Cryopreservation of *in vitro*-grown shoot tips of chrysanthemum by encapsulation-dehydration

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ABSTRACT

Chrysanthemums are amongst the most economically important flowers in the world. The protection and storage of these valuable genetic resources is of great importance. Today, cryopreservation, or the storage of biological material at the temperature of liquid nitrogen (-196°C), is believed to be the most promising long-term storage method. To optimise the cryopreservation protocol, the shoot tips of *Chrysanthemum* $\times$ *grandiflorum* /Ramat./ Kitam. 'Lady Orange' and 'Lady Salmon' mutants were cryopreserved using the encapsulation-dehydration technique. During the experiment, the influence of sucrose concentration (2, 3 and 6%) during preculture and the concentration of kinetin (0.25, 0.5, 0.75 and 1.0 mg dm^{-3}) in the regrowth medium were tested. A higher survival rate was observed for 'Lady Salmon'. In general, the media with higher sucrose levels provided the best survival and recovery rates (35-40%). Kinetin had no influence on the survival rate; however, it influenced the morphogenesis of the plants. The lowest number of explants forming multiple shoots was observed on the medium with the lowest sucrose (during preculture) and kinetin (in the recovery medium) concentration. On the other hand, the best rhizogenesis efficiency was observed when 0.25 mg dm^{-3} kinetin was added. In conclusion, the composition of both preculture and recovery media need to be adjusted to single cultivars. The use of 3% sucrose (preculture) and 0.25 mg dm^{-3} kinetin (recovery) seems reasonable, since it guarantees a satisfying recovery rate of the explants and at the same time prevents the formation of callus and multiple shoots, stimulating the rooting instead.

Key words: biodiversity, kinetin, mutants, preculture, recovery, storage, sucrose

INTRODUCTION

Chrysanthemum $\times$ *grandiflorum* /Ramat./ Kitam. of the Asteraceae family is one of the biggest floricultural crops in the world (Teixeira da Silva and Fukai 2003). In different countries it is used for various occasions and purposes. Not surprisingly, the demand for new attractive cultivars has been growing continuously every year (Zalewska et al. 2007). However, self-incompatibility, a high level

of ploidy and heterozygosity make generative reproduction and conventional breeding very difficult. Bearing that in mind, mutation breeding is the most frequently applied method of achieving new cultivars of vegetatively propagated species (Zalewska et al. 2007, Zalewska et al. 2011). Indeed, every year hundreds of new cultivars are produced, replacing the current assortment. Still the market of ornamental plants is subjected to periodical trend-driven changes (Halmagyi et al.

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2004). Not surprisingly then, the storage of valuable genetic resources for the purpose of protection and breeding are gaining importance.

In the past, plant material has been stored in gene banks under slow-growth (low temperature and light intensity) *in vitro* conditions. However, the maintenance of *in vitro* cultures is expensive, laborious and threatened with contamination loss and somaclonal variation (Engelmann 2011), previously observed with chrysanthemums (Zalewska 2010). With that in mind, cryopreservation; i.e. the storage of plant material at ultra-low temperatures of liquid nitrogen (LN, -196°C) is believed to be the most promising, safe and cost-effective biological material storage method. Over the years many cryopreservation techniques have been developed (Engelmann 2011). Among them, the encapsulation-dehydration technique seems more preferable and guarantees greater genetic stability, thanks to the presence of a protection bead and a lack of toxic cryoprotectants (typically required in other cryopreservation methods), which can be especially harmful for mutant-unstable, slow-growing under *in vitro* conditions, chimeric cultivars by disturbing their structure and leading to substantial losses in the horticultural industry. This technique is based on technology developed to produce synthetic seeds, i.e. the encapsulation of chrysanthemum explants in a protective capsule based on non-toxic calcium alginate beads (Pinker and Abdel-Rahman 2005). Encapsulated explants are then subjected to osmotic dehydration (in a liquid medium with a high sucrose concentration) and partially desiccated (physical dehydration – under laminar-air flow or over silica gel) before exposure to liquid nitrogen. The presence of the bead protects the explants from the toxic influence of concentrated sucrose solutions and allows for their desiccation to low moisture content (Engelmann 2008). However, previous studies with chrysanthemum encapsulation-dehydration have been rather unsatisfactory, resulting in low survival (20–45%) or poor regrowth rates (12%) and regeneration of multiple shoots through callus (Halmagyi et al. 2004, Martin and Gonzalez-Benito 2009, Osorio-Saenz et al. 2011 a, b).

There are numerous factors affecting cryopreservation protocol efficiency (Engelmann 2008, 2011). Many researchers underline the importance of proper preculture conditions (Suzuki et al. 2006, Antony 2011). Adding carbohydrates, usually sucrose, to the preculture media is recommended, since they are non-toxic cryoprotectants stimulating osmotic dehydration and the accumulation of

endogenous sugars, polypeptides, ABA (abscisic acid), proline and bounded water, which increases the plant's stress and LN tolerance (Suzuki et al. 2006). The concentration of the carbohydrate needs to be adjusted to the requirements of single cultivars. The composition of the recovery media is also important. Over time, however, no experiments have been carried out to optimise the regrowth medium composition. Presumably, the use of an optimal sucrose concentration during preculture and kinetin in the recovery media will result in more satisfying results of chrysanthemum cryopreservation (higher survival without callus formation or shoot deformation).

The aim of this study was to develop an easy, efficient and cost-effective chrysanthemum mutant encapsulation-dehydration cryopreservation protocol, without the need of applying toxic and expensive cryoprotectants, by optimising the preculture and recovery media composition, and to investigate the influence of this technique on the morphology of the plants produced.

MATERIAL AND METHODS

The two-week-old shoot tips of *Chrysanthemum* × *grandiflorum* /Ramat./ Kitam. 'Lady Salmon' and 'Lady Orange' mutants, with one or two leaf primordia, were used in the experiment, conducted from October through January.

The single-node explants of 'Lady Salmon' and 'Lady Orange' cultivars with leaves removed were cultured for one week on 30 ml of the modified MS (Murashige and Skoog 1962) medium with 0.06 (2% w/v) or 0.09 M (standard 3% w/v; control) sucrose concentration, 100 mg dm⁻³ myo-inositol, 0.5 mg dm⁻³ nicotinic acid, 0.5 mg dm⁻³ pyridoxine-HCl, 0.1 mg dm⁻³ thiamine-HCl, 2 mg dm⁻³ Glycine and an additional 330 mg dm⁻³ calcium chloride (CaCl₂ · 6H₂O), 13.9 mg dm⁻³ iron (II) sulphate (FeSO₄ · 7H₂O), and 20.6 mg dm⁻³ disodium ethylenediaminetetraacetic acid (Na₂EDTA · 2H₂O) were added, solidified with agar at a concentration of 8 g dm⁻³, at pH 5.8 prior to autoclaving in 350 ml glass jars (10 explants per jar). After one week the explants were transferred onto the same medium, and were supplemented with 10 µM (2.64 mg dm⁻³) of ABA. Moreover, half of the explants from the medium with 3% sucrose were cultured on the medium with 0.18 M (6% w/v) of the carbohydrate, also enriched with ABA. All of the explants were cultured in the growth room at 24°C ± 2°C, exposed to a 16-h photoperiod and photosynthetic photon

flux density of $27.40 \mu\text{mol m}^{-2} \text{s}^{-1}$ for another seven days.

The shoot tips had been isolated with the use of microscalpels, surgical tweezers (to minimise injury) and a microscope, incubated for 10 min in 3% sodium alginate (Na-alginate) based on the modified (as above) MS medium salts, without CaCl_2 , supplemented with 0.06 M, 0.09 and 0.18 M sucrose (respectively to the conditions from the preculture phase) and then dipped into 0.1 M CaCl_2 for 45 minutes, forming calibrated beads (4 mm in diameter), after which the beads were rinsed three times with distilled sterile water.

The beads were dehydrated in 25 ml of MS modified liquid medium in 100 ml Erlenmeyer flasks, gradually with the following sucrose concentrations: 0.3 M (48 h); 0.5 M (48 h); 0.7 M (48 h) and finally 0.9 M (24 h) at 130 rpm (4.5 amplitude) in the dark. Next the beads were desiccated for three hours under laminar airflow. At that point of the drying the beads reached about 40% of their initial mean fresh weight. The explants were placed in 2.0 ml cryovials (20 beads in a cryovial = a single replication) and immersed in LN. After one hour, the cryovials were removed from the LN, thawed rapidly in a water bath ($38 \pm 1^\circ\text{C}$ for 3 minutes) and the beads were inoculated on the modified MS medium with 0.09 M sucrose and 0.25 (control); 0.5; 0.75 or 1.0 mg dm^{-3} kinetin (Kin), and incubated in the growth room under reduced lighting conditions ($16.86 \mu\text{mol m}^{-2} \text{s}^{-1}$). Five days after thawing, the beads were cultured under the initial lighting conditions.

The survival (% of explants that survived freezing and remained green) and regrowth (% of explants that survived freezing and showed signs of further development) rates of the explants were estimated 14 and 30 days, respectively, after thawing. The fresh weight and the length of all of the shoots produced and the presence of roots were observed 10 weeks after thawing. The results

(completely randomised design) were statistically analysed using analysis of variance (ANOVA) for the three-factor experiment, and the comparisons of means were made with Tukey's Multiple Comparison Test ($p = 0.05$) using STATISTICA 10.0 and ANALWAR-5.2-FR tools. The experiment studied 24 combinations: 4 kinetin concentrations $\times$ 3 sucrose concentrations $\times$ 2 cultivars in 2 replications, 20 shoot tips in each replication.

RESULTS

During the experiment we observed that all of the factors investigated (cultivar, sucrose concentration in the preculture medium and kinetin in the recovery medium) had a significant influence on the cryopreservation protocol efficiency.

The composition of the media used during preculture had a significant influence on the survival ($F = 7.69$, $p = 0.0026$) and regrowth potential ($F = 8.69$, $p = 0.0014$, Tab. 1) of the explants. The highest survival was observed with 3 and 6% sucrose, while the lowest with 2% sucrose (Fig. 1). The situation was somewhat different when considering the regrowth potential (no difference was observed between the media with 2 and 3% sucrose). It was also observed that 'Lady Salmon' is more desiccation- and cold-tolerant since it showed better survival ($F = 8.32$, $p = 0.0081$) and regrowth potential ($F = 9.72$, $p = 0.0047$) in comparison to 'Lady Orange', although in the latter all of the explants that had survived grew into microshoots, which was not always observed with 'Lady Salmon' (Fig. 1). The kinetin concentration had no influence on the survival; however, it influenced the explants' recovery potential ($F = 2.42$, $p = 0.0904$ and $F = 5.24$, $p = 0.0063$, respectively, Fig. 1).

During the experiment the formation of both single and multiple shoots was observed. Higher kinetin concentrations increased the number of explants that survived freezing and produced multiple shoots ($F = 4.63$, $p = 0.0108$, Fig. 2). In

Table 1. Influence of the kinetin and sucrose concentration on the explants' regrowth potential (%) depending on the cultivar

Kinetin (mg dm^{-3})	Lady Orange			Lady Salmon		
				sucrose concentrate (%)		
	2	3	3/6	2	3	3/6
0.25	11 ab ¹	47 a	44 a	12 ab	13 ab	46 a
0.50	0 b	31 ab	28 ab	45 a	18 ab	42 a
0.75	15 ab	28 ab	30 ab	28 ab	42 a	38 ab
1.00	21 ab	31 ab	47 a	49 a	60 a	45 a

¹Values marked with the same letter do not differ significantly at $p = 0.05$

Values marked with the same letter do not differ significantly at $p = 0.05$; lowercase letters refer to survival; uppercase letters refer to regrowth rate

Figure 1. Influence of the sucrose level, cultivar effect and kinetin concentration on the chrysanthemum explants' survival rate and growth potential

addition, a higher sucrose concentration (6%) stimulated their formation more frequently in comparison to 2% ($F = 4.08$, $p = 0.0298$, Fig. 2). The cultivar effect was non-significant alone ($F = 0.0185$, $p = 0.8928$), but an interaction between the cultivars and the sucrose concentration ($F = 8.36$, $p = 0.0018$, Tab. 2) was demonstrated.

The shoots produced varied in length. After 10 weeks some remained very small (approx. 2 mm), while others were much longer (up to 16 mm). Kinetin had no influence on the weight of the shoots ($F = 0.62$, $p = 0.9022$, Fig. 3). However, the longest microshoots were obtained on the medium with the lowest cytokinin concentration ($F = 8.86$, $p = 0.0004$). It was also observed that 'Lady Salmon' produced significantly longer shoots of a bigger weight in comparison to 'Lady Orange' ($F = 33.83$, $p < 0.0001$ and $F = 16.14$, $p = 0.0005$, respectively, Fig. 3). Sucrose alone did not influence those parameters ($F = 0.76$, $p = 0.4804$ and $F = 0.33$, $p = 0.7194$, length and weight respectively). There were, however, some interactions observed between the cultivars and the sucrose or kinetin concentration applied ($F = 4.20$, $p = 0.0272$ and $F = 3.75$, $p = 0.0243$, respectively, Tabs 2 and 3).

Similarly, rhizogenesis efficiency was affected by the factors investigated. The medium with the lowest kinetin concentration significantly increased the number of explants that did survive freezing and

Values marked with the same letter do not differ significantly at $p = 0.05$

Figure 2. Influence of the kinetin and sucrose concentration on the percentage of chrysanthemum explants forming multiple shoots

Table 2. Influence of the sucrose concentration and the cultivar effect on the percentage of explants forming multiple shoots, shoot length and the percentage of shoots forming roots independently of the kinetin concentration

Characteristic	Sucrose (%)	Lady Orange	Lady Salmon	
Multiple shoots (%)	2	12.50 a ¹	13.96 b	ns
	3	41.24 a	24.79 b	ns
	3/6	22.70 a	57.09 a	*
Shoot length (mm)	2	5.73 a	16.25 a	*
	3	6.63 a	12.92 a	*
	3/6	7.87 a	11.13 a	ns
Rhizogenesis (%)	2	37.50 a	35.41 b	ns
	3	5.63 b	61.66 a	*
	3/6	12.91 b	50.41 a	*

¹Values marked with the same letter do not differ significantly at $p = 0.05$; lowercase letters refer to the sucrose concentration in the same cultivar; means signed with an asterisk (*) differ in terms of cultivar effect at the same sucrose concentration

created roots in comparison to the medium with the highest kinetin concentration ($F = 4.15$, $p = 0.0167$, Fig. 4). The cultivar effect was also relevant ($F = 6.22$, $p = 0.0199$). As for 'Lady Salmon' almost 50% of the explants formed roots, while 'Lady Orange' had less than 20%. There was also an interesting interaction demonstrated between the

Values marked with the same letter do not differ significantly at $p = 0.05$; lowercase letters refer to length; uppercase letters refer to weight

Figure 3. Influence of the kinetin concentration and cultivar effect on chrysanthemum shoot length and weight

Values marked with the same letter do not differ significantly at $p = 0.05$

Figure 4. Influence of the kinetin concentration on the percentage of chrysanthemum shoots forming roots independently of the sucrose concentration and the cultivar effect

cultivar effect and the sucrose concentration, since in 'Lady Orange' the largest number of roots was observed on the medium with the lowest sucrose concentration, while in 'Lady Salmon' the same

Table 3. Influence of the kinetin concentration and the cultivar effect on the shoot length (mm) independently of the sucrose concentration

Kinetin (mg dm ⁻³)	Lady Orange	Lady Salmon	
0.25	10.22 a ¹	25.64 a	*
0.50	5.14 c	13.25 ab	*
0.75	5.43 bc	12.21 ab	*
1.00	7.54 ab	9.91 b	ns

¹Values marked with the same letter do not differ significantly at $p = 0.05$; lowercase letters refer to the kinetin concentration in the same cultivar; means signed with an asterisk (*) differ in terms of cultivar effect at the same kinetin concentration

medium provided the least favourable results ($F = 13.74$, $p = 0.0001$, Tab. 2).

DISCUSSION

Due to its chimeric structure (observed in about 50% of all of the cultivars available), chrysanthemums are especially susceptible to various changes on the phenotype, karyotype, biochemical or molecular level (Fukai et al. 1991, Fukai et al. 1994, Hitmi et al. 1997). Furthermore, mutant cultivars (very popular in production) are very often sensitive to any stress and characterised by slow growth and poor rhizogenesis potential under *in vitro* conditions. These phenomena require particular consideration while developing a cryopreservation protocol. In the present experiment, the results were both procedure- and cultivar-dependent.

The survival and regeneration rates are key factors determining the cryopreservation protocols' success and commercial application. The results reported in the experiment (50-60% regrowth of the explants in the best conditions, depending on the cultivar) are quite satisfying since Sakai et al. (2000) and Halmagyi et al. (2004) recorded a 20-45% shoot regrowth. With droplet ultra-freezing, the results were even more diverse, reaching from 4 to 70% (Halmagyi et al. 2004).

Like with *Rosa* × *hybrida* L. (Halmagyi and Pinker 2006), the *Chrysanthemum* × *grandiflorum* shoot tips that did not survive freezing also demonstrated signs of browning after a few days. In 'New Dawn' roses, the addition of active charcoal prevented the explants from browning and dying (Pawłowska and Bach 2011), which could be considered in the future. Fukai et al. (1991) observed that not all of the explants that did survive freezing grew into shoots (a difference of even 40%). In our experiment, however, no such phenomenon was observed with 'Lady Orange', while with 'Lady Salomon' it was very rare, which additionally underlines the efficiency of our method.

Preculture is one of the most important factors in cryopreservation (Hitmi et al. 2000b). Usually higher concentrations of sucrose are recommended during that phase (Hitmi et al. 2000a, Mikula et al. 2005, 2009), however, our previous results with the cultivars tested revealed that a sucrose concentration higher than 0.25 M inhibits the proliferation of meristematic cells and therefore decreases the survival and regrowth potential of the frozen samples. The addition of cytokinins during that phase could improve this situation (Fukai 1991, Halmagyi et al. 2004), however,

their presence may disturb the chimeric structure of the plants. Moreover, as proved by Martin et al. (2011), treatment with a high sucrose concentration may be the cause of the genetic instability in cryopreserved *Chrysanthemum* × *grandiflorum* apices. Therefore, in this experiment we used a sucrose concentration lower than usual in cryopreservation and no plant growth regulators (PGRs) during preculture (reducing the costs of the procedure at the same time). In contrast to *Chrysanthemum cinerariaefolium* Vis. and *Gentiana scabra* Bunge var. *buergeri* Maxim., two-step preculturing with increasing sucrose levels did not significantly improve the explants' survival rate in comparison to standard 3% sucrose (Hitmi et al. 2000 b, Suzuki et al. 2006). The replacement of sucrose with other carbohydrates (e.g. glucose or trehalose) could change this situation.

Our previous research confirmed the necessity of applying cytokinins in the recovery medium (Zalewska and Kulus, unpublished). In all of the previous studies with chrysanthemum, benzyladenine (BA) was added to the recovery medium (Fukai et al. 1991, Fukai et al. 1994); however, this may result in callus formation and the regeneration of multiple shoots, which is in general unwanted and may lead to genetic disturbance (Halmagyi et al. 2004, Osorio-Saenz et al. 2011 b). In our experiment, kinetin in higher concentrations also stimulated the formation of multiple shoots, however it strongly limited their presence in lower levels (0.25 mg dm⁻³). The growth of single shoots without callus formation may indicate the maintenance of genetic stability, which, however, requires further molecular analysis.

In addition, the longest shoots were produced on the medium with the lowest (0.25 mg dm⁻³) Kin concentration. This indicates that cytokinins are necessary to stimulate the proliferation of the cells after freezing, since a PGRs-free medium is ineffective to stimulate the growth of the frozen explants (Zalewska and Kulus, unpublished), yet they inhibit the further growth of the plant (stimulating their proliferation instead) if added in higher levels. The kinetin concentration demonstrated no influence on the weight of the shoots, which possibly could be explained by the fact that lower cytokinin levels stimulated the growth of longer, but thinner shoots, in contrast to higher Kin concentrations, which stimulated the proliferation of shorter but thicker shoots (however, this requires additional measurements).

Some mutants show problems with rooting. We observed that the presence of the bead and a higher concentration of kinetin in the recovery media additionally inhibited root formation. Therefore, it could be beneficial to cut off the shoots produced when they break the capsule and to transfer them to a fresh medium without any PGRs. That way, the formation of callus and multiple shoots may be stopped, while the formation of roots stimulated. It may also increase the growth rate of the shoots thanks to the direct contact of the explant with the medium and better nutrients access.

The results reported in our experiment shed new light on the possibility of applying cryopreservation techniques for the purpose of storage, protection and breeding of valuable genetic resources of chrysanthemums and should be considered in further research on this subject.

CONCLUSIONS

1. The sucrose concentration during preculture and the cultivar effect have significant influence on the explants' survival and regrowth rate, while the concentration of kinetin in the recovery medium influences only the regrowth potential.
2. Higher levels (3 and 6%) of sucrose (preculture medium) and kinetin ($> 0.25 \text{ mg dm}^{-3}$; recovery medium) stimulate the formation of multiple shoots. However, it is the cultivar effect that mainly influenced the length and weight of the shoots produced.
3. Kinetin is necessary to stimulate the proliferation of shoots; however, at higher concentrations ($> 0.25 \text{ mg dm}^{-3}$) it inhibits their elongation and the formation of roots.

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KRIOPREZERWACJA PĄKÓW WIERZCHOŁKOWYCH CHRYSZANTEMY METODĄ KAPSUŁKOWANIA- DEHYDRATACJI

Streszczenie: Chryzantemy zaliczane są do najpopularniejszych i najbardziej dochodowych kwiatów na świecie (zarówno doniczkowych jak i ciętych). Z tych względów ochrona tych cennych

zasobów roślinnych nabiera szczególnego znaczenia. Obecnie to krioprezerwacja (przechowywanie materiału biologicznego w temperaturze ciekłego azotu; -196°C) uważana jest za najbardziej efektywną metodę ochrony roślinnych zasobów genowych. Celem badań była optymalizacja procedury krioprezerwacji metodą kapsułkowania-dehydratacji pąków wierzchołkowych mutantów chryzantemy wielkokwiatowej 'Lady Orange' oraz 'Lady Salmon'. Zbadano wpływ stężenia sacharozy w trakcie prekultury (2, 3 i 6%) oraz kinetyny w pożywce wzrostowej (0,25, 0,5, 0,75 oraz 1,0 mg dm⁻³). Uzyskane wyniki zależne były od odmiany. 'Lady Salmon' cechowała się większą przeżywalnością. Pożywki z wyższymi zawartościami sacharozy w trakcie prekultury zapewniły najwyższą przeżywalność oraz potencjał wzrostowy eksplantatów poddanych mrożeniu (35-40%). Stężenie kinetyny nie miało wpływu na przeżywalność eksplantatów, jednakże wpłynęło na morfogenezę uzyskanych roślin (procent eksplantatów tworzących zwielokrotnioną liczbę pędów oraz korzenie). Najmniej eksplantatów tworzących pędy wielokrotne zaobserwowano na pożywce z najniższym stężeniem sacharozy (w trakcie prekultury) oraz kinetyny (w pożywce wzrostowej). Natomiast ryzogeneza najwydajniej przebiegała właśnie po dodaniu 0,25 mg dm⁻³ kinetyny. Z przeprowadzonych badań wynika, iż skład zastosowanych pożywek (zarówno na etapie prekultury, jak i odtwarzania) musi być dostosowany nie tylko do indywidualnych wymagań gatunków, ale także poszczególnych odmian. Zastosowanie 3% sacharozy (prekultura) oraz 0,25 mg dm⁻³ kinetyny (odtworzenie) wydaje się być optymalne, gdyż zapewnia zadawalającą przeżywalność, chroniąc jednocześnie przed rozwojem kalusa i regeneracją pędów wielokrotnych, stymulując za to ukorzenianie roślin.

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