relative DNA content (using flow cytometry analysis) | Rooting frequency of 95–100 % after 6 weeks of postculture in 'Peak' and 'Baekma'. After acclimation in greenhouse 98–100 % of plantlets were survived without any differences between control and cryopreserved samples | Kim et al. (2009a, b); Lee et al. (2011a, b) |
| *Dendranthema morifolium* + 5 other species (*Ajanica pacificum*, *D. grandiflorum* Jinba, *D. indicum* Lishui yeju, *Dahlia pinnata* Cav.) | Shoot tips (1–2 mm) from 4-week old plantlets | MS | 0.1 mg/l BA + 0.01 mg/l NAA | 0.2 M sucrose | 6.0 g/l agar, 25 °C, 2,000–2,200 lux, | Vitrification, encapsulation–vitrification and encapsulation-programmed cooling procedure resulted in 85.7, 50.0 and 0 % survival, respectively. Vitrification resulted in 6–84 % survival from five other species | Direct regeneration or multiple shoots formation via callus phase | Rooting and acclimatization not performed | Liu et al. (2009) |
| Indianapolis | Shoot tips (0.5–1.0 mm long) from transgenic and non-transgenic 4-month-old plantlets, 15 days after their last sub-culture on SIM | MS | 1.0 mg/l IAA + 3.0 mg/l BA (SIM) | 3.0 % sucrose | 0.7 % agar; 25 °C; 16-h PP; 68 µmol $m^{-2}$ $s^{-1}$ (SIM) | 48–67 % regeneration for transgenic lines and 33–36 % for non-transgenic line | 101 RAPD banding profiles were identical in agarose gel electrophoresis | Flowering in vivo after 62 days. No acclimatization trials defined | Osorio-Saenz et al. (2011) |
| Dalmatian chrysanthemum (*Tanacetum cinerariifolium*) line '28v-75' + 6 other lines | Shoot tips | ½MS | 0.9 µM BA (SIM) | 3.0 % sucrose + 2.0 % PVP | 0.9 % agar; 25 °C; 16-h PP; 52 µmol $m^{-2}$ $s^{-1}$ (SIM) | 77 % regrowth for '28v-75' and 65–90 % regrowth for 6 other lines | NR | NR | Yamamoto et al. (2011) |

**Table 2** continued

| Species and/or cultivar(s) | Explant used (type, size, origin) | Basal medium | PGR (type and concentration, mg/l or molar)* | Other medium additives* | Other culture conditions | Effect on tissue culture, development, etc. (regeneration type)** | Productivity, somaclonal variation and abnormalities | Acclimatization (% survival) | References |
|---|---|---|---|---|---|---|---|---|---|
| Lady Orange, Lady Salmon mutants | 2-week-old shoot tips (size NR) | MS | 0.25 mg/l kinetin | 3.0 % sucrose | 0.8 % agar; 24 °C; 16-h PP; 27.4 µmol $m^{-2}$ $s^{-1}$ (SIM) | 60 and 47 % shoot regrowth for Lady Salmon and Lady Orange when the sucrose/kinetin ratio was 3 %/1 mg/l and 3 %/0.25 mg/l, respectively | NR | NR | Zalewska and Kulus, (2013) |
| *Chrysanthemum morifolium* Japanese Red + 5 other genotypes | Axillary shoot tips (2.0 mm) | MS | 0.05 mg/l $GA_3$ | 3.0 % sucrose | Agar conc. NR; 22 °C; 16-h PP; 50 µmol $m^{-2}$ $s^{-1}$ (SMM) | 83 and 43 % shoot regrowth rates for 'Japanese Red' and 'Xizi Qiuzhuang', with an average rate of 68 % in the six genotypes tested | SSR and FC analysis as well as histological analysis showed no significant differences between control and cryopreserved plantlets of these two genotypes | NR | Wang et al. (2014) |

BA, $N^6$-benzyladenine (also represents BAP or 6-benzylaminopurine if so reported in the literature originally, according to Teixeira da Silva 2012); CIM, callus induction (and development) medium; CRF, controlled rate freezing; ED, encapsulation–dehydration; FC, flow cytometry; $GA_3$, gibberellic acid; IAA, indole-3-acetic acid; MS, Murashige and Skoog (1962) medium; NAA, α-naphthaleneacetic acid; NOAA, α-naphthoxyacetic acid; NR, not reported; PPFD, plant photosynthetic photon flux density; PGR, plant growth regulator; PP, photoperiod; PVP, polyvinylpyrrolidone; RAPD, random amplified polymorphic DNA; SIM, shoot induction (and development) medium; SSR, simple sequence repeat; URF, ultra-rapid freezing; V, vitrification; Zea, zeatin

in cryopreservation research which is observed at the global level (Reed 2008; Normah et al. 2012). Progress in the cryopreservation of ornamental germplasm is also increasing (Lambardi et al. 2006: Ozudogru et al. 2010).

Most biological materials employed in plant cryopreservation, such as cell suspensions, calluses, shoot tips, or embryos contain high amounts of cellular water and are thus extremely sensitive to freezing injury, since most of them are not inherently freezing-tolerant. Cells have thus to be dehydrated artificially to protect them from the damage caused by the crystallization of intracellular water into ice (Mazur 1984). The techniques employed and the physical mechanisms upon which they are based vary between the two groups of existing cryopreservation techniques (Withers and Engelmann 1998). In controlled cooling techniques, dehydration of samples takes place both before and during cryopreservation (freeze-induced dehydration), whereas in more recent, vitrification-based techniques, dehydration takes place only before cryopreservation. In optimal conditions, all freezable water is removed from the cells during dehydration and the highly concentrated internal solutes vitrify upon immersion in liquid nitrogen. Vitrification can be defined as the transition of water directly from the liquid phase into an amorphous phase or glass, whilst avoiding the formation of crystalline ice (Sakai and Engelmann 2007).

Controlled cooling cryopreservation techniques involve slow cooling down to a defined prefreezing temperature, followed by rapid immersion in liquid nitrogen. With temperature reduction during slow cooling, the cells and the external medium initially supercool, followed by ice formation in the medium (Mazur 1984). The cell membrane acts as a physical barrier and prevents the ice from seeding the cell interior and the cells remain unfrozen but supercooled. As the temperature is further decreased, an increasing amount of the extracellular solution is converted into ice, thus resulting in the concentration of intracellular solutes. Since cells remain supercooled and their aqueous vapor pressure exceeds that of the frozen external compartment, cells equilibrate by loss of water to external ice. Depending upon the rate of cooling and the prefreezing temperature, different amounts of water will leave the cell before the intracellular contents solidify. In optimal conditions, most or all intracellular freezable water is removed, thus reducing or avoiding detrimental intracellular ice formation upon subsequent immersion of the specimen in liquid nitrogen, during which vitrification of internal solutes occurs. Rewarming should be as rapid as possible to avoid the phenomenon of recrystallization in which ice melts and reforms at a thermodynamically favorable, larger and more damaging crystal size (Mazur 1984).

Controlled cooling procedures include the following successive steps: pregrowth of samples, cryoprotection, slow cooling (0.1–2.0 °C/min) to a determined prefreezing temperature (usually around −40 °C), rapid immersion of samples in liquid nitrogen, storage, rapid rewarming, and recovery. Controlled cooling techniques are generally operationally complex since they require the use of sophisticated programmable freezers. These techniques are generally employed for apices of cold tolerant species and undifferentiated culture systems such as cell suspensions and calluses (Engelmann and Dussert 2012).

In more recent vitrification-based techniques, cell dehydration is performed prior to cryopreservation by exposing samples to highly concentrated cryoprotectant solutions and/or air desiccation, until most or all freezable water has been extracted from the cells, which results in vitrification of the aqueous compartment. Intracellular ice formation is thus avoided. This dehydration step is generally followed by direct immersion of samples in liquid nitrogen. These new procedures offer practical advantages compared to controlled cooling/freezing techniques. They are more appropriate for complex organs such as shoot-tips, embryos or embryonic axes. By precluding ice formation in the system, these procedures are operationally less complex than controlled cooling ones as they do not require the use of programmable freezers (Engelmann and Dussert 2012). Vitrification-based cryopreservation techniques include encapsulation–dehydration, vitrification, encapsulation–vitrification, droplet-vitrification and cryoplates.

Encapsulation–dehydration is based on the technology developed for synseed production. Explants are encapsulated in calcium alginate beads, pregrown in liquid medium enriched with sucrose for 1–7 days for osmotic dehydration, then physically desiccated with silica gel to a water content around 20 % (fresh weight basis), transferred into cryotubes and cooled rapidly by direct immersion in liquid nitrogen. For rewarming, beads are retrieved from the cryotubes and placed directly on recovery medium (González-Arnao and Engelmann 2006).

Vitrification involves treatment of samples with progressively more concentrated cryoprotectant solutions. Samples are firstly exposed to a loading solution with intermediate concentration, generally 2 M glycerol + 0.4 M sucrose (Matsumoto et al. 1994). Kim et al. (2009a) have devised a series of alternative loading solutions, which proved highly efficient with sensitive species such as chrysanthemum. They are then dehydrated with highly concentrated vitrification solutions. The most commonly employed vitrification solutions are the Plant Vitrification Solutions PVS2 (Sakai et al. 1990) and PVS3 (Nishizawa et al. 1993), which contain (w/v) 15 % ethylene glycol (permeable cryoprotectant; MW = 62) + 30 % glycerol + 15 % DMSO + 13.7 % sucrose and 50 % glycerol + 50 % sucrose, respectively. Kim et al. (2009b) have developed a series of alternative vitrification

solutions, derived from the original PVS2 and PVS3, some of which produced higher survival and recovery compared to the original vitrification solutions, for cryopreservation of sensitive species such as chrysanthemum. Explants are placed in cryotubes, in small quantities of vitrification solution (0.5–1.0 ml), which are cooled rapidly by direct immersion in liquid nitrogen. After rapid warming in a water-bath at 37–40 °C, the highly toxic vitrification solutions are removed and replaced by an unloading solution containing 0.8–1.2 M sucrose, thereby allowing progressive rehydration of the samples.

Encapsulation–vitrification is a combination of encapsulation–dehydration and vitrification. Samples are encapsulated in alginate beads, then treated following a standard vitrification protocol, as described in the previous paragraph. One interest of this technique is that encapsulated explants are not in direct contact with the highly concentrated vitrification solutions, thereby decreasing their toxicity (Sakai and Engelmann 2007). Another advantage is linked to the facilitated manipulation of encapsulated explants compared to naked ones.

Droplet-vitrification was developed more recently (Panis et al. 2005). In this technique, samples are treated following a standard vitrification protocol, except for the cooling step, for which explants are placed on an aluminum foil in minute droplets of vitrification solution, which are immersed rapidly in liquid nitrogen. The main advantage of this technique lies with the fact that explants are in direct contact with liquid nitrogen during cooling and with the unloading solution during rewarming, thereby ensuring very high cooling and rewarming rates.

Finally, the most recent technique is the cryoplate developed by Takao Niino's group in Japan (Yamamoto et al. 2011). In this technique, explants are placed in the wells of an aluminium cryoplate, to which they are made to adhere using minute droplets of calcium alginate. In the V-cryoplate procedure, explants are treated with a loading solution, then with a vitrification solution before their direct immersion in liquid nitrogen (Yamamoto et al. 2011). In the D-cryoplate procedure, after treatment with a loading solution, explants are physically dehydrated in the air current of a laminar flow cabinet before immersion in liquid nitrogen (Niino et al. 2013). In addition to the high cooling and warming rates achieved due to the direct contact between the explants and liquid nitrogen during cooling, and with unloading solution during warming, a significant advantage of this technique lies with the facilitated manipulation of explants which adhere to the cryoplates.

The experiments of Fukai (1990, 1992), Fukai and Oe (1990) and Fukai et al. (1991) were the first studies to focus on cryopreservation of chrysanthemum shoot tips using slow-freezing techniques, but these authors frequently found abnormal plant development as a result of the exposure to dimethylsulfoxide (DMSO). Fukai (1990) found that exposure to −196 °C affected shoot tip survival percentage more negatively than −40 °C and that the cooling rate, as well as the temperature to which shoot tips were exposed prior to exposure to LN, were important parameters that needed to be considered. Even though shoot tip survival and shoot regeneration values were high (60–90 %), the author claimed that cryopreserved shoot tips tended to form callus after replating on SIM, which was an undesirable result since "regenerated shoots from calli are less genetically stable". In a follow-up study, Fukai and Oe (1990) showed, using scanning electron microscopy (SEM), that only a small part of cryopreserved shoot tips survived and regenerated while as much as 82 % of cryopreserved shoot tips were abnormal or hyperhydric. Fukai et al. (1994) then cryopreserved shoot tips of a periclinal chimera 'Apricot Marble' and found that 70 % of plants had a pink colour instead of the original apricot colour, suggesting regeneration from the epidermis of the shoot tips.

The first studies to implement cryopreservation of *C. cinerariaefolium* (Dalmatian pyrethrin) callus were conducted by Hitmi et al. using undifferentiated (i.e., callus) tissue of a high pyrethrin-producing line (1997, 1999b, 2000b) or using shoot tips from in vitro-derived plants (1999a, 2000a). Their test material was a source of pyrethrins, which are a mixture of six monoterpene esters usually extracted from the capitula that are used as insecticides. Many members of the Asteraceae produce important compounds and essential oils (Teixeira da Silva 2003b, 2004b; Teixeira da Silva et al. 2005) but one of the weaknesses or risks of in vitro cultures used to derive economically important substances such as pyrethrin, as claimed by the authors, is the gradual weakening or total disappearance of such compounds with increasing subcultures. In this context, Hitmi et al. (1997, 1999b, 2000b) proposed cryopreservation as one way to sustain callus (or shoot tips; Hitmi et al. 1999a, 2000a) indefinitely and thus hopefully not lose the ability of high pyrethrin-producing clones to lose their ability to produce pyrethrin. In fact, the authors concluded, in their 1997 paper, that pyrethrin production was enhanced by cryopreservation, with sucrose and/or DMSO in the pre-treatment medium while the 1999a/2000a paper indicated no loss in pyrethrin production. To assess the mechanism by which callus lines acquired freezing tolerance after sucrose treatment and prior to cryopreservation, the authors tested for sucrose, glucose, fructose (reducing sugars) and starch content, as well as the water, ABA and proline content in the 1999b paper. The 1999b paper confirmed some results pertaining to sucrose in the 1997 paper. The 1999b paper also showed some new and important findings and trends: relative to treatment with 30, 90 or 180 g/l sucrose, and independent

of the preculture period (10, 20 or 30 days), callus that had not been cryopreserved had a significantly lower cell water content, a significantly higher unfrozen water content, amount of sucrose, glucose and fructose and endogenous levels of ABA and proline while no change was observed in starch content. These changes are the result of preculture itself and, of course, take place in both LNC and LN samples. These results are consistent with the increase in ABA and proline levels associated with abiotic stresses (Gusta et al. 2005; Hayat et al. 2012).

Sakai et al. (2000) established the first encapsulation–dehydration protocol for chrysanthemum. After preculture with 0.3 M sucrose at 5 °C in the dark for 3 days, shoot tips (1–1.5 mm long) were encapsulated with 2 % Na-alginate and 2 M glycerol + 0.4 M sucrose. Encapsulated apices were osmoprotected with loading solution (2 M glycerol + 0.4 M sucrose) for 60 min and then dehydrated for 3 h using silica gel prior to plunging into LN. With this procedure, named "New encapsulation–dehydration", shoot formation was 85 %, which was as high as with vitrification and significantly higher than with the classical "encapsulation–dehydration" procedure. The authors argued that glycerol, in combination with sucrose, may contribute to minimizing the injurious changes to the membrane resulting from severe dehydration. The encapsulation–dehydration procedure was also used by Zalewska and Kulus (2013) to cryopreserve'Lady Orange' and 'Lady Salmon' mutants.

Kim et al. (2009a, b) developed alternative loading solutions (LSs) and vitrification solutions (VSs) and applied those to chrysanthemum 'Peak', a multi-branch variety with relatively small flowers, which was sensitive to the cytotoxicity of cryoprotectants employed in the droplet-vitrification procedure. In studies with alternative LSs (Kim et al. 2009a), chrysanthemum axillary shoot tips were progressively precultured with 0.3 M sucrose for 27 h → 0.5 M sucrose 18 h → 0.7 M sucrose for 8 h. Due to the sensitivity of samples to osmotic stress induced by the high sucrose concentration, preculture with 0.7 M sucrose overnight was harmful. Kim and Lee (2012) thus classified chrysanthemum as being moderately sensitive to sucrose preculture. The highest survival and regeneration of cryopreserved shoot tips (89.2 and 65.3 %, respectively) were observed after treatment with C4-35 % (17.5 % glycerol + 17.5 % sucrose) LS. However, interestingly, endothermic enthalpies of dehydrated shoot tips were not significantly different between shoot tips treated, or not, with a LS and whatever the LS tested. This result indicates that the loading treatment did not affect ice-blocking properties of dehydrated samples, thus implying that loading had an indirect effect on the adaptation to toxic VS (decreasing osmotic shock) and to freezing injury (localization of cryoprotectants into the inner part of the explants) of samples associated with localization of cryoprotectants within samples, before dehydration with highly concentrated VSs.

In work with alternative VSs (Kim et al. 2009b), chrysanthemum axillary shoot tips were precultured and loaded with the same as those of loading solutions (Kim et al. 2009a), i.e., stepwise preculture with 0.3 M → 0.5 M → 0.7 M sucrose and loading with C4-35 %. The shoot tips were dehydrated with PVS2 [30 % glycerol + 15 % DMSO + 15 % EG + 22.5 % sucrose (w/v); Sakai et al. (1990)] and its variants (A-series, eight alternative vitrification solutions containing different glycerol, DMSO, EG and sucrose concentrations) for 20 min or with PVS3 [50 % glycerol + 50 % sucrose (w/v); Nishizawa et al. (1993)] and its variants (B-series, four alternative vitrification solutions containing different glycerol and sucrose concentrations) for 60 min at room temperature. PVS3 and its variants provided higher recovery compared to PVS2 and its variants and most PVS2 variants were as effective as the original PVS2. Because of the sensitivity of chrysanthemum shoot tips to the chemical toxicity of VSs, decreasing DMSO and EG concentrations compared to the original PVS2 [A7, 37.5 % glycerol + 10 % DMSO + 10 % EG + 32.5 % sucrose (w/v)] increased recovery of non-cryopreserved and of cryopreserved (LN) samples. By contrast, a higher DMSO and EG concentration [A9, 30 % glycerol + 20 % DMSO + 20 % EG + 15.05 % sucrose (w/v)] resulted in only 23.3 % regeneration for non-cryopreserved explants and 0 % for cryopreserved samples. The highest regeneration of 86.7 % for non-cryopreserved and 73.1 % for cryopreserved buds was achieved following dehydration with the original PVS3 (B1). However, dehydration of samples with PVS3 (B1) for more than 60 min was harmful, due to the sensitivity of explants to osmotic stress, while dilution of PVS3 (B2–B5, diluted to total concentration of 80–90 %) resulted in lower recovery, due to freezing injury. The authors (Kim et al. 2009b) thus concluded that chrysanthemum axillary shoot tips were very sensitive to chemical toxicity of PVS2 and its variants, and also sensitive to osmotic stress induced by PVS3 and its variants.

With the same variety, Lee et al. (2011a, b) tested the effect of age of donor plants (4–8.5 weeks), explant type (apical vs. axillary shoots), sucrose preculture, vitrification solutions in droplet-vitrification protocols, based on previous studies (Kim et al. 2009a, b). When comparing 7 week-old apical and axillary shoot tips submitted to four preculture treatments, sucrose preculture was crucial for both explant types and the highest regeneration of non-cryopreserved (83.3 %) and cryopreserved samples (70.6 %) was observed in axillary shoot tips progressively precultured with 0.3 M sucrose for 31 h → 0.5 M sucrose for 17 h → 0.7 M sucrose for 7 h. By contrast, shoot tips which had not been submitted to sucrose preculture

produced 0 % post-cryopreservation regeneration in apical shoots and 15.4 % in axillary shoot tips. This indicated that, for successful cryopreservation, chrysanthemum required a progressive preculture with 0.3 M → 0.5 M → 0.7 M sucrose to induce dehydration tolerance.

In several reports, donor plants were cold-acclimated at 10 °C for 3 weeks, and isolated explants were further acclimated at 5 °C for 3 days in vitrification and encapsulation–dehydration procedures (Sakai et al. 2000; Martín and González-Benito 2005) or at 5 °C for 20–40 days in aluminum cryo-plates (Yamamoto et al. 2011). In this study, cold acclimation of donor plants for 2–3 weeks at 4–5 °C did not result in higher post-cryopreservation regeneration, compared with optimized sucrose preculture, which implies that sucrose preculture can substitute cold acclimation.

A comparison between 7 week-old apical and axillary shoot tips treated with PVS2- and PVS3-based VSs showed that the highest regeneration of non-cryopreserved (84.3 %) and cryopreserved samples (70.9 %) were observed in axillary shoot tips dehydrated with PVS3 (B1) for 60 min (Lee et al. 2011a, b). These authors tested dehydration duration of 4 week-old apical shoots with B1-100 % at 0 and 25 °C and observed comparable regeneration of non-cryopreserved (98 %) and cryopreserved explants (83 %) at 0 °C. Figure 1 of Lee et al. (2011a, b) demonstrated that optimum dehydration duration for 25 °C was 60 min, while for 0 °C it was 90–120 min. The highest post-cryo regeneration with 0 and 25 °C was not different. Based on the previous work (see Fig. 2 of Lee et al. 2011a, b), apical or axillary shoots were sampled 4 or 7 weeks, respectively, after the last subculture, since those were optimum stages for sampling. Apical or axillary shoot tips, excised after 7 or 4 weeks from the last subculture, should be dehydrated with PVS3 for 90 or 60 min, respectively, to obtain highest post-cryo regeneration. The optimal dehydration duration at 25 and 0 °C was 60–90 and 90–120 min, respectively. This was not the case of PVS2-based VSs, and dehydration at 0 °C did not increase post-cryopreservation regeneration compared to 25 °C. As pointed out by the authors, the relatively low regeneration of cryopreserved apical shoot tips compared to axillary shoot tips sampled on 7 week-old plantlets, was attributed to their sub-optimal developmental stage. Dominance of apical and axillary buds is alternative, i.e., axillary shoots start to grow rapidly when apical shoots enter into the lag phase. At an early stage of in vitro culture (4 weeks), apical buds grew fast and thus responded well to cryopreservation. By contrast, axillary buds were not yet fully developed, and thus responded poorly to cryopreservation. Later on, the plants reached the top of the culture vessels; apical bud entered into lag phase, and simultaneously axillary buds developed and grew fast. The plants still looked healthy 7 weeks after the last subculture. This phenomenon has been notably reported in potato (Yoon et al. 2006). In further experiments on the effect of donor plant age (between 4 and 8.5 weeks), the optimal plant age for cryopreservation of apical shoots and axillary shoot tips was 4–5.5 and 5.5–7 weeks, respectively. In a direct comparison of apical shoots and axillary shoot tips at their optimal developmental stage and using the optimal dehydration duration, no significant differences were observed in post-cryopreservation regeneration (81.9 vs. 84.6 % for apical and axillary buds, respectively, when 4 week-old apical shoots and 7 week-old axillary shoot tips were dehydrated with PVS3 (B1) for 90 and 60 min, respectively). This result reflected the difference in maturation stage (4 vs. 7 weeks) and explant size (large vs. small) between apical and axillary shoots apices.

Lee et al. (2011a, b) observed that some non-cryopreserved and cryopreserved shoot tips turned brown 24 h after plating on recovery medium and that surviving shoot tips regained their green color within 48 h. Regrowth was observed 10–14 days after LN exposure, either by means of direct regeneration of individual apices or through intermediate callusing followed by multiple shoot formation. The implementation of cryobanking for chrysanthemum germplasm at the National Agrobiodiversity Center, Korea has been postponed until the confirmation of genetic stability of recovered plantlets can be confirmed (Haenghoon Kim, personal communication), even though cryopreserved apical shoots rooted normally within 6 weeks, developed into plantlets within 8 weeks, and could be acclimatized in the greenhouse with 98–100 % survival. No abnormal characteristics were identified in greenhouse plants derived from all experimental treatments. The authors applied this protocol to apical shoots of another chrysanthemum 'Baekma', a single-branch type variety with larger flowers, and achieved 90 % post-cryopreservation regeneration with direct and rapid shoot development without callus phase (Haenghoon Kim, unpublished results). With this single-branch type variety, regeneration took place mostly through direct regrowth of individual apices, unlike the multi-branch growth form of var. 'Peak', which produced multiple shoots in some cryopreserved samples.

The proposed cryopreservation procedure for chrysanthemum apical shoots or axillary shoot tips is as follows (Lee et al. 2011a, b): excision of apical or axillary shoot apices 4 or 7 weeks after the last subculture, respectively; progressive preculture with 0.3 M sucrose for 31 h → 0.5 M sucrose for 17 h → 0.7 M sucrose for 7 h; osmoprotection with LS C4-35 % (17.5 % glycerol + 17.5 % sucrose) for 40 min; cryoprotection with PVS3 for 60 min (axillary buds) or 90 min (apical shoots); cooling and warming using aluminum foil strips; unloading

in 0.9 M sucrose solution for 40 min. Based on Kim and Lee's classification (2012), chrysanthemum is sensitive to both osmotic stress and chemical toxicity of VSs. Two-mm long shoot tips should be treated with PVS3 (B1) at room temperature or smaller axillary shoot tips with PVS A3 [37.5 % glycerol + 15 % DMSO + 15 % EG + 22.5 % sucrose (w/v)] at 0 °C.

Liu et al. (2009) developed a vitrification procedure using *Dendranthema morifolium* shoot tips (1–2 mm) sampled from 4-week old in vitro plantlets. This procedure included preculture with 0.4 M sucrose for 2–3 days in the dark at 4 °C, osmoprotection with LS (2 M glycerol + 0.4 M sucrose) for 30 min, treatment with PVS2 for 15 min on ice, and cooling in cryovials. After storage in LN for 24 h, shoot tips were rewarmed in a 40 °C water-bath for 2 min and unloaded in 1.2 M sucrose solution for 20 min. With this protocol, 85.7 % post-cryopreservation survival was obtained, compared to 50.0 % with encapsulation–vitrification and 0 % with encapsulation-programmed cooling. Surviving shoots developed into plantlets through direct regeneration or through callusing followed by multiple shoot formation. The optimized vitrification protocol was applied to five other chrysanthemum species, i.e., *Ajanica pacificum*, *D. grandiflorum* 'Jinba', *D. indicum* 'Lishui yeju', and *Dahlia pinnata* Cav., with survival between 6 and 84 % (84 % with *Dahlia pinnata* Cav. and below 20 % with the four other species).

Osorio-Saenz et al. (2011) compared the regeneration of two trehalose-accumulating transgenic lines (35S-8 "TL1", 35S-19 "TL2") with the non-transformed wild-type ("control") chrysanthemum 'Indianapolis' following a vitrification procedure. The protocol included preculture on medium with 0.3 M sucrose for 4 days, osmoprotection with LS containing 2 M glycerol + 0.4 M sucrose for 20–30 min, treatment with PVS2 or PVS3 for 40 min at room temperature followed by immersion in LN in cryovials. Post-cryopreservation regeneration of shoot tips of the two transgenic lines was 48–67 % with PVS2 and 52–54 % with PVS3 compared to only 33–36 % for wild type shoot tips. The shoot tips of *Centaurea ultreiae*, a critically endangered wild species of the Asteraceae, were shown to be very sensitive to PVS2, which was cytotoxic when shoot tips were exposed at room temperature for 5 min (Mallon et al. 2008).

The genetic stability of regenerated plants was performed using eight RAPD primers, instead of using primers for the target gene (Osorio-Saenz et al. 2011). A total of 101 monomorphic loci were amplified from the 20 samples tested (10 cryopreserved and 10 non-cryopreserved) per primer. This study revealed no variation in banding profiles of PCR amplicons between cryopreserved and non-cryopreserved plantlets. This paper demonstrated the applicability of genetic engineering to increase plant tolerance to cryopreservation.

Yamamoto et al. (2011) developed a V-cryo-plate procedure, a combination of "droplet"-vitrification and encapsulation–vitrification but in which encapsulation plays an essential role. Shoot tips, dissected from Dalmatian chrysanthemum var. 28v-75 shoot cultures cold-hardened at 5 °C for 20–40 days, were precultured at 5 °C for 2 days on 0.5 M sucrose medium. Shoot tips were placed on aluminum cryo-plates and embedded in minute droplets of calcium alginate. After loading with LS (2 M glycerol + 1.4 M sucrose) for 30 min, shoot tips were treated with PVS 7 M (30 % glycerol + 19.5 % EG + 0.6 M sucrose) for 40 min. This procedure produced post-cryopreservation regrowth of 77 %. It was further applied to six additional lines (19v-KO4, 26v-84, 28v-173, 29v-289, 36v-34, 41v-2) with a regrowth range of 65–90 %. The authors indicated that the efficiency of the V cryo-plate technique was due the relatively low toxicity of the PVS employed and highlighted that it was a user-friendly procedure.

Recently, Wang et al. (2014) reported shoot recovery and genetic integrity using the conventional PVS2 droplet-vitrification procedure. After preculture with 0.5 M sucrose for 1 day, shoot tips were loaded with LS (0.4 M sucrose + 2 M glycerol) for 20 min at RT and dehydrated with PVS2 for 30 min at 0 °C. After cooling and thawing using aluminium foil strips, shoot tips were unloaded with 1.2 M sucrose for 20 min and postcultured on MS medium supplemented with 0.05 mg/l $GA_3$. The droplet-vitrification procedure resulted in 83 % shoot regrowth for *C. morifolium* 'Japanese Red' and 43 % for 'Xizi Qiuzhuang', with an average of 68 % among six genotypes tested. Genetic changes were not detected between control and cryopreserved plantlets using simple sequence repeats and flow cytometry analysis. This paper extended the application of the droplet-vitrification protocol to more genotypes.

## Conclusions and future objectives

LTS, as well as the use of synseed technology and cryopreservation have found complementary applications for the conservation and distribution of chrysanthemum germplasm, which is one of the best studied ornamental germplasm (Lambardi et al. 2006: Ozudogru et al. 2010). Encapsulated chrysanthemum shoot tips or nodal segments may be stored for several weeks or months to facilitate the management of large-scale production in commercial laboratories. They also represent very convenient propagules for international exchange and distribution of plant material between commercial laboratories and genebanks. As there is currently only one report of the application of synseed

technology to chrysanthemum (Pinker and Abdel-Rahman 2005), additional experiments will have to be performed before it becomes a routinely applicable technique.

As already mentioned in this paper, cryopreservation is currently the only technique that allows for the safe and cost-effective long-term conservation of chrysanthemum germplasm. Despite intensive cryopreservation studies, large-scale cryobanking of chrysanthemum collections has been hampered mainly by the sensitivity of shoot tips to chemically and osmotically toxic vitrification solutions (Kim and Lee 2012), although the National Institute of Agricultural Sciences (NIAS) of Japan has established a cryobank of edible chrysanthemum (personal comm. Dr. Takao Niino). Most solution-based vitrification procedures make use of PVS2 (Sakai et al. 1990). However, since chrysanthemum shoot tips are sensitive to PVS2, the use of PVS3 (Nishizawa et al. 1993) results in higher post-cryopreservation regeneration but only when shoots have acquired osmotic stress tolerance through a step-wise preculture in different sucrose concentrations for different time periods: 0.3 M for 31 h → 0.5 M for 17 h → 0.7 M for 7 h (Lee et al. 2011a, b). The use of this precisely timed preculture procedure and of the alternative LS C4-35 % (17.5 % glycerol + 17.5 % sucrose), guaranteed high post-cryopreservation regeneration. A droplet-vitrification procedure produced the highest post-cryopreservation regeneration of 82–90 % (Lee et al. 2011a, b), compared to other procedures applied for chrysanthemum shoot tips. This protocol should be tested with a wide range of chrysanthemum genotypes and fine tuning should take place before large-scale implementation of cryobanking can be envisaged. The V-Cryo-plate procedure may also be tested due to its advantage of combining encapsulation (alginate embedding) + droplet-vitrification and due to its user-friendliness for manipulating explants. Embedding explants in alginate may decrease the cytotoxicity of VSs at 25 °C, and thus the optimal dehydration duration may be up to 40–50 min, just like dehydration on ice. Finally, the VS 7 M can be useful for cytoxicity-sensitive tiny meristems (1 $mm^2$) rather than larger shoot tips, because of its low concentration (70 % w/v).

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