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Review article

Cryopreservation and genetic stability of *Dendranthema grandiflora* Tzvelev *in vitro* cultures

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Shoots apices of different chrysanthemum species and varieties have been cryopreserved following different procedures, which are reviewed and compared in this paper. The results obtained are not only method-dependant but also cultivar-dependant. When several methods were compared with a cultivar best results were generally obtained with vitrification procedures than with encapsulation-dehydration. Not much attention has been given to the stability of the recovered chrysanthemum shoots but there are indications of appearance of somaclonal variants, which in some cases has been explained as being due to the chimeric nature of the cultivar employed. Molecular-based stability studies have also shown the appearance of variants.

Key-words: chrysanthemum, cryopreservation, genetic stability, germplasm

Introduction

Chrysanthemum is one of the most popular cut flower and pot ornamental plants; it has been cultivated and improved for more than 2000 years. Therefore, it occupies an important position in the world cut flower trade. Commercial cultivars are propagated vegetatively by cuttings due to the presence of a strong sporophytic self-incompatible breeding system. *In vitro* culture techniques are commonly employed in the commercial production of chrysanthemum.

The ornamental market continuously demands new cultivars. Classical breeding in chrysanthemum has limitations mainly due to self-incompatibility problems, a restricted gene pool and the polygenic nature of growth and flowering. However, *sports*, spontaneous mutations in the vegetative reproduction, are a common source of new varieties for classical breeding. These variations, which are more or less frequent depending on the cultivar, seem to be related to the fact that many

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cultivars of chrysanthemum are periclinal chimeras, with one cell layer genetically different to the others (Dowrick and El-Bayoumi 1965, Martín et al. 2002). In the last few years, modern breeding techniques have been incorporated to obtain new cultivars: radiation-induced mutation (Broertjes et al. 1976), somaclonal variation induction (Malaure et al.1991, Rout and Das 1997) and, more recently, genetic transformation (see review of Teixeira da Silva 2003).

In this context, the application of cryopreservation methods can play an important role in the conservation of improved material or underutilized traditional cultivars compared to *in vitro* storage. Some cultivars (especially those which are periclinal chimeras) have shown high instability when they are under tissue culture conditions (Martín et al. 2002). Besides, the assessment of plant genetic integrity after *in vitro* culture and/or cryopreservation is a subject of increasing interest (Harding 2004).

Chrysanthemum cryopreservation

Cryopreservation methods of chrysanthemum shoot apices have been developed by several researchers following different approaches (Table 1). In two of those works, several cryopreservation methods were compared (Halmagyi et al. 2004 , Sakai et al. 2000). Among the ones employed by Halmagyi et al. (2004) the droplet ultra-rapid freezing method was tested in nine cultivars, with regeneration ranging from 4 to 70 %, although experiments were not very repetitive. Also in that work, the droplet-vitrification method resulted in the highest shoot regeneration percentage (60%) in the one cultivar more thoroughly studied ('Escort'). However, the authors indicated that recovery, with this and other methods, could be increased if rapid warming (at 30–40 ºC instead at room temperature) were employed. Using vitrification, also Sakai et al. (2000) obtained high recovery percentages with cultivar 'Shuhounochikara' (85%). An important difference in the two vitrification protocols studied was that on the latter plant material had been cold acclimated (nodal segments cultured at 10 ºC for 3 weeks, and then excised apices at 5 ºC for 3 days). Cold hardening has been shown to have an important role in cryopreservation by vitrification in many temperate species (Sakai and Engelmann 2007). Cultivar- or pretreatment-dependent factors may have also been the reason for the different response in these two studies after cryopreservation by encapsulation-dehydration. Sakai et al. (2000) obtained lower recovery with cv. 'Shuhounochikara' (20%) than Halmagyi et al. (2004) with cv. 'Escort' (45%). Besides the different cultivars employed for these studies, a finer protocol optimization could account for the results. Sakai et al. (2000) reported a new encapsulation-dehydration method, tested not only with chrysanthemum shoot apices but also in wasabi and mint, that resulted in similar or better recovery than the other two methods tested (encapsulation-dehydration and vitrification), but made the protocol shorter and less handling was required. The new encapsulation-dehydration method substituted the overnight culture of encapsulated apices in sucrose rich medium (usually 0.75–0.8 M) with osmoprotection of apices with a mixture of 2 M glycerol plus 0.4 M sucrose for 1 h carried out during the encapsulation process. Subsequently, air desiccation for only 3 h was required, compared to 9 h in conventional encapsulation-dehydration.

Previous to those works, shoot apices of *Dendranthema grandiflorum* and other chrysanthemum species had been successfully cryopreserved by controlled cooling (Fukai, et al. 1991, Fukai and Oe 1990). Recovery percentages ranged from 40 to 100%. Fukai and Oe (1990) demonstrated that after controlled freezing only a small part of the apex survived and that treatment with DMSO (2 days culture on medium with 5% v/v DMSO, and then incubated for 1h at 0 ºC with 10% DMSO) caused abnormal leaf development. In other cryopreservation works the possible adventitious origin of the recovered shoots is stated (Halmagyi et al. 2004, Fukai et al. 1991). Hitmi et al. (1999a, 2000a) reported no callus formation in recovered shoot from cryopreserved apices.

A standard cryopreservation protocol applicable to a variety of genotypes is desirable in order to establish an *in vitro* gene bank. Shoot tips of *C. morifolium* and related species native to Japan were

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Table 1. Chrysanthemum shoot-tip cryopreservation methods.

Species (as in the reference)	Method[1]	Shoot regeneration	Reference
Dendranthema grandiflorum Kitam. (Ramat) cv. 'Shuhounochikara'	**Controlled-rate** Precultured with 5% DMSO at 25 ºC for 2 days Incubation in 10% DMSO+ 3% glucose for 1 h at 0 ºC Cooling rate 0.2 ºC/min, inside straws	Not reported	Fukai and Masaharu (1990)
Chrysanthemum spp [2]	**Controlled-rate** Precultured with 5% DMSO at 25 ºC for 2 days Incubation in 10% DMSO+ 3% glucose at 0 ºC for 1 h Cooling rate 0.2 ºC/min, inside straws	40–100%[3]	Fukai et al. (1991)
Chrysanthemum morifolium Ramat. cvs. 'Shuhounotikara', 'Kenrokukougiku', 'Parliament'	**Controlled-rate** Precultured with 5% DMSO at 25 ºC for 2 days Incubation in 10% DMSO+ 3% glucose at 0 ºC for 1 h Cooling rate 0.2 ºC/min, inside straws	53–63%	Fukai et al. (1991)
Chrysanthemum cinerariefolium	**Preculture** Preculture with 0.55 M sucrose for 3 days Incubation in 7.5% DMSO for 1 h at 0 ºC Vials immersed in liquid nitrogen	60%	Hitmi et al. (1999a)
Chrysanthemum cinerariefolium	**Preculture** Preculture with 0.55 M sucrose + 4 μM ABA for 3 days Vials immersed in liquid nitrogen	75%	Hitmi et al. (2000a)
Chrysanthemum morifolium Ramat. cv. 'Shuhounochikara'	**New encapsulation dehydration** Preculture with 2M glycerol + 0.4 M sucrose for 1 h, of encapsulated shoot apices Dehydration with silica gel 3 h Vials immersed in liquid nitrogen	85%	Sakai et al. (2000)
	Vitrification Preculture with 2M glycerol + 0.4 M sucrose for 20 min Incubation in PVS2 for 20 min at 25 ºC Vials immersed in liquid nitrogen	85%	Sakai et al. (2000)
	Encapsulation-dehydration Encapsulated apices treated with 0.8M sucrose for 16 h at 25 ºC Dehydration with silica gel for 9 h Vials immersed in liquid nitrogen	20%	Sakai et al. (2000)
Chrysanthemum morifolium Ramat. cv. 'Escort'	**Controlled-rate** Preculture 0.5M sucrose for 24 h Cooling rate 0.25 ºC/min	46%	Halmagyi et al. (2004)
	Encapsulation-dehydration Preculture 0.75M sucrose , 4 h Air-flow desiccation 5 h Vials immersed in liquid nitrogen	45%	Halmagyi et al. (2004)
	Droplet ultra-rapid freezing[4] Incubation on 50 gl^{-1} sucrose for 24 h Incubation in 7.5%DMSO for 2 h Direct immersion in liquid nitrogen	36%	Halmagyi et al. (2004)
	Vitrification-droplet Incubation for 24 h in 0.5 M sucrose 5 min in PVS2 Direct immersion in liquid nitrogen	60%	Halmagyi et al. (2004)

[1]Method with best result reported
[2]Fifteen species and two hybrids studied.
[3]Excluded one species with very low shoot regeneration rate in control (unfrozen) shoot tips.
[4]Several cultivars were tested with this method, regeneration ranging 4–70%

cryopreserved using slow cooling (0.2 ºC/min), with 10% DMSO and 3% glucose, to –40 ºC prior immersion in liquid nitrogen (Fukai et al. 1991, Fukai 1995). High survival rates were observed in three cultivars, twelve species, and two hybrids of chrysanthemum, out of 20 tested. However, among the genotypes with high survival (83–100%), five showed shoot regeneration percentages lower than 40%. The variation in shoot regeneration could be attributed to differences in the degree of injury caused by freezing and thawing, the sensitivity to DMSO and the regeneration ability of the shoot tips. The authors of the present review have also observed differences in the morphology and structure of the shoot apices of several cultivars which could have influenced the different responses observed when cryopreserved by vitrification (Table 2).

A simple method based on sucrose pretreatment (3 days on 0.55 M sucrose, incubation for 1 h in 7.5% DMSO and direct immersion in liquid nitrogen) resulted in 60–75% shoot recovery in *C. cinerariaefolium* (Hitmi et al. 1999a, 2000a). The morphogenic potential, total chlorophyll contents and secondary metabolite biosynthetic ability of regenerated plants were similar to those of the unfrozen control.

Chrysanthemum cryopreservation studies have also been carried out in unorganized cultures. Hitmi et al. (1997) developed an efficient protocol for cryogenic storage of high-pyrethrin-producing cell lines of *Chrysanthemum cinerariaefolium*. Optimal survival (92%) was obtained with cells precultured in medium containing 180 gl^{-1} sucrose for 30 days, then incubated in 5% DMSO for 1 h at 0 ºC, cooled slowly to –20 ºC and immersed for 30 min in liquid nitrogen. After cryopreservation, the cells conserved the same growth pattern, but displayed different biochemical properties. The subculture derived from the thawed cells was characterized by lower chlorophyll content and higher pyrethrin biosynthesis ability. The preculture on sucrose medium increased ABA and sucrose endogenous contents, and the unfrozen water content (Hitmi et al. 1999b, 2000b).

Stability in cryopreserved chrysanthemum cultures

Probably, one of the most valuable characteristics of cryopreservation as an important tool for long-term germplasm conservation is the stability of the preserved material at these extremely low temperatures. Although cryopreservation is usually claimed as a guarantee for genetic stability, in comparison with other *in vitro* long term storage procedures,

Table 2. Survival percentage of five chrysanthemum cultivars using a vitrification method[1], after a month in culture, in parenthesis number of studied apices.

Cultivar	Survival (%) Duration of PVS2 treatment			
	20 min		40 min	
	Control	Cryopeserved	Control	Cryopeserved
Red Focus	25 (8)	0 (13)	45 (11)	0 (13)
Red Reagan	--	8 (26)	--	30 (30)
Sheena Select	88 (9)	0 (15)	25 (8)	72 (11)
Pasodoble	100 (13)	92 (20)	45 (13)	63 (19)
Davies	--	8 (13)	--	0 (18)

[1]Nodal segments were precultured at 10 ºC and 10 µmol $m^{-2}s^{-1}$ irradiance for 3 weeks. Subsequently, apices (ca. 2mm long) were excised and cultured on 0.3 M sucrose at 5 ºC in darkness for 3 days. Apices were then treated with loading solution (2M glycerol + 0.4 M sucrose), and, after removing it, with PVS2 at room temperature (20–21 ºC). Cryopreserved apices were then cooled by direct immersion in liquid nitrogen (inside the cryovial). After rewarming in a water bath at 40 ºC, PVS2 solution was substituted by a 1.2M sucrose solution for 20 min. Apices were cultured on recovery medium (for further details see Martin and González-Benito, 2005). Control apices were treated in a similar way, except for the cooling step. --: treatment not carried out.

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genetic variation in cryopreserved material has been occasionally reported (see Harding 2004). However, the cause of these variations is generally not attributed to the cryopreservation treatments themselves, but to the *in vitro* proliferation or regeneration steps, which are necessary before or/and during the cryoprotection process (Harding 1997). Exposure to extreme physical conditions (i.e. very low temperatures, high osmotic pressure) and to certain chemicals employed as cryoprotectans (i.e. DMSO) may result in physiological stress and, consequently in genetic instability.

There are very few studies on the stability of cryopreserved chrysanthemum cultures (in comparison with all the published works on cryopreservation, see Table 1). Fukai et al. (1994) using a periclinally chimeric cultivar ('Apricot Marble') detected changes in the flower color in plants derived from cryopreserved apices. Shoot tips were excised, loaded with cryoprotectants (including DMSO) and cryopreserved. Hardly any changes were detected in the *in vitro* cultivated control, only 10% of the cryoprotectant-treatment derived plants showed variation in flower color, but in near 70% of the plants derived from cryopreserved apices the flowers showed a different color to that of the cultivar. The authors suggested that these results may be due to some kind of damage suffered by the shoot tips during freezing and thawing that could disturb the chimeric structure of the shoot tips during the shoot regeneration process. Although no further genetic analysis of the variant material was done in that study, the authors concluded that these results should be taken into consideration when cryopreserving chrysanthemum. The same recommendation was done some years later by Halmagyi et al. (2004) in their work on cryopreservation of chrysanthemum using different approaches. They suggested that comparative investigations of genetic stability and cryo-injury should be carried out.

Genetic stability analysis of cryopreserved apices of chrysanthemum using molecular markers was carried out in the cultivar 'Pasodoble' comparing two different cryopreservation protocols: vitrification and encapsulation-dehydration (Martín and González-Benito 2005). Assessment of stability was evaluated, using RAPDs (Random Amplified Polymorphic DNA) markers, in pot-cultivated mother plants (from which buds were excised for micropropagation), in shoots (leave tissue) from which apices were extracted for cryopreservation, and in shoots regenerated from cryopreserved apices 30 days after recovery and after further 3 months in culture. Throughout the process the origin of the apices was recorded in order to evaluate the influence of the tissue culture procedure in the possible variation that could appear. Twenty one regenerants cryopreserved by vitrification and 25 by encapsulation-dehydration were assessed. Only one cryopreserved regenerant from the encapsulation-dehydration method (number C22), which was supposedly the most stable method, showed a different band pattern (Fig. 1).

The newly appeared DNA fragments in C22, together with the absent ones and other control fragments present in both control and cryopreserved samples were isolated, cloned and sequenced in order to obtain more information about the genetic changes suffered during the process (Martín and González-Benito 2006). The analyses of these sequences, checking all the possible alignments using MegAlign (DNAStar) program, confirmed the

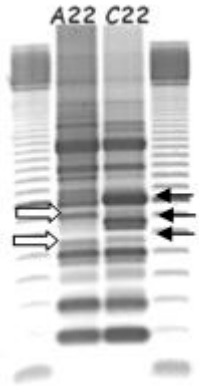

Fig. 1. RAPD banding profiles of DNA samples from control *in vitro* shoots (A) and cryopreserved material by encapsulation-dehydration (C) of *Dendranthema grandiflora* cv. ´Pasodoble' line 22. Amplification products were generated by primer OPO-15. Black arrows show new bands in C22 at 600, 650 and 800 bp. White arrows show the absent bands in the same sample (530 and 700 bp).

differences observed in the RAPD banding profile. Once the singularity of the sequences obtained from the variant fragments were checked, the study was focused on the analysis of each sequence in order to try to identify possible proteins that could be affected by the variation. DNA sequences were translated to protein sequences from all the possible ORF. Databases were consulted through BLAST program (Altschul et al. 1997). Only in two cases was a significant similarity found. A new fragment of approximately 600 bp in regenerant C22 matched significantly with a sequence corresponding to a reverse transcriptase from *Medicago truncatula*, with an identity of 42% and 62% similarity. The analysis of another new fragment (800 bp) showed a similarity of 62% and 48% identity with a kinase protein from *Arabidopsis thaliana.* The similarity of the 600 bp fragment with a reverse transcriptase is a phenomena previously described, since it is well known that stress situations may activate transposable elements present in the plant genome during the tissue culture process (Peschke and Phillips 1991).

Some studies have already revealed that cryopreservation techniques are associated with changes in the DNA methylation state (e.g. Hao et al. 2001), and those changes have been related to somaclonal variation. There are evidences that epigenetic changes, as differences in the methylation pattern of DNA, play a role in the occurrence of somaclonal variation through, for example, activation of transposable elements and silencing of genes, although the exact mechanism of this process remains unknown (Kaeppler et al. 2000). In order to evaluate the epigenetic stability of the regenerated plants from cryopreserved apices of cultivar 'Pasodoble', and the possible influence of each cryopreservation treatment the percentage of methylated cytosine (^{m}C) in each sample was calculated from HPLC histograms [%^{m}C = ^{m}C area / (C area + ^{m}C area)] (González-Benito et al. 2005). The percentage of ^{m}C in the samples studied prior cryopreservation ranged between 20.0 and 33.7 %. The corresponding samples after the cryopreservation process always showed lower values for ^{m}C content, except in one sample cryopreserved by the vitrification method. In some cases this reduction in the ^{m}C percentage, showed in the cryopreserved samples, was considerably high (Table 3; González-Benito et al. 2005). These results indicated a decrease in the methylation level of the DNA in the regenerated material after the cryopreservation process, independently of the method followed.

The different studies of genetic (and epi-genetic) stability of cryopreserved apices of chrysanthemum cv. 'Pasodoble' have revealed differences in the sequences and methylation status of the cryopreserved material compared with the unfrozen control. These variations occur at a low frequency, and they do not imply an invalidation of the cryopreservation technique in the conservation of chrysanthemum germplasm. For some types of germplasm (e.g. chimeras and genotypes inherently more prone to producing off-types) there is a greater need to exercise caution as to how cryop-

Table 3. Percentage of methylated cytosine (^{m}C) in cryopreserved samples of chrysanthemum cv. 'Pasodoble' calculated from HPLC histograms [%^{m}C = ^{m}C area /(C area + ^{m}C area)].

Cryopreservation Method	Samples prior cryopreservation	% ^{m}C	Samples after cryopreservation	% ^{m}C
Encapsulation-dehydration	A9	23.4	C9	21.9
	A16	29.7	C16	18.3
	A22	20.0	C22	17.2
Vitrification	A54	33.7	V54	18.5
	A68	20.2	V68	24.8
	A75	28.3	V75a	14.5
			V75b	24.6

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reservation is applied and to apply more stringent stability monitoring for quality control.

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