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EFFECT OF VARIOUS PRECULTURE AND OSMOTIC DEHYDRATION CONDITIONS ON CRYOPRESERVATION EFFICIENCY AND MORPHOGENETIC RESPONSE OF CHRYSANTHEMUM SHOOT TIPS

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ABSTRACT

The aim of this study was to evaluate the influence of sucrose concentration in the preculture medium, as well as the duration of osmotic dehydration on the efficiency of chrysanthemum 'Richmond', 'Lady Orange' and 'Lady Salmon' cryopreservation by encapsulation-dehydration technique. For all cultivars tested, the best regrowth of cryopreserved shot tips was recorded with 0.25 M sucrose concentration and 10 µM ABA during a two-week preculture, followed by a 4- or 7-day osmotic dehydration. The survival rate ranged from 56.8–58.0% (the Lady group) to 63.6% ('Richmond'). However, the ability to grow was smaller and reached 18.2–50.7%. It was found that higher sucrose concentration during the preculture slowed the growth of chrysanthemum shoot tips and led to an increased formation of multiple shoots (by activating axillary buds) or deformed adventitious shoots (incapable of further growth). The frequency of tissue hyperhydricity also increased, while the rhizogenesis efficiency decreased when higher sucrose concentration in the preculture medium was applied. The influence of osmotic dehydration duration on the explants morphogenetic response was cultivar-dependent.

Key words: ABA, chimeras, hyperhydricity, rooting, sucrose, survival evaluation

INTRODUCTION

Chrysanthemum is the second biggest floricultural crop in the world, following the rose. In order to meet the growing demands of the market, the novel biotechnological tools are applied in the reproduction and breeding of new cultivars [Teixeira da Silva et al. 2013]. The protection and storage of genetic resources of the species is of great importance. Chrysanthemum, however, is a particularly 'problematic' species since many cultivars are genetically unstable chimeras [Zalewska et al. 2011]. Such plants easily undergo variation caused by improper propagation or storage conditions [Jevremović et al. 2006]. Therefore, cryopreservation is believed to be the sole alternative to their long-term storage.

In order to protect the plant cells from the deleterious effect of cooling and thawing, the explants have to be properly prepared first; hence, cryopreservation protocols including preculture of explants in culture medium containing osmotically active compounds or abscisic acid (ABA) prior to exposure to liquid nitro-

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gen (LN) [Reed and Uchendu 2008]. Preculture can significantly enhance the *post*-thaw regeneration frequency by influencing the lipid metabolism, and stimulating the synthesis and accumulation of endogenous carbohydrates, polypeptides, ABA and proline [Kulus and Zalewska 2014a]. Therefore, it is very rarely observed that a plant material would require any preculture prior to storage in LN.

To evaluate the survival of the cryopreserved plant material, several viability tests were developed [Kulus and Zalewska 2014a]. In general, the chemical tests are not recommended for organized tissues, and instead, evaluation should be based on regrowth of plantlets [Reed and Uchendu 2008]. In the past, the survival of thawed explants was evaluated in a short time once they have been removed from LN [Fukai and Oe 1990]. Now it is well known that the longer time of plant material recovery is needed to properly estimate viability after cryopreservation. The survival of the chrysanthemum shoot tips is most often determined only once between weeks 2 [Lee et al. 2011] and 5 of recovery culture [Jeon et al. 2015a, b]. Research conducted by Kulus and Zalewska [2014b] showed that those parameters change depending on the culture duration, and the final deadline of their evaluation should be experimentally determined for the plant species tested. Sometimes authors provide only information about explants survival, however, they neglect to mention the recovery capacity of the shoot tips [Verleysen et al. 2005, Jevremović et al. 2006] or the other way around [Sakai et al. 2000, Shatnavi and Johnson 2004], while those parameters can vary greatly [Moges et al. 2004].

Shoot development and whole plant recovery after cryo-storage are another problem related to cryopreservation treatments. Sometimes the disturbances of the apical dome structure occur, and the shoots are recovered in a single or multiplied number [Sakai et al. 2000, Jeon et al. 2015a, b]. Moreover, the direct or indirect origin of shoots is not always distinguished, whereas the morphogenetic response may vary even within the same cryo-treatment [Halmagyi et al. 2004]. Also very little information on the rooting efficiency of the recovered plantlets is available. Therefore one should focus more on recovery capacity of the shoot tips after thawing.

Over time, various cryopreservation techniques have been successfully used. Halmagyi et al. [2004] obtained about 45% shoot regeneration of 'Escort' chrysanthemum after applying both the encapsulation-dehydration and controlled-rate freezing techniques. With vitrification techniques almost 60% of shoot tips were able to regenerate. Using the ultra-rapid droplet freezing technique, the lowest ability to regenerate was achieved [Halmagyi et al. 2004]. Halmagyi et al. [2004] and Lee et al. [2011] revealed that the preculture on the medium supplemented with increased sucrose concentration improves the survival and recovery of meristematic explants in at least some chrysanthemum cultivars. As for chrysanthemum 'Escort', the highest regeneration rate was achieved by the exposure of shoot tips to 0.50 or 0.75 M sucrose concentration in the dehydration medium [Halmagyi et al. 2004]. In chrysanthemum, the impact of high concentration of sucrose used during preculture and osmotic dehydration treatments on the morphogenetic potential and response of shoot tips in recovery culture is still not fully recognized. The aim of this study was to evaluate the influence of sucrose concentration in the preculture medium and of the duration of osmotic dehydration on the cryopreservation efficiency and morphogenetic response of chrysanthemum shoot tips.

MATERIALS AND METHODS

Plant material

Chrysanthemum (*Chrysanthemum* × *grandiflorum*/Ramat./Kitam.) 'Richmond', 'Lady Orange' and 'Lady Salmon' were the source of plant material. The cultivars were selected as donor plants due to their varied genetic composition. Purple-flowering 'Richmond' is a stable cultivar in terms of flower colour while 'Lady Orange' and 'Lady Salmon' are periclinal chimeras, valuable in breeding programmes [Zalewska et al. 2007].

Shoots were cultured on the modified MS medium [Murashige and Skoog 1962] supplemented with extra 330 mg·dm^{-3} calcium chloride, 13.9 mg·dm^{-3} iron sulphate and 0.09 M sucrose. The medium was solidified with 0.8 % agar (Biocorp), at pH 5.8 prior auto-

claving in 350 mL glass jars (10 explants per jar). Cultures were maintained in the growth room at 24 ±2°C, under 16/8 h (day/night) photoperiod conditions and photosynthetic photon flux density of approximately 27.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Cryopreservation procedure and survival assessment

Preculture. The single-node explants of chrysanthemums, with removed leaves, were cultured for one week on the modified MS medium (as described above) supplemented with 0.25 or 0.50 M sucrose. Next, the explants were transferred to the media supplemented with 10 µM of ABA.

Encapsulation/dehydration procedure. The biweekly shoot tips 2.0 mm in length, with two leaf primordia and two young leaves had been isolated and embedded for 10 min in 3% sodium alginate based on the modified MS medium salts, without $CaCl_2$, supplemented with 0.25 or 0.50 M sucrose (respectively to the conditions from the preculture treatment). Then beads 3–4 mm in diameter were hardened in 0.1 M $CaCl_2$ solution for 30 min, at room temperature). The beads were rinsed three times with distilled sterile water and dehydrated in 35 mL of MS modified liquid medium, gradually with the following sucrose concentrations: 0.30 M (24 or 48 h); 0.50 M (24 or 48 h); 0.75 (24 or 48 h) and finally 1.0 M (24 h) at 130 rpm (4.5 amplitude) in darkness. Next, the beads were desiccated for 3 hours under laminar air flow to a level of 40% of the initial fresh weight. The explants were placed in 2.0 mL cryovials, 20 beads per vial, and immersed in LN (–196°C).

After one hour, the cryovials were removed from the LN, thawed rapidly in water bath (38 ±1°C for 3 min) and the beads were inoculated on the 30 mL modified MS recovery medium with 0.09 M sucrose and 1.16 µM kinetin (KIN; 10 beads per 90 mm Petri dish). The cultures were kept as described above, in darkness. After 48 hours, the beads were transferred to a 16/8 photoperiod and kept at the light intensity of 12.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 5 days. One week after thawing, the beads were cultured under initial lighting conditions. After 30 days the beads were subcultured to a MS medium without plant growth regulators (PGRs) – MS0 for elongation.

Viability assessment. The cryopreservation effectiveness was evaluated by: 1) the explant survival 7 and 30 days after thawing (based on stereomicroscopic observations; green tissues were considered viable, the total number of cryopreserved shoot tips was considered 100%), 2) the ability of shoot tips for development and formation of shoots 60 days after thawing. Moreover, the percentage of the explants forming single, multiple (direct or indirect) and hyperhydrated shoots was also evaluated. Multiple shoots were the ones which formed in the number of at least two from a single shoot tip. The hyperhydrated ones were the shoots which showed a glassy water-soaked appearance with signs of deformation. Also the percentage of explants regenerating adventitious roots spontaneously was determined. As for that parameter the total number of explants forming shoots 60 days after thawing was considered 100%.

The control explants and shoots (i.e. recovered from encapsulated shoot tips precultured on the MS medium with 0.09 M sucrose and 10 µM ABA with no further cryo-treatment were also considered.

Statistical analysis

A two-factor experiment referred to the effect of 2 concentrations of sucrose in the preculture medium and 2 periods of osmotic dehydration for 3 cultivars. In every experimental combination 40 shoot tips were used in three replications; a total of 1440 explants.

For the data expressed as percentage, the Freeman-Tukey transformation was used. The results (completely randomised design) were statistically analysed with ANOVA, and the comparisons of means were made with Newman-Keuls test ($P \leq 0.05$) using Statistica 10.0 and ANALWAR-5.2-FR tools. The tables with results provide real numerical data, while alphabet letters point to homogeneous groups after the statistical calculations based on transformed data.

RESULTS

Effect of sucrose concentrations in preculture medium on the axillary shoot development

An increase in sucrose concentration from 0.09 to 0.50 M in the preculture medium resulted in a slower development of shoots, which was especially visible

with 'Lady Salmon' (fig. 1). The single-node explants inoculated on the media with 0.09 and 0.25 M sucrose were able to develop shoots already after one week of preculture (fig. 1a, b). As for the medium with 0.50 M sucrose only the anthocyanin discolouration of the explants was observed during that time. Visible shoots were developed during the second week of preculture (fig. 1c).

Effect of preculture and osmotic dehydration treatments on cryopreservation efficiency

As for the control, the survival of the explants reached 100% during the entire experiment. The greatest viability of the cryopreserved shoot tips of 'Richmond', 'Lady Orange' and 'Lady Salmon' was as high as 63.6%, 58.0% and 56.8%, respectively, at day 7 of recovery culture when 0.25 M sucrose concentration was used in preculture medium (tab. 1). The explant survival, however, decreased within 2 months of culture.

Survival of shoot tips and their ability for shoot formation in 'Lady Orange' were similar and reached 50.7 and 61.2%, respectively after 60 days of recovery culture (tab. 1). In 'Richmond', although even though 59.2% of shoot tips survived storage in LN, only 21.6% of them were capable of shoot formation. As for 'Lady Salmon', the shoot formation rate reached 32.2%.

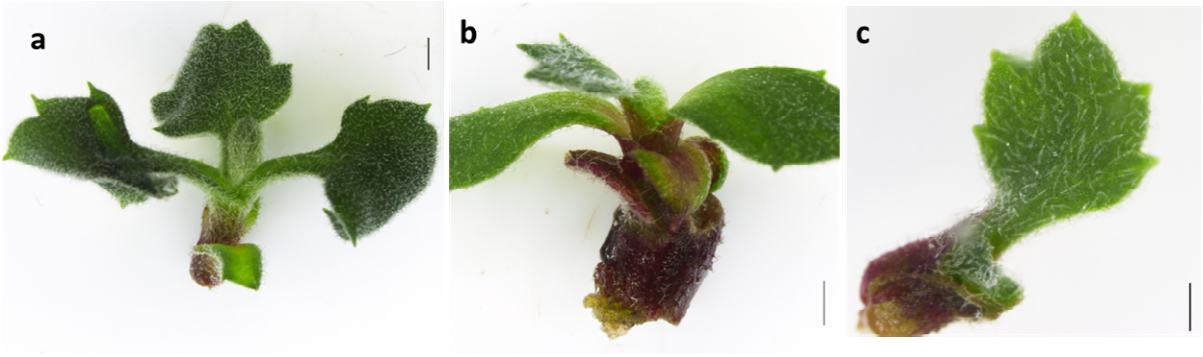

Fig. 1. Influence of various sucrose concentrations on the dynamic of the 'Lady Salmon' axillary shoot development after 2-week-long preculture treatment containing: a – 0.09 M (control), b – 0.25 M, c – 0.50 M sucrose (1 bar = 1 mm)

Table 1. Effect of sucrose and duration of osmotic dehydration treatments on the survival rate (%) of shoot tips determined 7 and 30 days after thawing, as well as shoot formation (%) and their rooting (%) after 60 days of culture

Sucrose concentration (M)	4-day-long osmotic dehydration				7-day-long osmotic dehydration			
	survival rate		shoots	roots	survival rate		shoots	roots
	7 d	30 d			7 d	30 d		
Richmond								
0.25	63.6 a	59.2 a	18.2 a	53.3 a	51.2 ab	23.6 b	10.3 ab	11.1 b
0.50	44.4 ab	42.2 ab	21.6 a	47.2 a	32.6 b	18.0 b	1.8 b	0.0 b
Lady Orange								
0.25	58.0 a	52.9 a	50.7 a	61.2 a	34.2 ab	33.4 ab	30.5 b	65.1 a
0.50	29.5 b	28.1 b	28.8 b	68.2 a	35.4 ab	34.5 ab	24.8 b	14.6 b
Lady Salmon								
0.25	39.2 b	30.0 ab	12.5 b	56.5 a	56.8 a	44.1 a	32.2 a	28.9 ab
0.50	43.9 b	33.9 ab	10.7 b	30.0 ab	37.7 b	29.8 b	21.3 ab	14.8 b

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

Regarding the influence of the sucrose concentration in preculture medium and the duration of osmotic dehydration on explant survival 7 and 30 days after thawing and their development (tab. 1) in 'Richmond' and 'Lady Orange', highest sucrose concentration (0.50 M) and longer (7-day) dehydration decreased the cryopreservation efficiency. In 'Lady Salmon', the survival of shoot tips 30 days after cryopreservation and shoot formation were the highest (44.1% and 32.2%, respectively) for explants pretreated with 0.25 M sucrose in preculture medium and dehydrated as long as 7 days (tab. 1). Similar relationships were confirmed for the rooting of shoots (tab. 1). Rooting was inhibited by the highest sucrose concentration in preculture medium and by longer osmotic dehydration, or even totally suppressed as observed in 'Richmond'.

Effect of preculture and osmotic dehydration treatments on shoot formation and development

The shoot tips subjected to different preculture conditions showed the adverse effect of higher sucrose concentrations on their growth rate after thawing (fig. 2). While the control explants precultured on the medium with 0.09 M sucrose formed shoots with several leaves within 30 days of culture (fig. 2a), the treatment with higher sucrose concentration slowed the elongation of stems, however, it did not decrease the number of leaves (fig. 2b, c). This negative effect was noticeable in all cultivars tested.

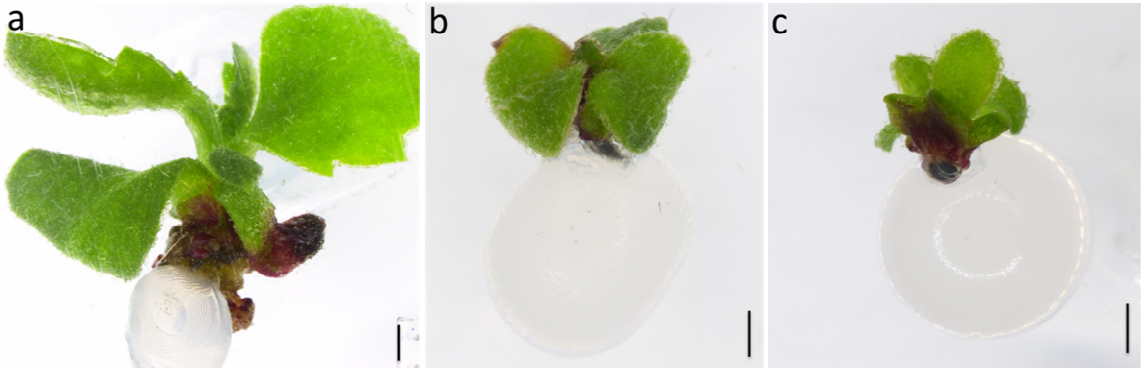

Fig. 2. Effect of various sucrose concentrations in preculture medium on the rate of 'Richmond' shoot tip development; 30 days after culture initiation (1 bar = 1 mm); a – 0.09 M (control), b – 0.25 M, c – 0.50 M

Table 2. Effect of sucrose and duration of osmotic dehydration on the percent of explants forming single and multiple shoots, and on shoot hyperhydration; 60 days after thawing

Sucrose concentration (M)	4-day-long osmotic dehydration				7-day-long osmotic dehydration			
	single shoots	multiple shoots		hyperhydrated shoots	single shoots	multiple shoots		hyperhydrated shoots
		direct	indirect			direct	indirect	
				Richmond				
0.25	53.3 a	43.3 a	3.3 ab	17.8 ab	66.7 a	33.3 b	0.0 b	0.0 a
0.50	29.2 ab	51.8 a	19.0 a	33.7 b	0.0 b	0.0 c	0.0 b	1.8 a
				Lady Orange				
0.25	54.3 a	25.6 b	20.1 a	17.9 a	57.7 a	24.4 b	17.9 a	21.4 a
0.50	59.3 a	30.4 ab	10.3 a	24.6 a	10.4 b	58.3 a	31.3 a	43.8 b
				Lady Salmon				
0.25	56.5 a	43.5 a	0.0 b	0.0 a	33.8 ab	17.9 b	48.3 a	8.0 a
0.50	45.0 ab	55.0 a	0.0 b	0.0 a	18.5 b	47.5 a	34.0 a	40.2 b

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

Nearly half the explants which survived freezing developed typical single shoots, similar with the control. Their formation was inhibited by the presence of the highest sucrose concentration followed by longer osmotic dehydration (in all cultivars tested) (tab. 2). Those explants were a source of multiple shoots, i.e. more than one shoot from a single shoot tip (fig. 3a–c, tab. 2). Such multiple structures occurred in two ways. Some grew directly in a number of two (rarely three) from a single explant and showed no signs of deformation – they had long internodes and typical leaves, usually one of them grew faster than the other(s) (fig. 3a). The second group regenerated indirectly through a callus phase in a bigger number (fig. 3b). Those adventitious shoots were usually deformed. Their internodes were very short and they had fasciated leaves. The shoots were unable of further development.

For all cultivars, the greatest percentage of multiple shoots (70.8–89.6%) was observed after applying 0.50 M sucrose (tab. 2). The influence of osmotic dehydration on the explant morphogenetic response was dependent on the preculture conditions and on the cultivar. As for 'Richmond' shoot tips, shorter dehydration stimulated the proliferation of multiple shoots (both direct and indirect), while longer dehydration, in combination with 0.50 M sucrose, nearly suppressed shoot formation. In 'Lady Orange' there was no influence of the tested factors on the indirect shoot formation observed, while most direct shoots (58.3%) were found after applying 0.50 M sucrose and 7-day-long osmotic dehydration treatment. As for 'Lady Salmon', no influence of sucrose concentration on the shoots proliferation was noticeable after applying 4-day osmotic dehydration period, while longer dehydration favored the formation of indirect shoots. Single shoot development from all control explants was solely observed.

A higher sucrose concentration contributed to excessive hydration of numerous shoots after cryopreservation (fig. 3c). In 'Richmond', the greatest number of glassy water-soaked appearance of shoots was observed after application of preculture with 0.50 M sucrose and after exposure of shoot tips to 4-day-long osmotic dehydration (33.7%). In 'Lady Salmon' and 'Lady Orange', excessive shoot malformation and hydration was associated with their treatment with higher than 0.25 M sucrose and longer than 4 days osmotic dehydration (40.2–43.8% hyperhydrated shoots) (tab. 2). Such structures were unable of further growth. As for the control plantlets no hyperhydration was observed.

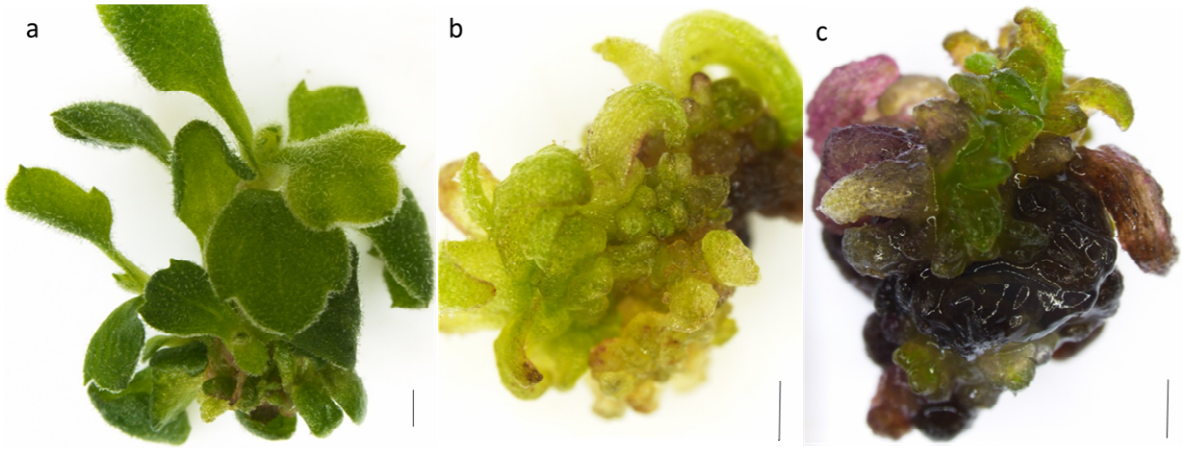

Fig. 3. Negative effect of sucrose on 'Lady Orange' shoot development after LN storage. a – the formation of not-deformed multiple shoots after preculture with 0.25 M sucrose; b – multiple adventitious shoots with short internodes and fascinated leaves after preculture with 0.50 M sucrose; c – hyperhydrated shoots regenerating from callus tissue after preculture with 0.50 M sucrose; 'Lady Salmon'; 60 days after shoot tips thawing (1 bar = 1 mm)

DISCUSSION

Survival and development in assessment of shoot viability after cryopreservation

In the present study screening the survival of shoot tips for 60 days after thawing showed their gradual decay. This was probably caused by the fact, that some of the explants only partially survived cryopreservation. Such phenomenon is caused by the heterogeneous structure of the shoot tips. Observing the cytoplasm density and the proliferation activity, as well as the cell size, three meristematic zones can be distinguished in the shoot apex [Yang et al. 2005]. This diversity may cause an unequal protection of the cells and, consequently, lead to necrosis of some parts of the cryopreserved meristem. The subsequent browning of the explants must have been due to the secretion of polyphenols, which are known to inhibit enzyme activity and kill the cells [Fukai and Oe 1990]. Thirty days after thawing, the survival rate of the shoot tips of some chrysanthemum cultivars studied here has even reached the level of 60%. However, the capacity for further development was lower and accounted from 21.6 to 50.7%. A similar regrowth potential (20–45%) of cryopreserved chrysanthemum shoot tips was recorded by Sakai et al. [2000] and Halmagyi et al. [2004]. A similar phenomenon was observed in chrysanthemum 'Indianapolis White' shoot tips, which reached a high survival rate of 60 and 74% for the encapsulation-dehydration and vitrification techniques, respectively. However, only few of them (10 and 12%) were able to develop shoots [Osorio-Saenz et al. 2011]. Quantitative differences in the survival and growth capacity of explants are related to the so-called cryoselection i.e. the development of only certain groups of cells best protected for the storage in LN [Mikuła et al. 2005]. The results confirmed that the evaluation of chrysanthemum explant viability after cryopreservation based on their green colour may be a non-reliable method and more attention should be paid to the possibility of forming shoots.

Role of preculture in cryopreservation of plant material

Sucrose is an essential compound in plant tissue culture medium [Yaseen et al. 2013]. It is also one of the most popular non-penetrating cryoprotectants applied during cryopreservation [Kulus and Zalewska 2014a]. Usually a higher sucrose concentration in the preculture medium has a positive impact on the ornamental plant cryopreservation efficiency [Kulus and Zalewska 2014a] and shoot development, especially when *in vitro* conditions are inadequate for significant photosynthesis [Giladi et al. 1977]. Hitmi et al. [2000] observed that the *post*-thaw viability rate of *Chrysanthemum cinerariaefolium* cell cultures increased with preculture duration (on the medium with 0.50 M sucrose). However, such conditions can lead to osmotic shock and cell plasmolysis. This could explain the decrease in quantity and quality of shoots produced from explants precultured at higher than 0.25 M sucrose in this work. Similarly, encapsulated-dehydrated *Ceratopetalum gummiferum* Sm. shoot tips demonstrated a 36 and 65% survival rate decrease after 3- and 5-day-long preculture with 0.75 M sucrose, respectively, as compared with the explants cultured at 0.09 M sucrose [Shatnawi and Johnson 2004]. According to Martín and González-Benito [2009] osmotic stress leads to the formation of reactive oxygen species that negatively affect the cellular activity. Another explanation of the positive influence of the lower sucrose concentration on the cryopreserved explant survival rate and a further recovery is that in such conditions a greater amount of endogenic ABA is accumulated in the cells [Giladi et al. 1977].

As for *Rabdosia rubescens* (Hemsl.) Hara the survival of shoot tips after cryopreservation increased (from 11.9 to 85.8%) with increasing time of dehydration [Ai et al. 2012]. However, in *Dioscorea* spp. shoot apices, a higher survival and regeneration rates were reported after applying 24-h dehydration, as compared to longer (40-h) treatment [Agbidinoukoun et al. 2013]. In the present study a short (4-day) osmotic dehydration proved more optimal in terms of explants survival, their regrowth potential and the quality of the microshoots produced than that 7-day-long one, which is in conjunction with a poor response of the explants to higher sucrose concentrations during preculture.

Relationship between sucrose pretreatment and shoot formation in recovery culture

In the present research the formation of single, multiple (direct or indirect) and hyperhydrated shoots

was observed. Our studies showed that a high sucrose concentration in the preculture medium and prolonged osmotic dehydration may result in the malformation of recovered shoots. Halmagyi et al. [2004] also observed the formation of multiple shoots, although their share and origin were not evaluated. The recovery of two-three typical shoots could have been a result of axillary buds activation and the presence of KIN in the recovery medium. The activation of axillary buds can be considered positive since it increases the number of available shoots after thawing. The formation of numerous deformed shoots, on the other hand, is probably a result of meristem extensive damage and regeneration of meristemoids from other viable cells. In comparison Karimi et al. [2002] obtained a multiple-shoots regeneration from 91% of non-cryopreserved chrysanthemum shoot tips on the MS medium with 0.09 M sucrose and 4.44 µM BA, while Wang et al. [2014] obtained 48% of multiple shoots regenerated via callus after cryopreservation of 'Japaneese Red' chrysanthemum via the droplet-vitrification method.

An increase in the quantity of hyperhydrated shoots on media with higher sucrose concentration (even 40.2% in the presence of 0.50 M sucrose) can be due to carbohydrates being mainly engaged in the formation of the so-called biological glass/vitrification state. The number of hyperhydrated shoots produced in the present study is not large since Wang et al. [2014] observed 94% of deformed structures in chrysanthemum 'Japanese Red' after applying the droplet-vitrification technique.

In the past little attention was paid to rhizogenesis of the cryopreserved explants. In our research, the root regeneration was affected by the sucrose concentration during preculture. According to Roycowicz and Malay [2012] sucrose enhances the root formation, which, however, was not confirmed by our observations. The observed negative impact of higher sucrose concentration and prolonged dehydration on the rhizogenesis efficiency is in line with the reports by Nilanthi and Yang [2014], and may result from the high osmotic potential of such media. Removing the explants from the bead shortly after thawing could improve that situation.

CONCLUSION

In conclusion, we emphasize that despite the positive role of high sucrose concentrations in stimulating the cellular resistance that was recorded in several plant species, it was not confirmed with 'Richmond', 'Lady Orange' and 'Lady Salmon' chrysanthemums. The application of the 2-week-long preculture with an increased sucrose concentration, followed by longer osmotic dehydration, lead to developing shoots deformation, which may have a negative impact on maintaining the chimerical structure of the apical dome. Such conditions also decreased the rhizogenesis efficiency. Therefore, maintaining the usable parameters is arguable. The long-term impact of cryopreservation will be the scope of further research.

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