Cryopreservation of Rye Protoplasts by Vitrification

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ABSTRACT

A procedure has been developed for the vitrification of mesophyll protoplasts isolated from leaves of nonacclimated (NA) and cold-acclimated (ACC) winter rye seedlings (Secale cereale L. cv Puma). The procedure involves (a) equilibration (loading) of the protoplasts with an intermediate concentration (1.5, 1.75, or 2.0 molar) of ethylene glycol (EG) at 20°C; (b) dehydration of the protoplasts in a concentrated vitrification solution made of 7 molar EG + 0.88 molar sorbitol + 8% (w/v) bovine serum albumin (BSA) at 0°C; (c) placing the protoplasts into polypropylene straws and quenching in liquid nitrogen (LN2); and (d) recovery of the protoplasts from LN2 and removal (unloading) of the vitrification solution. For NA protoplasts, 47 ± 1% survival was obtained following recovery from LN2, if the protoplasts were first loaded with 1.75 molar EG prior to the dehydration step. However, to achieve this level of survival, NA protoplasts had to be unloaded in a hypertonic (2.0 osmolar [osm]) sorbitol solution. If they were unloaded in an isotonic solution (0.53 osm), survival was 3 ± 2%. In contrast, survival of ACC protoplasts following recovery from LN2 was 34 ± 10% when the protoplasts were loaded in a 2.0 molar EG solution and unloaded in an isotonic sorbitol solution (1.03 osm). If ACC protoplasts were unloaded in a hypertonic sorbitol solution (1.5 osm), survival was 51 ± 9%. These results indicate that the osmotic excursions incurred during the procedure are a major factor affecting survival.

Conventional cryopreservation protocols generally involve (a) equilibration of the cells or tissues in molar concentrations of CPAs, (b) cooling at an optimum rate to an intermediate subzero temperature (approximately -40°C) to effect freeze-induced cell dehydration, and (c) quenching in liquid nitrogen (LN2) (14). Following cooling to the intermediate temperature, the solute concentration in the unfrozen fraction of the suspending medium and in the cytosol is such that vitrification (formation of an amorphous glass) occurs during quenching in LN2 (8). With such a procedure, the rate of cooling to the intermediate subzero temperature must be sufficiently slow so as to minimize the incidence of intracellular ice formation (7). However, vitrification can also be achieved by direct immersion into LN2 without going through the freeze-concentration step by exposing the cells or tissues to extremely concentrated solutions of permeating and nonpermeating CPAs (9). Such procedures are referred to as vitrification procedures to distinguish them from conventional cryopreservation procedures.

Although use of the vitrification procedure has been limited so far to animal systems (6, 9, 10, 13), there is no a priori reason why it would not be applicable to plant systems. The objective of the present study was to develop a procedure for vitrification of plant protoplasts. Because vitrification procedures result in a high degree of osmotic stress, attention was given to potential injuries resulting from the osmotic excursions incurred during the loading, dehydration, and unloading steps of the procedure. For these studies, the behavior of protoplasts isolated from cold acclimated and nonacclimated rye leaves (referred to as ACC and NA protoplasts, respectively) was contrasted because cold acclimation alters the cryobehavior of the plasma membrane and results in a greater tolerance of the protoplasts to osmotic excursions, dehydration, and freezing stresses (2, 3, 11, 12).

MATERIALS AND METHODS

Plant Material

Seeds of Secale cereale L. cv Puma were sown in vermiculite and germinated in a controlled environment at 20°C (day) and 15°C (night) temperatures (16-h photoperiod). Nonacclimated plants were maintained in this environment for 10 to 15 d. Acclimation was achieved by exposing 1-week-old plants to 13°C (day) and 7°C (night) temperatures (11.5-h photoperiod) for 1 week, and then to a 2°C controlled environment (10-h photoperiod) for 4 weeks.

Protoplast Isolation

Before isolation, Carborundum powder (320 grit; Fisher) was used to abrade the leaves to facilitate infiltration of the isolation medium. The leaves (10 g fresh weight) were then rinsed thoroughly with tap water and isotonic sorbitol (0.53 osm for NA protoplasts and 1.03 osm for ACC protoplasts) to remove the Carborundum and were cut into 0.5 cm sections using a razor blade. The isolation was started by transferring the tissue sections into 50 mL of isotonic sorbitol containing 1 mm CaCl2, 1 mm Mes (pH 5.5), 1% w/v BSA, 0.3% (w/v) potassium dextran sulfate, 0.5% (w/v) Macerase (Calbiochem Corp., LaJolla, CA), and 1.5% (w/v) Cellulysin (Calbiochem). The leaf tissues were incubated at 30°C in the dark for 2 to 4 h. The resulting protoplast suspension was filtered through two layers of Miracloth to remove cell debris and protoplasts were pelleted by centrifugation at 50g for 10 min at 2°C. Protoplasts were washed twice with 20 mL of suspending
medium (isolation medium minus dextran sulfate and enzymes). The LT50 for NA protoplasts is -25 to -30°C; for ACC protoplasts it is -25 to -30°C (2).

Survival Determinations

The number of viable protoplasts in the suspension was estimated after fluorescein diacetate (FDA) staining using a hemocytometer. FDA was used at a final concentration 3.6 \times 10^{-3} \text{ M}. For example, for a 950 \muL suspension, 5 \muL of a stock solution of FDA (7.2 \times 10^{-3} \text{ M} \text{ in acetone}) was added (final acetone concentration = 0.5% v/v). Protoplasts showing bright fluorescence after a minimum of 10 min incubation with FDA at 20°C were counted as viable.

Effect of Manipulation of the Protoplasts

In preliminary studies, the effect of the manipulation of the protoplasts was quantified to determine the loss resulting from multiple centrifugation/resuspension of the protoplasts and transfer into and out of the straws. For this, all manipulations were done using only isotonic solutions (untreated manipulation control). A 50 \muL aliquot of the concentrated protoplast suspension in isotonic suspending medium was transferred into 1 mL of isotonic suspending medium. The suspension was centrifuged at 100g for 5 min, the supernatant was discarded, and the tube containing the pellet was brought into a cold room at 2°C. Following resuspension of the pellet in the residual liquid, 900 \muL of cold isotonic suspending medium was added and 300 \muL of the suspension was drawn into a 0.5 mL polypropylene straw (unsterilized, clear mini-straw; catalog No.A201, IMV Corp., Minneapolis, MN). The contents of the straw were expelled into 2.5 mL of cold, isotonic sorbitol containing 1 mM CaCl2, 1 mM Mes, and 0.2% w/v BSA (unloading medium). An additional 0.5 mL of this solution was used to rinse the straw and the suspension was warmed to 20°C. After 15 min of incubation, the suspension was centrifuged at 100g for 8 to 10 min at 20°C, and the pellet resuspended in 300 \muL of isotonic unloading medium.

Vitrification Procedure

The complete vitrification procedure involves (a) loading of the protoplasts with EG, (b) dehydration of the protoplasts by exposing them to a concentrated vitrification solution, (c) transfer of the specimens into a polypropylene straw (0.5 mL), (d) quenching in LN2, (e) removal from LN2 and warming the straw in water at 0°C, and (f) unloading the CPAs by transferring the protoplasts to sorbitol solutions.

Estimation of the amount of injury occurring at the different steps required the determination of survival after five different treatments. The treatment numbers correspond to the different columns in the histograms presented with the results.

Treatment 1. Untreated Control

A 50 \muL aliquot of the concentrated protoplast suspension in isotonic suspending medium was transferred into 900 \muL of the isotonic suspending medium. The density of the concentrated stock suspension was adjusted to obtain a final concentration of at least 3 \times 10^2 protoplasts/mL in the untreated control.

Treatment 2. Loading

To determine the toxicity of EG during the loading step of the procedure, a 50 \muL aliquot of the concentrated protoplast suspension in isotonic suspending medium was transferred into 900 \muL of isotonic sorbitol containing 1.5, 1.75, or 2.0 mM EG and 1% (w/v) BSA (equilibration solutions). EG (Mal- linckrodt Inc. Paris, KY) was used without further purification. BSA (Sigma Chemical Co., St. Louis, MO), was a certified 98 to 99% albumin content. The suspension was gently mixed and incubated for 20 min at 20°C. To determine the appropriate incubation time, the volumetric behavior of individual protoplasts exposed to different concentrations of EG was analyzed in a microdiffusion chamber (5). When incubated in isotonic suspending medium containing EG, the protoplasts responded in a predictable manner, i.e. there was an initial volumetric contraction (because water efflux is more rapid than solute influx) followed by a gradual return to the initial volume as solute entered the protoplasts. A computerized video image analysis system was used to observe the behavior of individual protoplasts and to estimate the average equilibration time for EG (12-15 min). Because solutions containing BSA tend to foam when agitated, the solutions used in the diffusion chamber experiments did not contain BSA.

Treatment 3. Loading/Unloading

To determine the effect of unloading following loading with EG, a 50 \muL aliquot of the concentrated protoplast suspension in isotonic suspending medium was transferred into 1 mL of the equilibration solution (isotonic sorbitol + 1.5, 1.75, or 2.0 mM EG + 1% [w/v] BSA) and the resulting suspension was incubated at 20°C for 20 min. The suspension was then centrifuged at 100g for 5 min, and the supernatant was discarded using a Pasteur pipette. The pellet was gently resuspended, and 3 mL of the unloading solution was added. The toxicity of the unloading solution was either 0.53, 1.03, 1.5, 2.0, 2.5, 3.0, or 3.5 osm according to the experiment. The suspension was further incubated at 20°C for 15 min. After 15 min, the suspension was then centrifuged at 100g for 8 to 10 min, the supernatant was discarded, and the pellet was finally resuspended in 900 \muL of the same unloading solution.

Treatment 4. Loading/Dehydration/Unloading

To determine the effect of dehydration in the final concentrated vitrification solution, a 50 \muL aliquot of the concentrated protoplast suspension was transferred into 1 mL of the equilibration solution (isotonic sorbitol + 1.5, 1.75, or 2.0 mM EG + 1% [w/v] BSA) and incubated for 20 min at 20°C. The suspension was then centrifuged, the supernatant discarded, and the pellet resuspended in the residual liquid (20-30 \muL). At this point the suspension was brought into the cold room at 2°C. Following thermal equilibration of the sample, 900 \muL of cold vitrification solution (7 mM EG + 0.88 mM sorbitol +...
6% [w/v] BSA = 40% [w/w] EG + 15% [w/w] sorbitol + 5.5% [w/w] BSA) was added to the protoplast suspension. After mixing of the suspension, 300 μL was drawn into a polypropylene straw and immediately expelled into 2.5 mL of the unloading solution. An additional 0.5 mL of the unloading solution was used to rinse the straw. The protoplasts remained in the presence of the vitrification solution for an average time of 60 s. This time was determined to be sufficient for water efflux to be nearly completed as based on studies of osmotic contraction of the protoplasts in the diffusion chamber (data not shown). After addition of the unloading solution, the suspension was thoroughly mixed and brought to 20°C for 15 min before being centrifuged at 100g for 8 to 10 min. The pellet was finally resuspended in 300 μL of the unloading solution.

Treatment 5. Loading/Dehydration/LN2/Warming/Unloading

To determine the effect of the excursion into and out of LN2 (vitrification), protoplasts treated as described in treatment 4 were used. Both ends of the polypropylene straw were heat sealed. The straw was rapidly plunged into LN2. After 10 min in LN2, warming of the samples was done by holding the straw in air at 2°C for 10 s followed by 10 s in ice water. Both ends of the straw were then cut, and the contents of the straw expelled into cold unloading solution. The rest of the procedure was exactly the same as previously described. All data are the means of at least three different experiments.

RESULTS AND DISCUSSION

Effect of BSA on Protoplast Recovery

Loss of protoplasts occurred as a result of the physical manipulation of the protoplasts, i.e. multiple centrifugation/resuspension steps and the transfer into and out of the straws, even in absence of any of the treatments in the vitrification procedure (manipulation control). In the absence of BSA, 67 ± 10% of the ACC and 60 ± 11% of the NA protoplasts initially present in the suspension were recovered after the centrifugation/resuspension and transfer into and out of the straws. This loss may be a result of electrostatic interactions between the protoplast surface and the surfaces of the containers. When BSA (1% w/v) was added to the isotonic suspending medium, a greater percentage of the protoplasts was recovered (92 ± 6% and 87 ± 7% for ACC and NA protoplasts, respectively). The use of higher concentrations of BSA did not increase the percentage of protoplasts that were recovered. Rather, preliminary studies have shown that BSA concentrations as low as 0.2% (w/v) are equally effective (data not shown). However, because the addition of BSA can reduce the amount of EG required in the vitrification solution the concentration of BSA was raised to 6% (w/v) in the final vitrification solution.

Vitrification of ACC and NA Protoplasts

In the initial studies, the protoplasts were loaded with 2.0 M EG, which corresponds to approximately one-fourth times the EG concentration in the final vitrification solution as prescribed by Rall and Fahy (9). Following recovery from LN2, the protoplasts were unloaded in an isotonic solution (1.03 and 0.53 osm for ACC and NA, respectively). Following the complete vitrification procedure (treatment 5), 34 ± 10% of the ACC protoplasts survived after return to isotonic conditions. In contrast, only a small percentage of the NA protoplasts (3 ± 2%) survived the excursion into and out of LN2. Determination of survival after each of the steps in the procedure (Fig. 1) allowed for a systematic analysis of the factors responsible for the low survival following the entire procedure.

Following the loading step (a 20-min exposure to the 2.0 M EG solution at 20°C), 93 ± 6% of the ACC protoplasts and 75 ± 8% of the NA protoplasts survived. If the protoplasts were immediately unloaded in an isotonic solution without going through any further steps in the vitrification procedure, there was a substantial decrease in survival (compare treatment 2 with treatment 3 of Fig. 1). The direct transfer of the protoplasts to an isotonic unloading solution results in a rapid increase in volume because the efflux of EG is slower than the influx of water. The greater survival of ACC protoplasts than NA protoplasts following unloading in an isotonic solution (54 ± 11% compared to 7 ± 4%) is consistent with the fact that ACC protoplasts have a greater tolerance to osmotic excursions than NA protoplasts (11). Dowgert and Steponkus (2) have previously determined that ACC protoplasts have a mean tolerable surface area increment (TSAI) of 1.7. In their experiments, 50% of the protoplasts lysed when the surface area was increased by 70% as a result of a dilution of the suspending medium. In contrast, NA protoplasts have a TSAI of only 1.33. Therefore, although the efflux of EG will rapidly diminish the gradient in osmotic concentration, the probability of lysis following transfer to the unloading solution remains high, especially for NA protoplasts. The decrease in protoplast survival following just a loading/unloading sequence suggests that osmotic excursions incurred during loading and unloading are a major limitation to the success of the entire vitrification procedure.

The decrease in survival following dehydration of the protoplasts in the concentrated vitrification solution at 0°C and unloading in an isotonic solution (treatment 4 in Fig. 1, A and B), was minimal for both ACC and NA protoplasts if one uses treatment 3 as the reference, indicating that the effect of the dehydration stress alone was small. Exposure to the very concentrated vitrification solution for more than 60 s greatly decreased protoplast survival (data not shown). The large variability in survival of ACC protoplasts following the dehydration step (treatment 4) is a consequence of the time effect. To reduce variability in subsequent experiments and to provide a basis for comparison, the duration of the dehydration step was precisely controlled at 60 s. Since most of the NA protoplasts (Fig. 1B) did not survive unloading in isotonic medium following loading (treatment 3), the effect of the dehydration stress was not apparent.

Optimization of the Vitrification Procedure for ACC Protoplasts

The results presented in Figure 1 emphasize the need to reduce the loss in survival that occurs during the osmotic
excursions associated with the loading/dehydration/unloading steps in the procedure. This can be achieved either by increasing the osmolality of the unloading solution and using a step-wise dilution procedure or by reducing the intracellular concentration of the CPA in the equilibration step. In addition, the toxicity of the equilibration solution for NA protoplasts could be diminished by reducing the concentration of EG to less than 2.0 m.

Survival of ACC protoplasts after loading/unloading was increased significantly by increasing the osmolality of the unloading solution from 1.0 to 1.5 osm (cf. treatment 3, Fig. 2A versus treatment 3, Fig. 1A). However, there was no further improvement when a 2.0 osm solution was used (treatment 3, Fig. 2B). As a result, survival following both the dehydration step and the vitrification step was also increased. Following dehydration and unloading in a 1.5 osm solution, survival was 74 ± 4% (treatment 4, Fig. 2A) compared to 54 ± 11% if the protoplasts were unloaded in an isotonic solution (1.03 osm) (treatment 4, Fig. 1A). Survival following vitrification was 51 ± 9% when unloading was done in a 1.5 osm solution (treatment 5, Fig. 2A) compared to 34 ± 10% when unloading was done in an isotonic solution (treatment 5, Fig. 1A). These survival values following unloading in 1.5 osm sorbitol are statistically greater than those following unloading in an isotonic medium at the 95% probability level using a $t$-test for the difference between the means.

Figure 1. Survival of ACC (A) and NA (B) rye protoplasts following different steps in a complete vitrification procedure: treatment 1, untreated control; treatment 2, loading: ACC protoplasts (A) equilibrated with 2.0 m EG + 0.88 m sorbitol + 1% (w/v) BSA; NA protoplasts (B) equilibrated with 2.0 m EG + 0.44 m sorbitol + 1% (w/v) BSA for 20 min at 20°C; treatment 3, loading/unloading: loading with 2.0 m EG followed by direct transfer of the protoplasts to isotonic sorbitol solution (1.03 osm for ACC protoplasts [A], or 0.53 osm for NA protoplasts [B]); treatment 4, loading/dehydration/unloading: loading with 2.0 m EG solution, dehydration by transfer to a vitrification solution of 7 m EG + 0.88 m sorbitol + 6% w/v BSA and unloading in isotonic solution; treatment 5, vitrification: quenching into LN$_2$ and unloading after rapid warming. Means ± SD. For more details see "Materials and Methods."

Figure 2. Effect of increasing the osmotic concentration of the dilution solution on the different steps of a complete vitrification procedure for ACC protoplasts. The protoplasts were unloaded in either a 1.5 (A) or 2.0 (B) osm sorbitol solution. Treatment 1, untreated control; treatment 2, loading: protoplasts equilibrated with 2.0 m EG + 0.88 m sorbitol 1% (w/v) BSA for 20 min at 20°C; treatment 3, loading/unloading: loading with 2.0 m EG followed by unloading of the protoplasts using 1.5 (A) or 2.0 (B) osm sorbitol solution; treatment 4, loading/dehydration/unloading: loading with 2.0 m EG, dehydration by transfer to a vitrification solution of 7 m EG + 0.88 m sorbitol + 6% (w/v) BSA and unloading with 1.5 osm (A) or 2.0 osm (B) sorbitol; treatment 5, vitrification: quenching into LN$_2$, and unloading after rapid warming. Means ± SD. For more details see "Materials and Methods."
In spite of the effect of increasing the osmolality of the unloading medium to 1.5 osm, a decrease in survival was still apparent after the excursion into and out of LN₂ (cf. Fig. 2A, treatment 4 versus treatment 5). The difference between the mean survival values was significant at the 95% probability level. Thus, when the decrease in survival resulting from the osmotic excursions is minimized, the limitation to the success of the vitrification procedure is the injury occurring during the excursion into and out of LN₂, i.e., during cooling and warming. For example, damage can occur if the glass fractures, i.e., if a network of fissures and cleavages develops in the glass because of the limited fracture strength of the vitreous material coupled with inhomogeneous thermal contraction or expansion during cooling or warming. Injury can also occur if the extracellular medium devitrifies (crystallizes during warming) (5). For a given vitrification solution, such changes depend on the stability of the glassy state and on the cooling and warming rates (1, 5). The lower survival of protoplasts carried through the entire vitrification procedure (treatment 5) relative to that of protoplasts that were carried through the dehydration step (treatment 4) indicates that there is the need for the formulation of more stable vitrification solutions at subzero temperatures.

Optimization of the Vitrification Procedure for NA Protoplasts

Optimization of the vitrification procedure for NA protoplasts involved both an increase in the toxicity of the unloading solution and a decrease in the EG concentration in the loading step. Table I shows the effect of increasing the osmolality of the unloading solution following loading of NA protoplasts with 2.0, 1.75, or 1.5 M EG. When the protoplasts were loaded with 2.0 M EG, maximum survival was achieved following unloading in a 2.0 osm solution. Increasing the osmotic concentration of the unloading solution did not result in any further increase in survival. Unloading in solutions of either 2.5, 3.0, or 3.5 osm resulted in a lower survival than if the protoplasts were unloaded in 2.0 osm sorbitol. One possible reason for the lower survival at the higher osmolalities is that a high external tonicity could reduce the water uptake by the protoplasts in the initial stage of unloading, reducing the rate of dilution of the intracellular EG. This suggests that the optimum conditions for unloading could correspond to the situation where water uptake is allowed to take place up to the osmotic expansion limits of the protoplasts so as to rapidly dilute the intracellular EG. Apparently, for NA protoplasts loaded with 2.0 M EG, this corresponds to unloading in a 2.0 osm solution. However, in spite of the reduction in the osmotic expansion of the protoplasts, the survival of protoplasts after unloading remained at 72 ± 4%.

Survival of NA protoplasts following loading/unloading was further increased by reducing the concentration of EG used for loading (Table I). Following loading with 1.75 or 1.5 M EG, survival (before unloading) was 84 ± 7% and 95 ± 7%, respectively. For protoplasts loaded with 1.75 M EG, the highest survival after unloading was obtained by unloading in a 2.0 osm solution, and decreased following unloading in a 3.0 osm solution. In the case of the protoplasts loaded with 1.5 M EG, there was no difference in survival regardless of the toxicity of the unloading medium over the range of 1.5 to 3.5 osm, presumably as a result of the lower internal solute concentration of the protoplasts. This is different from results obtained for protoplasts loaded with 1.75 or 2.0 M EG, in which there was an optimum unloading concentration.

For protoplasts loaded with 1.75 or 2.0 M EG, the existence of an optimum unloading concentration (maximum survival peak at about 2.0 osm, Table I) suggests that an equivalent decrease in survival can result from different forms of injury. For example, when the protoplasts are unloaded in a solution that is less than 2.0 osm, expansion-induced lysis appears to contribute to the reduced survival, whereas, when unloaded in a solution greater than 2.0 osm, the reduced survival is a result of either osmotic-induced dehydration or the attainment of an injurious intracellular concentration of EG. Therefore, vitrification procedures must be optimized with respect to these different possible situations, the relative importance of which may vary according to the particular system used.

The combined effect of a reduction in the concentration of EG used during loading and optimization of the unloading solution is illustrated in Figure 3, A, B, and C, which show the percentage survival of NA protoplasts after the different steps in the complete vitrification procedure. In the results presented in Figure 3A, the protoplasts were loaded with 2.0 M EG and unloaded in a 2.0 osm sorbitol solution. In this experiment, the survival of NA protoplasts following recovery from LN₂ was limited to approximately 30 ± 2%. This is less than that for ACC protoplasts for which we obtained 41 ± 7% survival using exactly the same treatments (Fig. 2C). The difference seems to be the result of the toxicity of the 2 M EG solution (cf. Fig. 2C versus Fig. 3A, treatment 2). When loaded with 1.75 M EG, survival of NA protoplasts after vitrification increased to 49 ± 12% if the protoplasts were unloaded in a 2.0 osm sorbitol solution (Fig. 3B, treatment 5). This value appears to be maximal with respect to the optimization of the unloading solution and the reduction of the intracellular concentration of EG. Higher survival of NA protoplasts was not obtained for protoplasts loaded with 1.5 M EG and unloaded in 1.5 osm solution. Although the decrease in

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Table I. Survival of NA Protoplasts following Loading with EG and Unloading in Sorbitol Solutions of Increasing Concentration (Treatment 3; loading/unloading)

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<tr>
<th>Osmolality of Unloading Solution</th>
<th>EG Concentration in Loading Solution</th>
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* Not determined.
dehydration injury during concentration 4), tional loss (B), loading: protoplasts treatment that suggests was within. Treatment (B), unloading: loading vitrification: transfer to B) in loaded specified in Figure 3. Survival of (B), ± of a a 4 2.0 osm M osm EG/2.0 M osm EG/1.75 M EG (A), 1.75 M EG (B), or 1.5 M EG (C); treatment 3, loading/unloading: loading as specified in treatment 2 followed by unloading with either 2.0 osm (A and B) or 1.5 osm (C) sorbitol solution; treatment 4, loading/dehydration/unloading: loading as specified in treatment 2, dehydration by transfer to a vitrification solution of 7 M EG + 0.88 M sorbitol + 6% (w/v) BSA, and unloading as specified in treatment 3; treatment 5, vitrification: quenching into LN2 and unloading after rapid warming. Means ± SD. For more details see “Materials and Methods.”

Figure 3. Survival of NA rye protoplasts following different steps in the vitrification procedure. The protoplasts were either loaded/unloaded in 2.0 M EG/2.0 osm sorbitol (A), 1.75 M EG/2.0 osm sorbitol (B), or 1.5 M EG/1.5 osm sorbitol (C). Treatment 1, untreated control; treatment 2, loading: protoplasts loaded with 2.0 M EG (A), 1.75 M EG (B), or 1.5 M EG (C); treatment 3, loading/unloading: loading as specified in treatment 2 followed by unloading with either 2.0 osm (A and B) or 1.5 osm (C) sorbitol solution; treatment 4, loading/dehydration/unloading: loading as specified in treatment 2, dehydration by transfer to a vitrification solution of 7 M EG + 0.88 M sorbitol + 6% (w/v) BSA, and unloading as specified in treatment 3; treatment 5, vitrification: quenching into LN2 and unloading after rapid warming. Means ± SD. For more details see “Materials and Methods.”

solutions of osmotic concentration as close as possible to the isotonic conditions. This is especially important for NA protoplasts which undergo deletion of plasma membrane material via endocytotic vesiculation during osmotic contraction (2, 3, 11). A reduction in the surface area of the plasma membrane can result in expansion-induced lysis during subsequent dilution of the suspending medium (2, 3, 11). In the present study, however, recovery of the NA protoplasts in isotonic medium was not attempted and survival was determined in hypertonic solutions.

In summary, these results indicate that osmotic excursions incurred during loading/dehydration/unloading are limiting factors in a vitrification procedure for isolated protoplasts. However, by reducing the osmotic stresses associated with loading and unloading, 50% survival following vitrification was attained. Further improvements now require the formulation and application of more stable vitrification solutions.

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LITERATURE CITED