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Cryopreservation of wheat (*Triticum aestivum L.*) egg cells by vitrification

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Abstract A procedure has been developed for the cryo-preservation of wheat female gametes. The procedure involves loading the cells with 25% concentrated vitrification solution consisting of 30% glycerol, 10% sucrose, 120 mM ascorbic acid (AA) and 5% propylene glycol (PG), dehydration in 80% concentrated vitrification solution, droplet vitrification and storage in liquid nitrogen, unloading and rehydration of the cells by gradual addition of isolation solution. Supplementation with AA significantly increased the proportion of viable egg cells after de- and rehydration. During the early phase of rehydration AA reduced the probability of membrane damage caused by rapid water uptake. Maintaining the temperature of the cells at 0°C during the de- and rehydration processes increased cell survival. Microscopic examination of the semi-thin sections of untreated and viable cryopreserved cells revealed that the vitrification process might cause changes in cell structure.

Keywords Cryopreservation · Wheat · Egg cell · Propylene glycol · Dimethyl sulphoxide

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Introduction

Even today, cryopreservation is the method most frequently used for the long-term preservation of the genetic pool of plant species without alteration. In this method liquid nitrogen serves as a coolant, in which protoplasts, cells, shoot tips, embryos, pollen and seeds are maintained at a temperature of −196°C. As cell division and metabolic processes are arrested in cryopreserved cells at this temperature, plant material can theoretically be stored without any changes for unlimited periods (Bajaj 1995). Since biological samples contain a high amount of water, which can cause mechanical damage to the cells due to intra- and extracellular ice crystal formation during freezing and thawing, a reduction in the water content of cells and tissues are needed prior to cryopreservation. Vitrification is the rapid cooling of a highly concentrated solution, in which intracellular ice formation cannot take place on account of the extreme elevation in viscosity during cooling (Fahy et al. 1984). In vitrification protocols, the viscosity is elevated by water removal through an increase in the concentration of osmotically active permeate and non-permeate cryoprotective agents (CPAs) in vitrification solutions. Droplet vitrification method (Sakai 2000, Panis et al. 2005) uses small quantities of cryopreservation media, which provides the high velocity of freezing and thawing. This supports the formation of non-crystalline ice and ensures the avoidance of crystallization during thawing.

With respect to plants the long-term cryopreservation of egg cells could be a valuable research tool, particularly for cell manipulation techniques (e.g. in vitro fertilisation), genetic transformation procedures and the preservation of genetically valuable stocks. Although methods have been elaborated for the cryopreservation or cold storage of

pollen (Barnabás and Kovács 1997), microspores (Chen and Beversdorf 1992) and isolated sperm cells (Roeckel and Dumas 1993) and for the in vitro fertilisation of wheat egg cells (Kovács et al. 1995) and the regeneration of wheat zygotes (Bakos et al. 2003), no attempt at the cryopreservation of female plant gametes has previously been made. This is probably because the female gametophytes are deeply embedded in the sporophytic tissues and are produced seasonally in a limited number. The excision of ovules and the isolation of egg cells in a viable condition require a sophisticated microsurgical procedure (Kovács et al. 1994). The elaboration of an efficient method for the long-term storage of wheat female gametes would allow the all-year round accessibility of adequate amounts of egg cells without seasonal variations in quality. The expensive cultivation of donor plants in the phytotron would thus be avoidable.

The gene encoding ascorbate peroxidase was found to be downregulated after *Arabidopsis* ovules committed to abort (Sun et al. 2005), indicating that ascorbic acid (AA) may have a physiological role during the development of female gametophytes and embryos in plants. Lane et al. (2002) verified the stimulatory effect of AA during cryopreservation on subsequent mammalian embryo development. Moreover, AA supplementation was found to be an effective treatment for luteal phase defect in humans (Henmi et al. 2003). AA is a small, water-soluble antioxidant molecule, which acts as a primary substrate for the detoxification of reactive oxygen species (ROS), including singlet oxygen, superoxide and hydroxyl radicals (Padh 1990; Smirnoff and Wheeler 2000; Conklin and Barth 2004). AA also maintains the membrane-bound antioxidant α-tocopherol in the reduced state (Liebler et al. 1986); Packer et al. 1979), thereby protecting both cellular and organellar membranes against destructive lipid peroxidation, which subsequently leads to membrane leakage and cell lysis (Montillet et al. 2005). Although cell levels of ROS under optimal environmental conditions are controlled by protective antioxidant activity, under stress conditions it has been calculated that there is a 3- to 10-fold increase in ROS levels (Polle 2001). AA is more than just a defence molecule; it serves as a co-factor for many enzymes (Arrigoni and De Tullio 2000), regulates cell elongation (González-Reyes et al. 1998) and cell proliferation (De Gara and Tommasi 1999) and is an important factor in regulating the transition of cells through the cell cycle (Potters et al. 2004).

The investigation reported here examined the effect of various CPAs on the de- and rehydration dynamics and post-vitrification survival of wheat egg cells. The protective effect of AA on cell membranes during the vitrification procedure was also studied.

Materials and methods

Plant material and cultivation

Plants of a winter-hardy wheat genotype (*Triticum aestivum L.* cv. Mironovskaya 808) were used as egg cell donors. The plants were grown until anthesis under controlled conditions in phytotron chambers using a spring climatic programme (Tischner et al. 1997).

Egg cell isolation

Spikes were emasculated and insulated with cellophane bags to avoid self- and cross-pollination 5 days prior to anthesis. Egg cells were excised from the ovaries and isolated at the time of anthesis according to the method described earlier by Kovács et al. (1994). The isolation solution (IS) contained 0.4 M mannitol, 0.2 M glucose and 2 mM $CaCl_2$ at pH 5.8. In each treatment 40 egg cells were transferred into a small droplet of IS, placed in the centre of a sterile cell culture dish ($d = 35$ mm) lined with a wet, rolled filter paper.

Dehydration and rehydration procedure

Basic vitrification solution (BVS, 30% glycerol, 10% sucrose in water, pH = 5.8) and BVS variants VS1 (BVS supplemented with 5% DMSO) and VS2 (BVS supplemented with 5% propylene glycol) were used to identify the most suitable CPA for the dehydration of wheat egg cells. AA at concentrations of 60, 120 and 240 mM was added to BVS, VS1 and VS2 in order to determine the optimum membrane-protective concentration of the compound. BVS, VS1 and VS2 supplemented with 120 mM AA named as BVSA, VS1A and VS2A, respectively. The dehydration process included two steps. The first dehydration step (loading) was performed by the addition of a 2.5 μl vitrification solution to 7.5 μl droplets of IS containing the egg cells, giving a 25% proportion of the hypertonic vitrification solution. After 1 min, an additional 27.5 μl of vitrification solution was added until the proportion of hypertonic solution reached 80%. After 5 min of exposure the cells were transferred to 15 μl droplets of unloading solution containing 80% BVS or BVSA and 20% IS for 15 min. The cells were gradually rehydrated by six additions of isotonic IS at intervals of 5 min. As the temperature of the microscope stage could not be kept at 0°C, volume changes during the de- and rehydration processes were monitored at 25°C. Survival rates were determined 1 h after treatments carried out at both 0 and 25°C.

Droplet vitrification procedure

The basic vitrification solution (BVS) and BVS variants supplemented with various concentrations of either dimethyl sulphoxide (DMSO) or propylene glycol (PG) were used for the procedure together with counterparts containing 120 mM AA. To maximise the velocity of freezing and thawing, the egg cells were vitrified in small, 15 μl droplets. After loading and dehydration, the cell culture dishes were rapidly plunged into liquid nitrogen. The vitrified egg cells were thawed by floating the dishes on a 40°C waterbath for 10–15 s and transferred into 15 μl droplets of 80% unloading solution (BVS or BVSA). After 15 min of unloading the cells were rehydrated by six additions of 5 μl isotonic IS every 5 min. The loading, dehydration and rehydration procedures were carried out at 0°C. During 1 h of incubation the temperature of the cells was gradually warmed to 25°C and the proportion of viable egg cells was determined.

Measurement of relative volume changes

Microscopic images were recorded using a CoolView CCD camera (Photonic Science) connected to an Opton Axiovert 35 microscope. The cross-sectional area of the egg cells was measured using image analysis software (Lucida; Kinetic Imaging Ltd.). The relative cross-sectional area, A, was expressed by dividing the cross-sectional area by the initial area of the same cell in isotonic IS solution. The relative volume changes of the cells were calculated using the equation $V = S^{3/2}$, where S is the relative cross-sectional area and V is the relative volume (Pedro et al. 2005).

Cell viability assay

Survival rates were determined by fluorescein diacetate (FDA) staining (Widholm 1972) 1 h after treatment, at a final FDA concentration of 3.4×10^{-7} M. After 10 min of incubation at 25°C, egg cells showing clear fluorescence were taken as viable.

Examination of structural changes in egg cells caused by the vitrification procedure

Untreated and cryopreserved egg cells were examined in this study. Untreated cells were fixed in 2% glutaraldehyde in 0.5 M Sorensen phosphate buffer (pH = 7.2; 0.6 Osm) for 2 h at 4°C, washed and embedded in 4% low melting point agarose solution. Agarose blocks was cut into cubes (containing 1–2 cells per cube) then dehydrated in an ethanol series, embedded in Unicryl (BioCell) at −20°C, and polymerised under ultraviolet irradiation. For cryopreserved cells, the same solutions used, supplemented by

BVSA to provide the appropriate osmolarity (1.8 Osm) for these cells. Semi-thin (1 μm) sections were made from the blocks using an RMC MT-7000 microtome, stained by 0.5% Toluidine Blue O (TBO) dye, mounted and examined using an Olympus BX51 microscope.

Statistical analysis

All the data were pooled means from the four replicates and statistically evaluated by the Student's t test using SPSS for Windows, version 10.0.

Results

Cell survival after the de- and rehydration procedure

The fact that the survival rates of untreated egg cells only decreased slightly in isotonic IS (2.5%) 2 hours after isolation indicates the high efficiency of the isolation method, with only a small proportion of the cells suffering severe mechanical damage. Compared to BVS, the addition of 120 mM AA resulted in a significant 20% increase ($P < 0.05$) in the survival rates (Fig. 1). The percentage of viable cells in BVS supplemented by 60 or 240 mM AA did not differ significantly from that of the control. Compared to the survival rate of cells in VS2 (22%), the 120 mM concentration of AA significantly ($P < 0.05$) increased the proportion of viable cells by 22.4%. A slight but not significant increase (3.4%) occurred when 60 mM AA was added to VS2 (Fig. 1). Although 240 mM AA increased the proportion of surviving cells by 20.6% ($P < 0.05$) compared to the control, the proportion of viable cells was lower than at an optimum AA concentration of 120 mM (Fig. 1). When dehydration was carried out in vitrification solutions VS1 or VS1A containing 5% DMSO, no FDA-positive cells were observed 1 h after rehydration.
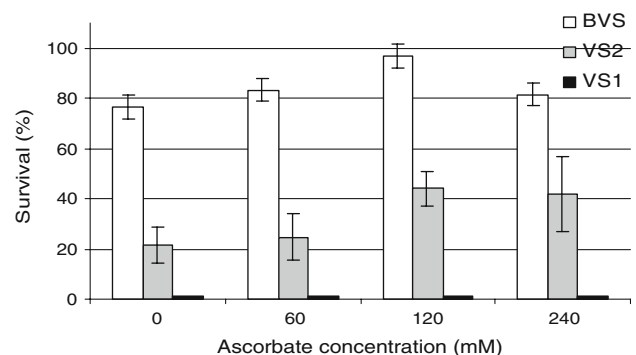

Fig. 1 Effect of various AA concentrations on post-rehydration viability ($N = 40$)

Changes in relative cell volume and morphology
induced by dehydration and rehydration

Prior to dehydration, the isolated egg cells were spherical
(Fig. 2a), and the organelles and cytoplasmic streaming
were clearly visible. Osmotic contraction occurred imme-
diately after exposure to the 25% dehydration solutions
(BVS, VS1 and VS2). The plasma membrane became
flaccid (Fig. 2b), the organelles could not be distinguished
and cytoplasmic streaming stopped immediately. The
addition of 5% PG to BVS significantly reduced the osmotic
contraction of the cells 1 min after exposure to 25% vitri-
fication solution (Fig. 3a). After increasing the proportion
of dehydration solution to 80%, cell volumes decreased by
additional 12 ± 3, 12 ± 2 and $18 \pm 4\%$ for BVS, VS1 and
VS2, respectively. Figure 3a shows the volume changes in
egg cells dehydrated in BVS, VS1 and VS2, where the mean
decrease in relative cell volumes was 58–61% 1 min after
exposure to 80% vitrification solution. After 5 min of
dehydration a slow increase in cell volumes was observed,
the extent of which was only significant ($P < 0.05$) in the
case of VS2 (Fig. 3a). During the unloading step between
dehydration and rehydration, there was a slight non-sig-
nificant increase in relative cell volume, which is not
depicted. The egg cells remained flaccid during the first step
of gradual rehydration, and regained their original spherical
shape only after the fourth dilution step. When BVS and
VS2 were used, a sharp increase in cell volume occurred
5 min after the rehydration process began and the final
volume of the rehydrated cells was 77 and 87%, respec-
tively, of the initial value (Fig. 3b). In the case of VS1, a
continuous linear increase in cell volume was observed until
the cells became spherical and regained 71% of their initial
volume. During the first 20 min, there was a significant
difference ($P < 0.05$) between the extent of osmotic
re-expansion observed in BVS and VS2 or VS1 solution.
After 35 min of rehydration, cytoplasmic streaming became
visible. When a higher rate of IS was added to the cells or
dehydrated cells were transferred directly into IS, the
volume of the cells increased sharply, causing membrane
rupture and the disintegration of the cells. According to
Fig. 3a, c, supplementation with 120 mM AA did not
significantly affect the extent of cell dehydration, though it
slightly lowered the water uptake of the cells between 3 and
5 min. During rehydration, the water uptake of cells dehy-
drated in BVSA and VS2A solutions was significantly
slower ($P < 0.005$) after the first addition of IS, if compared
to those dehydrated in BVS and VS2 (Fig. 3b, d), but no
significant difference was observed in the final cell volume.
The addition of 120 mM AA to VS1 improved rehydration;
the final cell volumes were 15% higher ($P < 0.005$) than in
VS1 (Fig. 3b–d). Irrespective of the CPAs applied during
dehydration, all the cells regained their spherical shape

immediately after the first rehydration step when 120 mM
AA was applied.

Effect of temperature on cell survival after loading
and unloading

There was a significant difference in survival rates of the
egg cells de- and rehydrated in VS2A at 0 or 25°C at a
$P < 0.05$ level of probability. Proportion of viable cells
after de- and rehydration procedures carried out at 0°C
($62 \pm 15.2\%$) was significantly higher than survival rates
after treatments at 25°C ($38 \pm 8.3\%$).

Comparison of various vitrification solutions

No FDA-positive wheat egg cells were observed 1 h after
vitrification without AA supplement. Irrespective of their
AA content, vitrification solutions containing DMSO had a
fatal effect on the cells. Egg cells vitrified in VS2A con-
taining 5% PG showed the highest, $12.7 \pm 2.04\%$ post-
thaw viability. The cytoplasm of thawed viable cells was
bright, the plasma membrane remained intact (Fig. 2c) and
the cells gave an intense fluorescent signal after the FDA
staining (Fig. 2d). Survival rates decreased significantly
($P > 0.005$) when PG was added to BVSA at higher con-
centrations. Mean viability was $7.34 \pm 1.93\%$ and
$3.25 \pm 1.06\%$ when 10 and 15% PG used, respectively.

Examination of structural changes in egg cells caused
by the vitrification procedure

Untreated egg cells showed spherical shape (Fig. 2e), with
a large, central nucleus with nucleolus, and dense cyto-
plasm with a large number of small vacuoles. The
organelles were clearly visible and placed equal density in
the cytoplasm. Cryopreserved cells using VS2A vitrifica-
tion solution (Fig. 2f) remained in spherical shape after the
process, without any visible membrane damage. The
structure of the cytoplasm changed: organelles and vacu-
oles concentrated around the nucleus and the peripheral
region of the cytoplasm became less dense and deficient in
organelles.

Discussion

To the best of our knowledge, this is the first report to date
on the successful cryopreservation of unfertilised wheat
egg cells. In animals, besides the existence or lack of
cumulus cells, oocytes are surrounded by a formidable
multi-layer egg coat (as in the mammalian zona pellucida
or in the abalone vitelline envelope) and by the egg
membrane. By contrast, isolated plant egg cells lack the

Fig. 2 Morphology of wheat egg cells during different stages of the cryopreservation process. **a**, **b** Changes in size and morphology of the egg cell caused by dehydration (VS2) and rehydration. **a** Untreated egg cell, **b** dehydrated, flaccid egg cell. *Bar* represents 20 μm. **c**, **d** Viable wheat egg cell after vitrification and rehydration. **c** Bright-field image of the egg cell; **d** the same cell showing clear FDA fluorescence. *Bar* represents 20 μm. **e**, **f** Structural changes in egg cells caused by the vitrification procedure. **e** Untreated egg cell (*N* nucleus, *n* nucleolus, *V* vacuoles), **f** cryopreserved egg cell (*N* nucleus, *n* nucleolus, *V* vacuoles, *p* pale-stained cytoplasm region, *d* dense cytoplasm region). *Bar* represents 10 μm
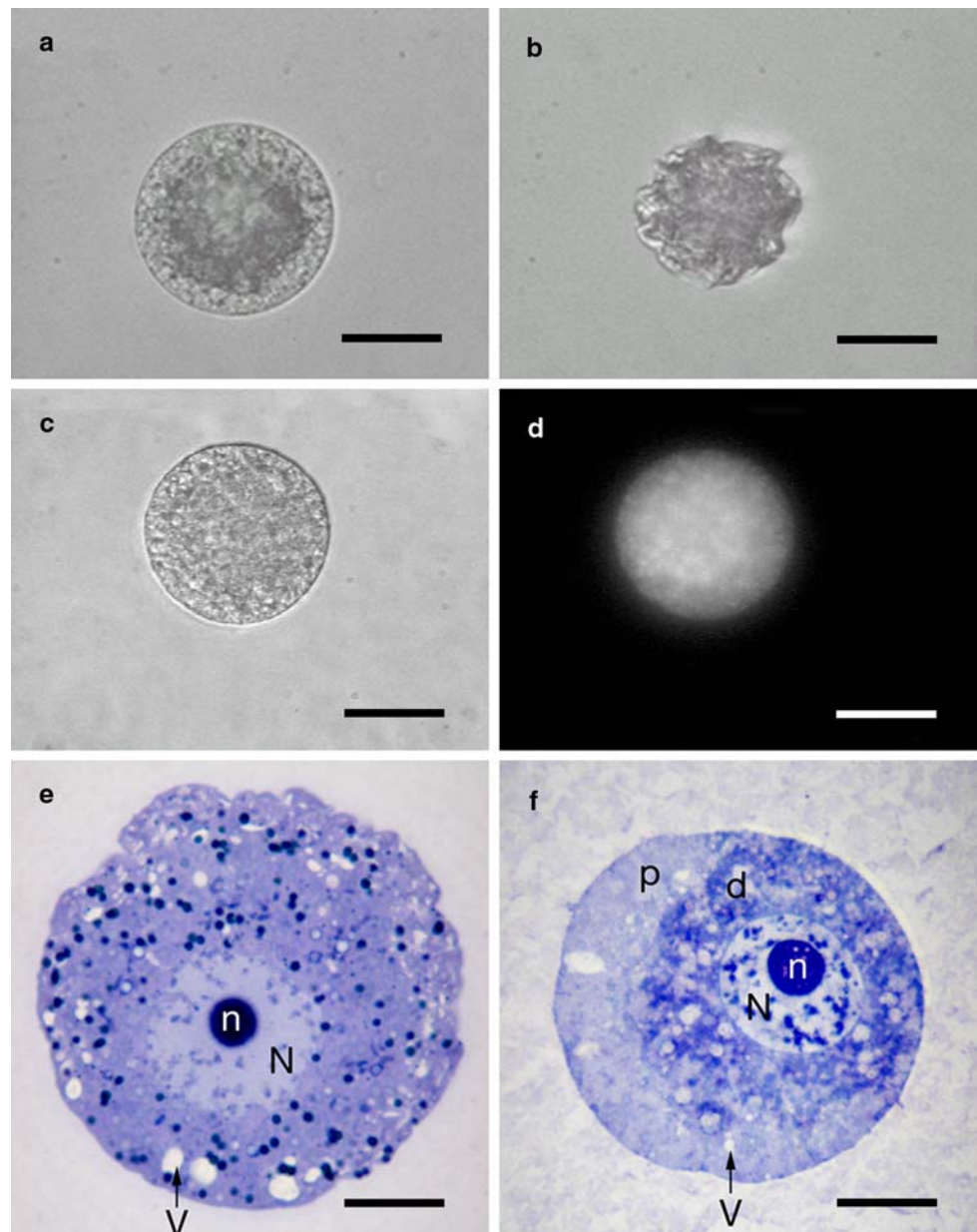

egg coat typical of animal oocytes; they are protected from the environment only by a single barrier, the plasma membrane. Although the permeability of mammalian oocytes to various CPAs has been widely studied, there has been no report on the permeability of plant cells to the CPAs used for vitrification. In agreement with the observations, published by Pedro et al. (2005) on mammalian oocytes and embryos, and Amorim et al. (2006) on primordial follicles, the present results show that during dehydration wheat egg cells are moderately permeable to PG and DMSO and less permeable to glycerol. Differences were found in the permeability of the plasma membrane to various CPAs during hyperosmotic contraction and hypoosmotic re-expansion. The ranking of the rehydration solutions on the basis of cell volume increase was as follows: DMSO, PG, and glycerol.

The supplementation of BVS with 5% of PG or DMSO resulted in 72 and 100% loss of viability, respectively. This indicates the toxic effect of the CPAs applied, confirming the results of Finkle and Ulrich (1979), who described the toxicity of DMSO to sugarcane cells after the addition and removal of the compound with or without freezing. The present results contradict the fact that DMSO is widely and successfully used for the cryopreservation of plant protoplasts and cell suspensions (Chen and Wang 2003; Huang et al. 1995; Langis and Steponkus 1990; Sakai et al. 1990); however, it should be noted, that the sensitivity of cells to various CPAs varies according to species (Blanco et al.

Fig. 3 Alterations in relative cell volume occurred during (**a**) dehydration in different vitrification solutions and (**b**) gradual rehydration by isotonic IS and effect of 120 mM AA added to vitrification solutions on volume changes during dehydration (**c**) and gradual rehydration (**d**). Note that unloading took place between dehydration and rehydration (*n* = 5)
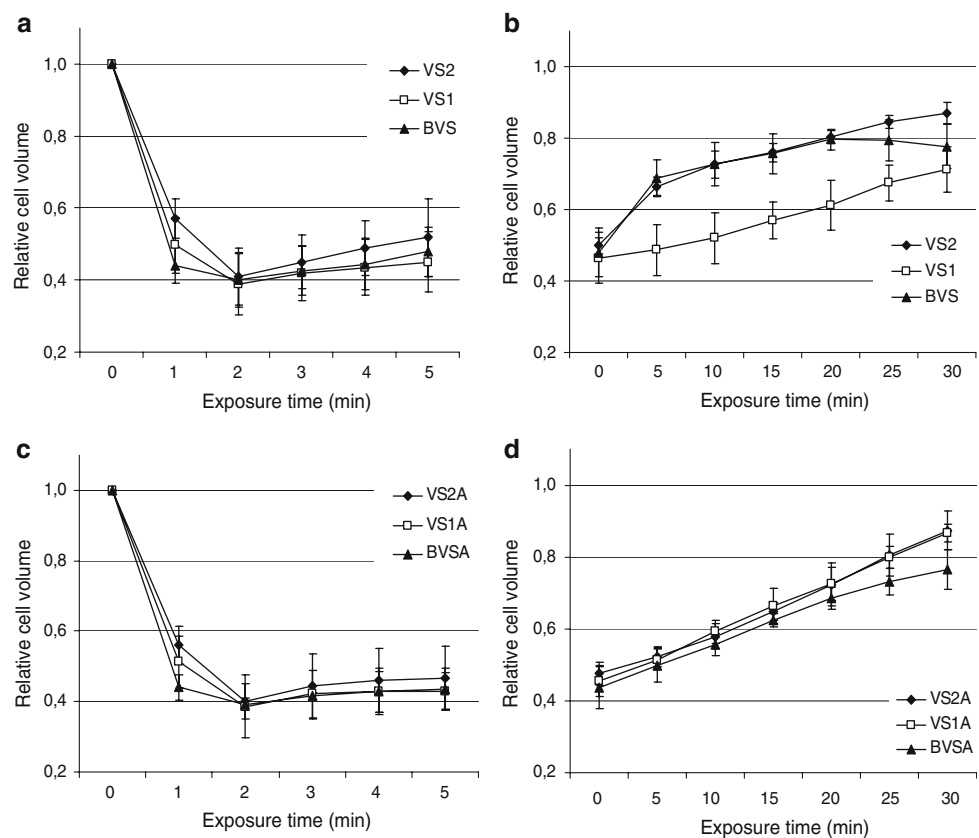
It was previously demonstrated that antioxidants have a positive effect on in vitro cultures of plant protoplasts, cells or embryos (Earnshaw and Johnson 1987; Hoque et al. 2007). The addition of AA to the solutions used for the vitrification of wheat egg cells was expected to have a beneficial effect on cell survival via its membrane-protective effect. The addition of AA to the vitrification solution did not affect the penetration rate of the CPAs (Fig. 3a, c). By contrast, during the early phase of rehydration AA increased the efflux of polyhydroxy CPAs (glycerol, PG), thereby reducing the probability of membrane damage caused by rapid water uptake (Fig. 3b, d). Although AA reduced the extent of DMSO efflux during rehydration and significantly increased the final cell volume (Fig. 3d), it could not compensate for or reduce the toxicity of the CPA, which resulted in 100% lethality. Supplementation of vitrification solutions BVS and VS2 with 120 mM AA significantly increased the proportion of viable egg cells after de- and rehydration. This indicates the general protective nature of AA provided that no compound with a lethal effect is present in the vitrification solution.

The cells generally suffered expansion-induced lysis when they were rehydrated up to the basic osmotic equilibrium level (0.6 Osmol kg^{-1}), confirming the results of Dowgert and Steponkus (1984); Dowgert et al. (1987) and Steponkus (1984). A gradual darkening of the cytoplasm and subsequent cell death was observed when osmolarity of the rehydration solution dropped to below 1.8 Osmol. Bright-field microscopic examinations showed that, except in a single case, no exocytotic extrusion of the plasma membrane occurred after the dehydration process. As the shape of the cells at the end of the progressive volume increase during rehydration (1.8 Osmol kg^{-1}) became spherical without any detectable surface irregularity, and the post-rehydration cell volumes were significantly reduced, it is assumed that the increased osmotic demand of the cells was the result of irreversible endocytotic vesiculation, and that the membrane material deleted during osmotic contraction was not readily incorporated into the plasma membrane during re-expansion. Similar endocytotic vesiculation was observed earlier in cryopreserved maize pollen (Barnabás et al. 1988) and non-cold-pretreated rye protoplasts (Dowgert and Steponkus 1984).

The survival rates of dehydrated wheat egg cells increased significantly when the temperature was reduced to 0°C during de- and rehydration procedures. Consistently with these results, reducing the loading temperature to 0°C was previously reported to reduce osmotic shock and the

toxic effect of CPAs in plants (Nishizawa et al. 1993; Vidal et al. 2005; Wang et al. 2005) and mammals (Agca et al. 1998).

Adding a combination of 5% PG and 120 mM AA to BVS was confirmed to be the most effective formula for the successful vitrification of wheat egg cells, resulting in 12.7% mean cell survival. The fact that post-vitrification cell survival increased from 0 to 12.7% when VS2 was supplemented with the AA indicates, that the antioxidant tempered the toxic effect of PG, probably by providing protection against oxidative membrane damage. The lower survival rate of vitrified egg cells compared to rehydrated ones points out the need to formulate more efficient vitrification solutions. Although DMSO is widely used for the cryopreservation of somatic plant cells, in the present experiments egg cells did not survive in solutions containing this compound irrespective of whether de- and rehydration were accompanied by vitrification. Despite the fact that the highest post-rehydration survival rates were recorded for the cells de- and rehydrated in BVS; the amount of glycerol penetrated was not sufficient to avert intracellular ice formation during ultra-rapid freezing and thawing.

Microscopic examination of the semi-thin sections of untreated and viable cryopreserved cells revealed that the vitrification process might cause changes in cell structure. Structural study of the untreated wheat egg cells confirmed the results of Pónya et al. (1999). Compared to these cells, the most conspicuous difference in cryopreserved cells is the sickle-shaped peripheral pale-stained cytoplasm region. It may be the result of the considerable water loss occurred in the course of dehydration. During this step the cytoplasm become highly concentrated. At the time of rehydration, the extensive water influx could dilute the peripheral cytoplasm region, causing the formation of an external, diluted, organelle-poor cytoplasm area and a highly dense, organelle- and vacuole-rich central cytoplasm region.

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