

Effects of various preculture, pretreatment, and recovery conditions on the morphogenetic response of cryopreserved Lady Orange chrysanthemum shoot tips

Dariusz KULUS*

Department of Ornamental Plants and Vegetable Crops, Faculty of Agriculture and Biotechnology,
UTP University of Science and Technology, Bydgoszcz, Poland

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Abstract: The aim of this research was to optimize the encapsulation-dehydration cryopreservation protocol of Lady Orange chrysanthemum explants on the preculture, pretreatment, and post-rewarming recovery steps. Shoot tips were precultured on MS medium with various abscisic acid concentrations (0–30 μ M). Next, the encapsulated explants were osmotically dehydrated for 3 or 5 days and desiccated for 3–4 h and, after rewarming, they were subcultured on recovery media of various compositions (control and cytokinin/auxin-supplemented). A high explant survival rate, even up to 100%, was observed. The value of this parameter, however, changed depending on the post-rewarming culture duration. Moreover, not all the viable explants were capable of forming shoots. A lower ABA concentration (15 μ M) during preculture and the presence of 4.65 μ M kinetin in the post-rewarming recovery medium enhanced cryopreservation efficiency with a high survival rate and typical microshoot formation. A higher ABA concentration and the presence of 6-benzylaminopurine in the recovery medium resulted in shoot multiplication, abundant callus formation, and root formation inhibition.

Key words: Abscisic acid, dehydration, desiccation, encapsulation, liquid nitrogen, plant growth regulators, regeneration

1. Introduction

Chrysanthemum has a great share in the floristic market (Zalewska et al., 2010). The number of available cultivars is, in fact, impossible to estimate. In order to protect the genetic resources in a small area and at reduced costs, cryopreservation techniques can be applied. Attempts to store chrysanthemum plant material in liquid nitrogen (LN) have been made since 1990 (Fukai and Oe, 1990). In vitro-grown shoot tips are most suitable for cryopreservation (Lee et al., 2011; Kaya et al., 2013; Souza et al., 2016). Unfortunately, there are still some problems, such as low viability or regeneration potential of the cryopreserved chrysanthemum tissues (Osorio-Saenz et al., 2011), their hyperhydricity (Wang et al., 2014), disturbance of the chimeric structure (Fukai et al., 1994), or DNA sequence alternations (Martín and González-Benito, 2009; Kaya and Souza, 2017). With that in mind, optimization research on chrysanthemum cryopreservation procedures is still being carried out to make the process more efficient.

Previous results clearly underline that the preculture and pretreatment of tissues prior to storage in LN, as well as post-rewarming (recovery) culture conditions, significantly influence the cryopreservation protocol

success (Zalewska and Kulus, 2013, 2014; Kaya et al., 2016; Souza et al., 2017). Preculture (hardening) on a properly modified, usually solid, medium increases the stress tolerance of explants, associated with their further pretreatment: dehydration (osmotic and/or physical – air desiccation/drying) and storage in LN (Ozudogru et al., 2010, 2011; Kulus, 2015).

The survival rate and, most importantly, the recovery efficiency (a further shoot development potential) of LN-stored explants are essential factors determining the cryopreservation efficiency. Over time several cryopreserved cell viability tests were developed: determination of volatile hydrocarbon stress markers (ethylene, hydroxyl radicals, and lipid peroxidation products); peptide profile evaluation; chlorophyll content and phosphatase or peroxidase activity analysis (Verleysen et al., 2004; Benson, 2007); acetocarmine, fluorescein diacetate (Ishikawa et al., 2006), 2,3,5-triphenyl tetrazolium chloride (Lurswijidjarus and Thammasiri, 2004), or Evans Bleu assays (Zainuddin et al., 2011); and histological and electron or confocal microscopy observations (Gallard et al., 2009; Jeon et al. 2015a, 2015b). All these approaches, however, require tissue killing, which makes

* Correspondence: dkulus@gmail.com

their further analysis (morphogenetic response or genetic stability) impossible. Visual observation based on the number of explants with living, i.e. green, tissues is still a popular technique. That parameter, however, may change depending on culture duration, as observed by Kulus and Zalewska (2014). Therefore, the evaluation of explant survival rate should be repeated with time.

The aim of this study was to assess the influence of abscisic acid (ABA) during preculture, the duration of pretreatment (osmotic and physical dehydration), and the composition of the recovery medium on Lady Orange chrysanthemum cryopreservation efficiency. Moreover, the effect of each of the cryopreservation steps on the explant morphogenetic response (formation of single and multiple shoots, roots, and calli, as well as their morphometric parameters) was evaluated for the first time. An attempt was also made to address the question of whether the survival rate of the LN-recovered shoot tips changes depending on the post-rewarming culture duration. It was assumed that by optimizing the protocol steps it would be possible to achieve high cryopreservation efficiency (i.e. high survival of explants and further development of typical shoots with no signs of malformation). The results will have a significant impact on the understanding of the influence of various factors on cryopreservation efficiency and in improving the storage methods for chrysanthemum and other plant species, which is especially important in the era of advancing genetic erosion.

2. Materials and methods

2.1. Optimization of pretreatment conditions and recovery medium composition

Single-node explants of *Chrysanthemum × grandiflorum* (Ramat.) Kitam. Lady Orange, with removed leaves, were cultured on 30 mL of modified MS medium (Murashige and Skoog, 1962) supplemented with 9% (w/v) sucrose and additional 330 mg dm⁻³ calcium chloride (CaCl₂·6H₂O), 13.9 mg dm⁻³ iron(II) sulfate (FeSO₄·7H₂O), 20.6 mg dm⁻³ disodium ethylenediamine-tetraacetic acid (Na₂EDTA·2H₂O), and 4.65 µM kinetin (N6-furfuryladenine, KIN, Sigma-Aldrich) added, solidified with 0.8% agar (Biocorp) at pH 5.8 prior to autoclaving in glass jars of 350 mL (10 explants per jar). The explants were cultured in the growth room at 24 ± 2 °C, exposed to a 16-h photoperiod and photosynthetic photon flux density of an average of 37.0 µmol m⁻² s⁻¹.

After 9 days, shoot tips of 2.0 mm in length with two or three leaf primordia were isolated with the use of a binocular, embedded for 10 min in 3% sodium alginate (Na-alginate, CARLO ERBA) based on the modified (as described above) MS medium salts without CaCl₂, supplemented with 9% sucrose, and then dipped into 0.1 M CaCl₂ for 30 min, forming calibrated beads (5 mm in

diameter), after which the beads were rinsed three times with distilled sterile water.

The beads were dehydrated in 30 mL of modified MS liquid medium in 100-mL Erlenmeyer flasks gradually with the following sucrose concentrations: 0.3 M (24 or 48 h), followed by 0.6 M (24 or 48 h), and finally 0.9 M (24 h) at 130 rpm (4.5 amplitude) in the dark (3- or 5-day osmotic dehydration; 1st experimental factor). Next, the beads were desiccated (physical dehydration) for 3 or 4 h (42.9% and 40.8% initial moisture content, respectively) under laminar air flow at room temperature (2nd experimental factor). The explants were placed in 2.0-mL cryovials (10 beads per vial) and immersed in LN (-196 °C).

After 1 h, the cryovials were removed from the LN and rewarmed rapidly in a water bath (39 ± 1 °C for 3 min), and the beads were inoculated on modified MS recovery medium with 3% sucrose, 4.44 µM 6-benzylaminopurine (BA) + 0.54 µM 1-naphthalene acetic acid (NAA), or 4.65 µM KIN + 0.54 µM NAA, or hormone-free (MS0) control (10 beads per Petri dish) and sealed with Parafilm (3rd experimental factor). The cultures were kept in the same growth room under reduced lighting conditions (approximately 12.0 µmol m⁻² s⁻¹) for 7 days. Next, the beads were cultured under initial lighting conditions. After 30 days the explants were transferred to MS0 medium. A precultured and dehydrated but non-LN-stored control (-LN) was also considered. The experiment was repeated at least twice.

Based on the described above study, optimized pretreatment and recovery conditions were used in the following experiment.

2.2. Optimization of ABA concentration during preculture

Single-node explants of Lady Orange chrysanthemum were cultured on 30 mL of modified MS medium supplemented with 3% (w/v) sucrose, 9.29 µM KIN, and 15 µM or 30 µM abscisic acid (ABA, Sigma-Aldrich), with 10 explants per jar. The explants were cultured in the same growth room.

After 2 weeks, shoot tips were isolated and encapsulated as described above. The beads were dehydrated in modified MS liquid medium gradually with the following sucrose concentrations: 0.3 M (48 h), followed by 0.6 M (48 h), and finally 0.9 M (48 h). Next, the beads were desiccated for 3.5 h under laminar air flow (41.8% initial moisture content). The explants were placed in cryovials and immersed in LN.

After 1 h, the cryovials were removed from the LN and rewarmed in a water bath (as above), and the beads were inoculated on modified MS recovery medium with 3% sucrose and 1.16 µM KIN + 0.54 µM NAA (Zalewska and Kulus, 2013) and sealed with Parafilm. The cultures were kept in the same growth room. After 30 days the explants were transferred to MS0 medium. The experiment was repeated five times.

2.3. Statistical analysis

The survival of the shoot tips (%; based on microscopic observations; explants with green tissues were recognized as viable) was estimated 7, 30, and 90 days after rewarming. After 90 days the explant regeneration potential (%) was recorded. The total number of cryopreserved shoot tips was considered 100%. The percentages of the explants forming single, multiple (i.e. those that formed in the number of at least two from a single cryopreserved shoot tip), and hyperhydrated (deformed and of glossy appearance) shoots as well as calli were evaluated. The percentage of explants spontaneously regenerating adventitious roots was also determined. As for those parameters, the total number of explants viable 90 days after rewarming was considered 100%. The fresh weight and length of regenerated tissues produced after 90 days was also estimated.

The number and percentage data were not normally distributed; therefore, for the data expressed as percentages, Freeman-Tukey transformation was used. After the

transformation, the results (completely randomized design) were statistically analyzed with analysis of variance (ANOVA) and the comparisons of means were made with the Newman-Keuls multiple comparison test ($P = 0.05$) using Statistica 10.0 and ANALWAR-5.2-FR tools. The tables with results provide real numerical data, while letters mark homogeneous groups following the statistical calculations based on transformed data.

3. Results

3.1. Influence of pretreatment and recovery medium composition

3.1.1. Explant survival and shoot formation

A lower survival rate of the LN-stored samples (+LN) was observed in comparison to the nonfrozen control (-LN). As for the control shoot tips, 100% survival was observed, while with the LN-stored explants a maximal viability of 35% was recorded after 1 week in culture (Table 1). No

Table 1. Influence of osmotic and physical dehydration duration and recovery medium composition on the survival rate (%) and shoot formation (%) of cryopreserved (+LN) and nonfrozen control (-LN) Lady Orange chrysanthemum shoot tips 7, 30, and 90 days after rewarming.

Recovery medium	Dehydration period							
	3-day osmotic dehydration				5-day osmotic dehydration			
	3-h desiccation		4-h desiccation		3-h desiccation		4-h desiccation	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
	7-day survival (%)							
MS0	90.9 a	5.0 b	84.6 a	25.0 b	83.3 a	0.0 b	100 a	5.0 b
BA + NAA	100 a	28.4 b	90 a	21.8 b	100 a	5.6 b	100 a	24.1 b
KIN + NAA	100 a	26.7 b	100 a	35.0 b	100 a	20.0 b	100 a	28.3 b
	30-day survival (%)							
MS0	63.6 ab	0.0 d	69.2 ab	5.0 d	58.3 ac	0.0 d	27.3 bd	0.0 d
BA + NAA	100 a	68.6 ab	90.0 ab	74.1 ab	100 a	42.2 ad	87.5 ab	90.4 ab
KIN + NAA	100 a	63.3 ab	100 a	100 a	100 a	80.0 ab	77.8 ab	78.3 ab
	90-day survival (%)							
MS0	54.5 ab	0.0 c	69.2 ab	0.0 c	50.0 ab	0.0 c	27.3 bc	0.0 c
BA + NAA	88.9 a	76.0 a	90.0 a	84.9 a	100 a	52.2 ab	87.5 a	100 a
KIN + NAA	90.9 a	55.0 ab	100 a	90.0 a	100 a	80.0 a	77.8 a	82.9 a
	90-day shoots initiation (%)							
MS0	36.4 ad	0.0 d	38.5 ad	0.0 d	25.0 bd	0.0 d	18.2 cd	0.0 d
BA + NAA	88.9 ab	19.5 cd	90.0 ab	46.1 ad	100 a	15.6 cd	87.5 ab	56.8 ad
KIN + NAA	90.9 ab	35.0 ad	100 a	55.0 ad	100 a	11.1 cd	77.8 ac	34.3 ad

Means in rows and in columns marked with the same letter do not differ significantly at $P = 0.05$.

influence of dehydration duration or recovery medium composition was noticeable after that period. However, the survival rate of the LN-stored shoot tips increased with time since some of the explants, which were primarily brown and therefore considered dead, were eventually able to regenerate. An interaction of the analyzed factors was then evident. After 1 month in post-rewarming culture, an even 100% survival rate of explants was noticed after applying a 3-day osmotic dehydration, followed by 4 h of desiccation and by adding KIN to the recovery medium (Table 1). Eventually, 90 days after rewarming, 100% viability of LN-stored shoot tips was obtained after applying 5-day osmotic dehydration, followed by 4 h of desiccation and recovery on the BA-supplemented medium, although other treatments also provided very good results. No living tissues after storage in LN were observed on the recovery medium free of plant growth regulators (PGRs), regardless of all other experimental conditions.

In general, a significantly higher survival rate of the LN-stored explants was observed 30 days (52.2%) and 90 days (55.0%) after rewarming in comparison to 7 days (19.1%) (Figure 1). As for the nonfrozen explants, the opposite was often observed; a longer time of culture led to a survival rate decrease (especially on the MS0 medium; Table 1).

Not all of the LN-stored explants that survived freezing were capable of further growth (Table 1). Longer (4-h) desiccation provided a greater percentage of cryopreserved explants capable of forming shoots (35.8%) in comparison to 3-h desiccation (14.0%) (Figure 2A). The presence of PGRs during the post-rewarming culture guaranteed over 30% shoot recovery regardless of the cytokinin type, while no shoots were obtained after 90 days of culture on the MS0 medium (Figure 2B).

3.1.2. Morphogenetic response of the LN-stored shoot tips

It was observed that some of the cryopreserved shoot tips developed only a single leaf (Figure 3A) or callus (Figure 3B). As for shoot development, besides typical single shoots (Figure 3C), some of the viable explants recovered multiple shoots (in a number greater than one; Figure 3D) or deformed hyperhydrated shoots (Figure 3E). The formation of single shoots was supported by the presence of KIN (19.2%) in the recovery medium, followed by BA (5.8%) (Figure 4). The greatest percentage of viable explants forming typical single shoots 90 days after rewarming (16.7%–35.0%) was observed after applying a 3-day osmotic dehydration, followed by 3- or 4-h desiccation or with 5-day dehydration and 4-h desiccation, all in the presence of KIN (Table 2).

The presence of PGRs in the recovery medium favored the development of multiple shoots; BA was more effective in stimulating the regeneration of multiple shoots than

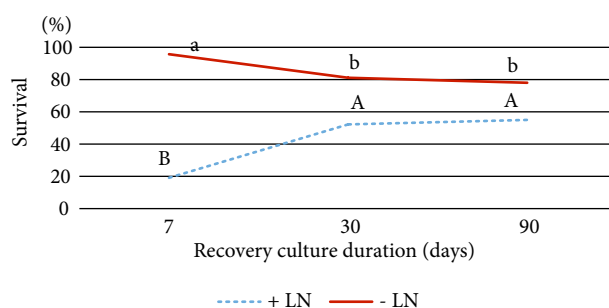


Figure 1. Influence of recovery culture duration on the survival (%) of LN-stored (+LN) and control (-LN) chrysanthemum shoot tips. Means marked with the same letter do not differ significantly at $P = 0.05$; lowercase letters refer to control samples and uppercase letters refer to LN-stored samples.

KIN (35.0% and 21.3% of explants, respectively; Figure 4). The greatest percentage of viable explants forming multiple shoots (56.8%) was observed after applying longer osmotic and physical dehydration and in the presence of BA during post-rewarming culture (Table 2).

As for deformed hyperhydrated shoots of glossy appearance, their presence 90 days after rewarming (18.3%) was observed only after applying 3-day osmotic dehydration, followed by 3-h desiccation and regeneration in the presence of KIN (Table 2). Those structures were incapable of further development.

The regeneration of adventitious roots was stimulated by the presence of KIN in the recovery medium, while no rooting occurred on the MS0 medium (Table 3). The greatest percentage of viable explants forming roots 90 days after rewarming (45.4%) was observed after applying 3-day osmotic dehydration and recovery medium supplemented with KIN.

Approximately 37.5% of shoot tips viable 90 days after rewarming regenerated a brown compact callus only. Its formation was stimulated by the presence of PGRs in the recovery medium (callus regeneration was not observed on the MS0 control medium); however, no influence of the cytokinin type was identified (59.2% explants for BA and 55.4% for KIN) (Table 3). Furthermore, no influence of the dehydration duration was recorded on this parameter.

3.1.3. Influence of pretreatment duration and recovery medium composition on the physical parameters of the regenerated biological material

The development of LN-stored shoots was much slower in comparison to the control, regardless of pretreatment conditions.

Shorter (3-h) desiccation guaranteed faster growth of the LN-recovered shoots in comparison to 4-h desiccation (13.1 and 8.3 mm in length, respectively; Figure 5A). The presence of KIN in the recovery medium also provided a

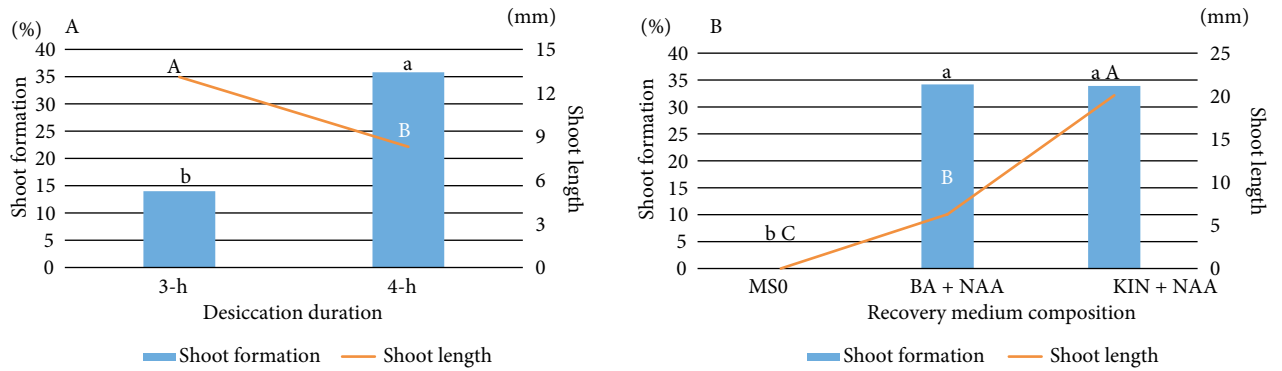


Figure 2. Influence of desiccation duration (A) and recovery medium composition (B) on the shoot formation rate (%) 90 days after rewarming, regardless of all other experimental factors. Means marked with the same letter do not differ significantly at $P = 0.05$.

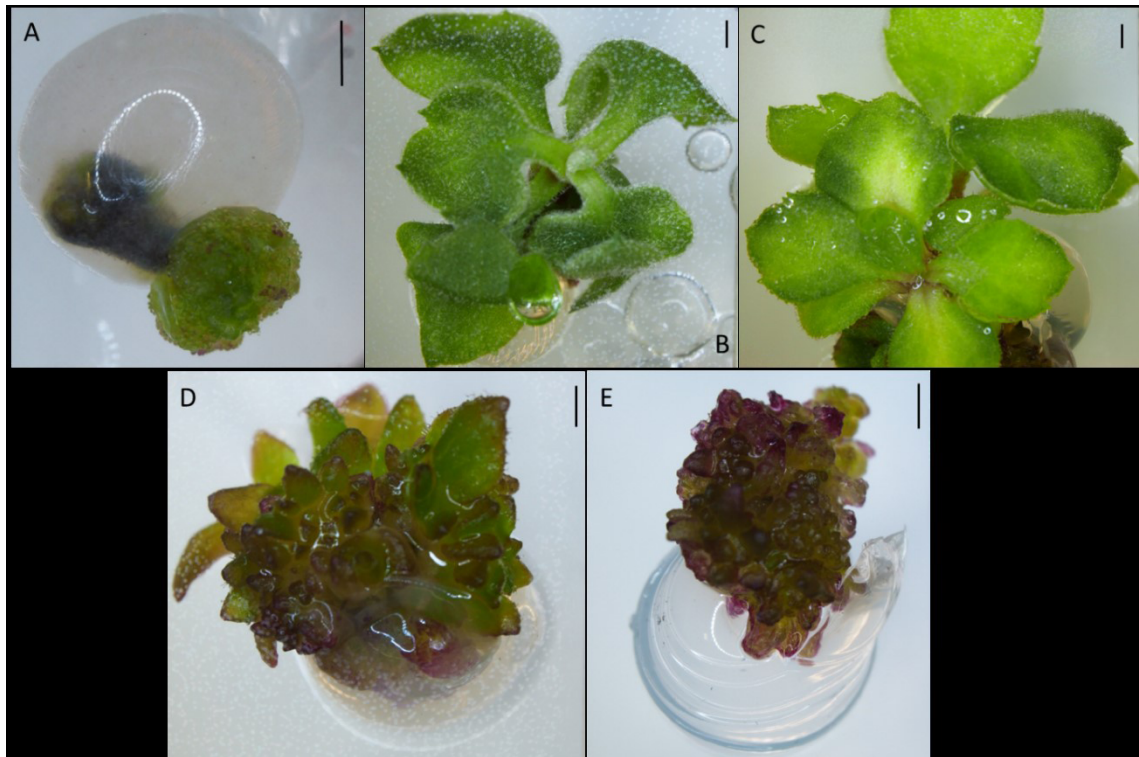


Figure 3. Morphogenetic response types of cryopreserved Lady Orange chrysanthemum shoot tips. A- Development of a single leaf; B- callus 30 days after rewarming; C- typical single shoot 30 days after rewarming; D- multiple shoots 30 days after rewarming; E- hyperhydrated shoots 30 days after rewarming (1 bar = 1 mm).

better elongation of shoots than BA (20.1 and 6.3 mm after 90 days, respectively; Figure 5B). The greatest mean length of shoots (28.8 mm) 90 days after rewarming was observed after applying a 5-day osmotic dehydration followed by 3-h desiccation and in the presence of KIN in the recovery medium (Table 4). The greatest fresh weight of shoots was produced after applying 3-day osmotic dehydration followed by 3- or 4-h desiccation and a BA-supplemented

recovery medium or 5-day dehydration, 3-h desiccation, and KIN after rewarming (Table 4).

As for the fresh weight of roots, the presence of KIN during post-rewarming culture provided better results (134.8–242.5 mg) than BA (0.0–57.8 mg) (Table 4). On the other hand, a higher callus fresh weight was observed after adding BA (271.0–386.2 mg) in comparison to KIN (83.4–142.4 mg). No calli regenerated on the control PGRS-free medium.

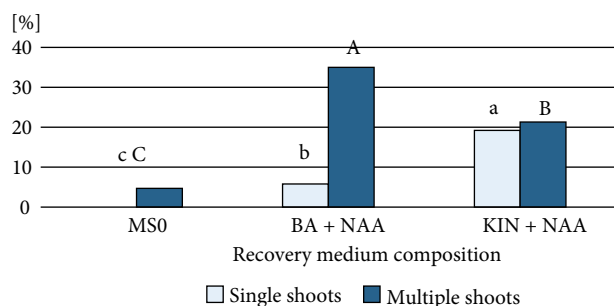


Figure 4. Influence of recovery medium composition on the single and multiple shoot formation rates (%) 90 days after rewarming, regardless of all other experimental factors. Means marked with the same letter do not differ significantly at $P = 0.05$; lowercase letters refer to single shoots and uppercase letters refer to multiple shoots.

3.2. Influence of ABA during preculture

During the entire experiment a lower ($15 \mu\text{M}$) ABA concentration provided higher explant survival and shoot formation rates than the higher one (Table 5), although still they were similar to results of the first experiment (with no ABA during preculture). Similarly to the first experiment (which included optimization of dehydration duration and recovery medium composition), it was observed that the

explant survival efficiency changed with time. One week after rewarming only 49.2% of shoot tips remained green; however, eventually the survival rate reached 67.7% and 71.4% after 30 and 90 days of culture, respectively. Three months after rewarming 90.2% of shoot tips remained viable in the presence of $15 \mu\text{M}$ ABA during preculture (Table 5). A lower ABA concentration also enhanced the shoot formation (50.0% of cryopreserved shoot tips) in comparison to the higher one (2.5%) (Table 5).

Similarly to the first experiment, besides typical single shoots, the formation of multiple or hyperhydrated shoots, or regeneration of brown-green or brown-red compact calli, was noted 90 days after rewarming (Table 6). More single shoots (18.3%) of a greater mean fresh weight of 620 mg recovered when explants were exposed to a lower ABA concentration during preculture (Table 6). In those conditions a greater percentage of explants regenerating multiple shoots (28.9%) was also observed, although no influence of ABA concentration on their fresh weight was noticeable (706–1014 mg). ABA also did not influence the formation of hyperhydrated shoots (0.0%–5.0%). On the other hand, a greater explant capacity to form adventitious roots was observed with $15 \mu\text{M}$ ABA in comparison to $30 \mu\text{M}$ ABA (25.8% and 6.2%, respectively). A higher ABA concentration, on the other hand, led to more frequent callus formation, although the ABA concentration did not

Table 2. Influence of osmotic and physical dehydration duration and post-rewarming recovery medium composition on the morphogenetic response (%) of cryopreserved Lady Orange chrysanthemum shoot tips 90 days after rewarming.

Recovery medium	Dehydration period			
	3-day osmotic dehydration		5-day osmotic dehydration	
	3-h desiccation	4-h desiccation	3-h desiccation	4-h desiccation
Single shoots (%)				
MS0	0.0 b	0.0 b	0.0 b	0.0 b
BA + NAA	0.0 b	13.9 ab	8.3 b	0.0 b
KIN + NAA	16.7 a	35.0 a	5.6 b	19.4 a
Multiple shoots (%)				
MS0	18.8 ab	0.0 b	0.0 b	0.0 b
BA + NAA	24.4 ab	33.3 ab	20.8 ab	56.8 a
KIN + NAA	17.8 ab	30.0 ab	5.6 b	22.3 ab
Hyperhydrated shoots (%)				
MS0	0.0 a	0.0 a	0.0 a	0.0 a
BA + NAA	0.0 a	0.0 a	0.0 a	0.0 a
KIN + NAA	18.3 b	0.0 a	0.0 a	0.0 a

Means in rows and in columns marked with the same letter do not differ significantly at $P = 0.05$.

Table 3. Influence of osmotic dehydration duration and post-rewarming recovery medium composition on the percentage of viable explants regenerating roots and calli 90 days after rewarming, regardless of the desiccation period.

Osmotic dehydration	Recovery medium			Mean
	MS0	BA + NAA	KIN + NAA	
	Roots (%)			
3-day dehydration	0.0 b	8.6 ab	45.4 a	16.6 A
5-day dehydration	0.0 b	12.0 ab	38.6 ab	18.6 A
Mean	0.0 B	10.0 B	41.6 A	
	Calli (%)			
3-day dehydration	0.0 b*	60.7 a	36.7 a	36.5 A
5-day dehydration	0.0 b	57.0 a	70.5 a	44.6 A
Mean	0.0 A	59.2 B	55.4 B	

Means marked with the same letter do not differ significantly at $P = 0.05$. Lowercase letters refer to the interaction analysis and uppercase letters refer to main effects.

affect its fresh weight, which reached approximately 80.2–106.4 mg per explant.

4. Discussion

4.1. Survival rate of cryopreserved explants

The survival rate of LN-stored accessions (100% in the best conditions after 90 days in culture) is very high since Zalewska and Kulus (2013) recorded a maximal 60% of viable chrysanthemum shoot tips 30 days after rewarming. The results suggest that even though the evaluation of cryopreservation efficiency based on explant color was often used in the past (Pawłowska, 2008; Zalewska and Kulus, 2013, 2014), it may not be a precise survival indicator due to its variation with time. It can be concluded that at least 30-day-long observation is required in order to obtain reliable results. Possibly in the present study often the young leaf covering the meristem did not survive freezing and turned brown; however, the tissues below (i.e. meristem and/or leaf primordia) remained viable and were capable of further development, which could account for the changeable visually evaluated survival rate. Explant browning might have also been due to nonlethal phenol secretion (Shibli, 2000; Pawłowska, 2008).

4.2. The role of abscisic acid in cryopreservation

Absciscic acid proved to be essential in the preservation of explants of numerous plant species (Hitmi et al., 2000; Burritt, 2008). Suzuki et al. (2006) observed a significantly lower desiccation and cryopreservation tolerance of *Gentiana scabra* Bunge axillary buds by introducing fluridone (an inhibitor of ABA synthesis) into the preculture medium, while an addition of 10 μM ABA led to a twofold

higher survival rate of *Betula pendula* Roth. shoot tips as compared to the untreated control (Ryynänen, 1998). By applying 10 μM ABA during preculture, Pence (2000) recorded an increase in *Cibotium glaucum* (Sm.) Hook. & Arn. explant survival rate from 10% to 88%. This could have been due to the phytohormone affecting the activity of genes responsible for stress tolerance and the expression of the proteome (Himmelbach et al., 2003), as well as enhancing the uptake and accumulation of sucrose from the medium (Giladi et al., 1977). The necessity of applying ABA in low concentrations during preculture was also observed by Zalewska and Kulus (2014) with Lady Salmon chrysanthemum. In the present study, on the other hand, there was no difference in the recovery potential of the cryopreserved shoot tips after applying 0 μM (56.8%) and 15 μM ABA (50.0%). Likewise, in the research conducted by Zalewska and Kulus (2014), a higher (30 μM) level of this PGR had a deleterious effect on cryopreservation efficiency since the hormone is also responsible for cell senescence. Moreover, higher concentrations of ABA may stimulate the regeneration of undesirable calli (Giladi et al., 1977; Ryynänen, 1998), which may account for its presence in the present research.

4.3. Importance of dehydration in cryopreservation

Water removal is the most basic condition that has to be fulfilled in order to obtain high tissue survival after cryopreservation. That way, the formation of lethal ice crystals is prevented (Kulus and Zalewska, 2014). This could explain a higher shoot formation rate observed after applying longer (4-h) desiccation. On the other hand, excessive dehydration is stressful for tissues. For that

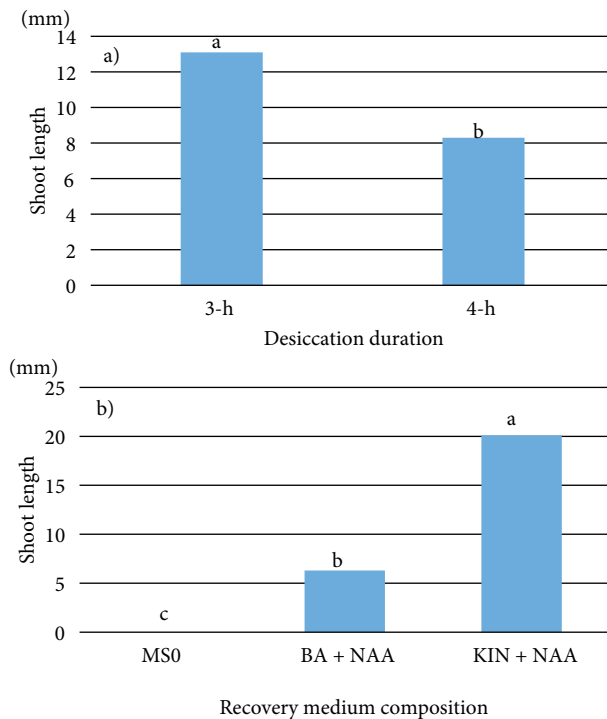


Figure 5. Influence of desiccation duration (A) and recovery medium composition (B) on the shoot length (mm) 90 days after rewarming, regardless of all other experimental factors. Means marked with the same letter do not differ significantly at $P = 0.05$.

reason, the same desiccation period could lead to a slower elongation of shoots after rewarming in comparison to 3-h air drying. A more precise analysis of the effect of pretreatment duration (osmotic and physical dehydration) on the other parameters was not possible, due to the complex interaction of all the factors tested.

4.4. Influence of recovery medium composition on the morphogenetic response of cryopreserved explants

The present research confirms the necessity of applying PGRs to the recovery medium since no recovery of LN-stored shoot tips was observed on the control (MS0) medium after 90 days in culture. On the other hand, the presence of KIN stimulated the development and elongation of vigorous shoots. Similar results were recorded by Zalewska and Kulus (2014). One of the biggest drawbacks of cryopreservation is the fact that many explants, even though they survive freezing, are incapable of further proper development (Osorio-Saenz et al., 2011). In the present study, 35% of explants formed typical single shoots in the best conditions, whereas over 40% of the shoot tips regenerated solely a callus. The fact that not all the viable explants were able to regenerate shoots may indicate that the meristem, or at least a significant part of it, did not survive freezing. This hypothesis is supported

by the fact that sometimes only the development of a single leaf was observed, although more histological observations are required. The survival of leaf primordia (or other nonmeristematic tissues), on the other hand, could account for the activation of axillary/adventitious buds and the development of multiple shoots. Still, the percentage of such structures was not high and reached a maximum of 56.8% in the presence of BA. In comparison, Karim et al. (2003) reported multiple-shoot regeneration from 91% of noncryopreserved chrysanthemum shoot tips on MS medium with 0.09 M sucrose and 4.44 μ M BA. Wang et al. (2014) observed that 48% of cryopreserved chrysanthemum shoot tips regenerated multiple shoots via calli.

As for hyperhydrated shoots, their formation could be explained by excessive tissue damage or mutation occurrence during freezing/rewarming, although this hypothesis requires further molecular analysis. It can be assumed that storage in LN did influence this parameter since no hyperhydration was observed within the nonfrozen control explants. Fortunately, in the present work the percentage of such malformed shoots was minor, especially after applying ABA during preculture (only 0%–5%). In comparison, Wang et al. (2014) recorded 48% of hyperhydrated shoots with cryopreservation-recovered Japanese Red chrysanthemum. Moreover, over 50% of the shoot tips cryopreserved with the droplet-vitrification technique developed only single leaves on MS medium with 1.0 mg dm⁻³ (4.44 μ M) BA and 0.1 mg dm⁻³ (0.54 μ M) NAA (Wang et al., 2014). Much better results were recorded after adding gibberellic acid (GA₃) to the recovery medium (60% initiation of typical shoots), which could also be used with Lady Orange.

It was observed that cryopreservation decreased rooting efficiency (only 25.8% of explants). Usually chrysanthemum forms roots easily (100% on a typical MS medium), but Kulus and Zalewska (2014) concluded that encapsulation itself inhibits rhizogenesis. The addition of an auxin (NAA) to the recovery medium in the present research did not improve that situation. However, similarly to the results from 2014, it was noted that a greater percentage of explants regenerated roots on the KIN-supplemented medium (41.6%) in comparison to the control (0.0%). A different result was observed by Ai et al. (2012) with *Rabdosia rubescens* (Hemsl.) Hara, which showed better rooting efficiency after cryopreservation (encapsulation-dehydration technique) in comparison to the untreated control.

The present results clearly indicate that chrysanthemum shoot tips can be stored in LN; however, still more work is required to stimulate their further growth in vitro. Moreover, Martin et al. (2014) reported that the encapsulation-dehydration protocol can be a source

Table 4. Influence of osmotic and physical dehydration duration and post-rewarming recovery medium composition on the length (mm) and fresh weight (mg) of shoots, roots, and calli recovered from cryopreserved Lady Orange chrysanthemum shoot tips 90 days after rewarming.

Recovery medium	Dehydration period			
	3-day osmotic dehydration		5-day osmotic dehydration	
	3-h desiccation	4-h desiccation	3-h desiccation	4-h desiccation
	Shoot length (mm)			
MS0	0.0 c	0.0 c	0.0 c	0.0 c
BA + NAA	9.6 ac	3.7 bc	2.5 bc	6.0 bc
KIN + NAA	23.5 ab	19.6 ac	28.8 a	11.8 ac
	Shoot fresh weight (mg)			
MS0	0.0 c	0.0 c	0.0 c	0.0 c
BA + NAA	1332.0 a	1024.0 a	107.5 b	663.1 b
KIN + NAA	989.3 b	551.8 b	1018.4 a	797.6 b
	Root fresh weight (mg)			
MS0	0.0 c	0.0 c	0.0 c	0.0 c
BA + NAA	57.8 b	3.0 b	0.0 c	20.5 b
KIN + NAA	242.5 a	148.3 a	172.4 a	134.8 a
	Callus fresh weight (mg)			
MS0	0.0 a	0.0 a	0.0 a	0.0 a
BA + NAA	337.0 c	275.2 c	386.2 c	271.0 c
KIN + NAA	142.4 b	83.4 b	100.5 b	93.8 b

Means in rows and in columns marked with the same letter do not differ significantly at $P = 0.05$.

Table 5. Influence of ABA on the survival rate (%) of cryopreserved Lady Orange chrysanthemum shoot tips 7, 30, and 90 days after rewarming.

ABA (μM)	Survival rate (%)			Shoot initiation (%)
	7 days	30 days	90 days	90 days
15	68.7 a	86.4 a	90.2 a	50.0 a
30	35.0 b	52.5 b	52.5 b	2.5 b
Mean	49.2 B	67.7 A	71.4 A	

Means in columns (lowercase letters) and in rows (uppercase letters) marked with the same letter do not differ significantly at $P = 0.05$.

Table 6. Influence of ABA on the recovery potential (percentage and fresh weight of regenerated structures) of cryopreserved chrysanthemum shoot tips 90 days after rewarming.

ABA (μM)	Single shoots		Multiple shoots		Hyperhydrated shoots		Calli		Roots
	Share (%)	Mass (mg)	Share (%)	Mass (mg)	Share (%)	Mass (mg)	Share (%)	Mass (mg)	Share (%)
15	18.3 a	620 a	28.9 a	706 a	5.0 a	96.9 a	47.8 a	106.4 a	25.8 a
30	0.0 b	0.0 b	6.2 b	1014 a	0.0 a	0.0 b	93.8 b	80.2 a	6.2 b

Means in columns marked with the same letter do not differ significantly at $P = 0.05$.

of some minor genetic variation in chrysanthemum. Therefore, it is recommended to monitor genetic stability after cryopreservation.

In conclusion, cryopreservation conditions have a significant impact on explant survival, morphogenetic response, and shoot rooting. A lower ABA concentration

(0–15 μ M) during preculture and the presence of KIN in the recovery medium enhance the cryopreservation efficiency (a high survival rate and the formation of typical shoots). A higher ABA concentration and the presence of BA in the recovery medium result in abundant callus formation and inhibit root formation.

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