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Qualitative assessment of rice : novel strategies for improving post-thaw recovery of cryopreserved cells

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Abstract

Supplementation of culture medium with oxygenated perfluorocarbon increased the post-thaw viability, following cryopreservation, of suspension cultured cells of the japonica rice, Oryza sativa cv. Taipei 309. The mean viability, as assessed by triphenyl tetrazolium chloride (TTC) reduction, of cells at 4 d after thawing was increased by 29% over control by oxygenated perfluorodecalin ($P < 0.05$).

The effects were also assessed of supplementing culture media with Pluronic® F-68 [0.01-0.2% (w/v)], on post-thaw growth of suspension cultured cells of the cvs. Taipei 309 and Tarom. The mean absorbance, following TTC reduction, of Taipei 309 cells at 4 d after thawing was increased 2-fold over control by 0.01% (w/v) of Pluronic® F-68 ($P < 0.01$) and almost 2-fold by 0.1% (w/v) of the surfactant. In the case of cv. Tarom, all concentrations of surfactant promoted significant increases in mean cell viability. Both the perfluorodecalin and Pluronic® F-68 treatments also promoted an increase in biomass, measured as fresh weight gain 30 d after thawing, to a maximum of 38% above control ($P < 0.05$).

Supplementation of culture medium with a commercial haemoglobin solution (Erythrogen™) at 1:50-1:500 (v:v), had beneficial effects on the growth, following cryopreservation, of cells of the indica rice cv. Pusa Basmati 1. The mean absorbance, as assessed by TTC reduction, of cells at 8 d post-thawing, was

increased by up to 60% (P < 0.05), compared to cells recovered in the absence of haemoglobin. Erythrogen™ (1:50-1:500 v:v) promoted an increase in biomass, of up to 25% over control (P < 0.05), at 24 d post-thawing. These results demonstrate the marked cytoprotectant effects of oxygenated perfluorodecalin, Pluronic® F-68 and Erythrogen™ for plant cells recovered from cryo-storage.

Keywords

- Oryza sativa, cryopreservation, oxygenated perfluorocarbon, haemoglobin, Pluronic® F-68, Erythrogen™, post-thaw growth
- world

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Introduction

Embryogenic cell suspension cultures are used routinely as source materials for the enzymatic isolation of totipotent plant protoplasts which have been exploited in genetic manipulation studies of rice (Wan and Vasil, 1996). Additionally, such suspensions are an alternative source to immature zygotic embryos for transgenic rice production using biolistics (Sivamani et al., 1996). However, the establishment and maintenance of embryogenic suspensions is technically difficult with, for example, morphogenic competence declining during extended sub-culture at physiologically normal temperatures (Abe and Futsuhara, 1991). Cryopreservation is a routine procedure for the stable, long-term storage of biological tissues at ultra-low temperatures (Grout, 1995). This technique negates the need to re-initiate and to characterize new cell lines and thus provides a constant supply of competent cells (Lynch et al., 1994). Whilst the successful and reproducible recovery of frozen cells depends upon pre-freeze, cryogenic and post-freeze conditions, the transition of cells between ultra-low and physiologically normal temperatures can cause respiratory imbalances leading to the production of toxic oxygen radicals (Cella et al., 1982; Benson et al., 1992, 1995).

One novel approach for enhancing oxygen supply to post-thaw cryopreserved rice cells is the use of chemically-inert, oxygen-carrying perfluorochemical (PFC) liquids. These compounds dissolve substantial volumes of key respiratory gases and have been studied in, for example, emulsified form as vehicles for oxygen transport in vivo (Lowe, 1994). PFC liquids have also been routinely used to facilitate oxygen supply to cultured plant protoplasts and protoplast-derived cells (Lowe et al., 1995, 1997). Additionally, PFCs and their emulsions have been used to prolong the fertilising capability of fish semen (McNiven et al., 1993) and turkey spermatozoa (Thurston et al., 1993) during low temperature storage and promoted hypothermic preservation of mammalian organs (Urushihara et al., 1994).

The non-ionic, polyoxyethylene (POE)-polyoxypropylene (POP) surfactant, Pluronic® F-68 (Poloxamer 188), has been employed extensively as a low cost, non-toxic, cell-protecting agent in both animal (Handa-Corrigan et al., 1989; Papoutsakis, 1991) and plant (Lowe et al., 1993) culture systems. The cytoprotectant properties of Pluronic® F-68 make this compound a strong and obvious candidate for use in plant cell cryopreservation protocols. Early studies showed that the Pluronics could prevent haemolysis of human red blood cells in response to freeze-thawing procedures (Glauser and Talbot, 1956), whilst subsequent studies demonstrated that Pluronic® F-68 was an effective cryoprotectant of cultured Chinese Hamster cells (Ashwood-Smith et al., 1973).

A further novel approach to facilitate oxygen supply to post-thaw cultured cells is supplementation of the recovery medium with a commercial bovine haemoglobin (Hb) preparation (Erythrogen™). Indeed, chemically-modified Hb preparations, including the products of recombinant technology, have been widely evaluated as respiratory gas carriers in animal systems (Zuck and Riess, 1994; Winslow et al., 1995). However, to date,

there have been relatively few corresponding studies with cultured plant cells. In the present investigation, embryogenic suspension cells of rice were used to assess the potential beneficial effects of oxygenated perfluorodecalin, Pluronic® F-68 and haemoglobin (Erythrogen™) on post-thaw growth following both short-term (30 d) and long-term (ca. 3 years) cryopreservation.

Materials and methods

Plant materials and cell suspensions

Cell suspensions of Oryza sativa L. cvs. Taipei 309, Pusa Basmati 1 and Tarom were initiated from embryogenic calli derived from mature seed scutella (Finch et al., 1991). Cell suspensions of cv. Taipei 309 were maintained in AA2 medium (Abdullah et al., 1986) and those of Pusa Basmati and Tarom in R2 medium (Ohira et al., 1973) in 100 ml Erlenmeyer flasks with shaking (120 r.p.m., 2.5 cm throw) at 28 ± 1°C in the dark. Rice cell suspensions were sub-cultured every 7 d by transferring 1 ml of settled cells with 7 ml of spent medium to 22 ml aliquots of fresh medium. Prior to cryopreservation, cells were cultured for 3-4 d in their respective liquid medium supplemented with 60.0 g l^{-1} mannitol.

Cryopreservation and post-thaw recovery

The cryopreservation protocol was based on that of Lynch et al. (1994). Cells were harvested onto a nylon mesh (45 m m pore size) and placed into 2 cm^3 polypropylene vials (Sarstedt, Leicester, UK) with approximately 0.2 g fresh weight of cells per vial. Approximately 0.75 ml of a cryoprotectant mixture [46.0 g l^{-1} glycerol, 39.0 g l^{-1} dimethyl sulphoxide, 342.0 g l^{-1} sucrose, 5.0 g l^{-1} proline] was added to each vial. The cryoprotectant mixture was prepared in the liquid culture medium appropriate for each cultivar and the pH adjusted to 5.8, prior to filter sterilisation (0.2m m pore size; Sartorius, Göttingen, Germany). All cryoprotectants were of Analar grade except dimethyl sulphoxide, which was spectroscopically pure.

Cells were cryoprotected for 1h on iced water. Vials containing the cells were transferred to aluminium canes and the cells frozen at a controlled rate (-1°C min^{-1}) from 0°C to -35°C and held at this temperature for 35 min in a programmable freezer (Planer Cryo 10 Series, Planer Biomed, Sunbury-on-Thames, UK), prior to storage in liquid nitrogen at - 196°C. Cells of cvs. Tarom and Pusa Basmati 1 were stored for 30 d, whereas those of cv. Taipei 309 were cryopreserved for 3 years.

Cells (all cvs.) were thawed by immersing the vials into sterile water at 45°C; excess cryoprotectants were removed under axenic conditions from the cells using a Pasteur pipette. Cells of cv. Taipei 309 from individual vials were placed onto the top of two superimposed 2.5 cm diameter Whatman No. 1 filter paper disks overlaying 5.0 ml aliquots of AA2 medium made semi-solid with 0.4% (w/v) SeaKem LE agarose (FMC Bioproducts, Rockland, ME, USA). In some treatments, the semi-solidified AA2 medium was overlaid onto 20 ml aliquots of oxygenated (10 mbar, 15 min) perfluorodecalin (Flutec® PP6; F2 Chemicals Ltd., Preston, UK) contained in 100 ml capacity screw-capped glass jars (Beatson Clark and Co. Ltd., Rotherham, UK).

In a separate assessment, cells of cvs. Taipei 309 and Tarom were placed onto two superimposed 5.5 cm diameter Whatman No. 1 filter paper discs overlaying 20 ml aliquots of the appropriate culture medium made semi-solid with 0.4% (w/v) SeaKem LE agarose in 9 cm Petri dishes. In some treatments, the medium was supplemented with 0.01%, 0.1% or 0.2% (w/v) of Pluronic® F-68 (Sigma, Poole, UK). These concentrations of Pluronic were selected on the basis of previous studies demonstrating the stimulatory effects of this compound on the growth in culture of protoplasts isolated from cell suspensions of Solanum dulcamara (Kumar et al., 1992).

In a further assessment, cells of cv. Pusa Basmati 1 were placed onto two superimposed 5.5 cm diameter Whatman No. 1 filter paper discs overlaying 20 ml aliquots of R2 medium in 9 cm Petri dishes. R2 medium was semi-solidified with 0.4% (w/v) SeaKem LE agarose. In some treatments, the medium was supplemented with 1:50, 1:100 or 1:500 (v:v) of Erythrogen™ (Biorelease Corporation, USA), a commercial Hb solution. Following the addition of Hb solution to the medium, the resultant pH was 6.1 and, therefore, control medium lacking Hb was re-adjusted to this value.

Cells were cultured for all treatments in the dark for 3 d at 28 ± 1°C, prior to transfer of the upper filter disk containing the cells to the respective fresh medium lacking Pluronic F-68 or overlaying ungassed perfluorodecalin, as appropriate. In the case of cells recovered in the presence Erythrogen™ , the upper filter disks were transferred to fresh R2 medium containing Erythrogen™ as before. Cells were cultured for a further 24-96 h prior to viability assessments and, where appropriate, for an additional 20-26 d, under the same conditions, for biomass determinations. Each of the treatments was replicated using cells of the same cell line taken from 10-20 individual vials.

Measurement of post-thaw viability and biomass

The post-thaw viability and metabolic capacity of cells was assessed by reduction of TTC based on a modification of the method of Steponkus and Lamphear (1967). The wetting agent used in the original assay was replaced with 0.05% (v/v) Tween 80 (Sigma) and the absorbance was read spectrophotometrically at 490 nm. The same protocol was also employed for unfrozen cells at 4 d following sub-culture. The fresh weight of thawed cells was recorded after 24 d (cv. Pusa Basmati 1) and 30 d (cvs. Taipei 309, Tarom) to determine biomass changes (Lynch et al., 1994).

Re-initiation of cell suspensions and subsequent isolation of protoplasts

Five cell suspensions were re-established for each of the treatments after 24 d (cv. Pusa Basmati 1) and 30 d (cv. Taipei 309) of post-thaw culture, and placing into 22 ml aliquots of the appropriate liquid medium in 100 ml Erlenmeyer flasks. Suspensions were sub-cultured thereafter every 7 d for 28 d by removing all the spent medium and replacing with the equivalent volume of fresh R2 or AA2 liquid medium. Subsequently, suspensions were maintained as described earlier. After 8 passages, protoplasts were isolated enzymatically and cultured in the presence of Lolium multiflorum nurse cells, as described by Jain et al. (1995). Protoplast-derived colonies were transferred from the membranes to 20 ml aliquots of MSKN medium and regenerating plants rooted, prior to transfer to the glasshouse, as described by Azhakanandam et al. (1997).

Statistical analyses

Means and standard errors (s.e.m.) were used throughout. Statistical significance between mean values was assessed using conventional analysis of variance and Student's t-test, as appropriate (Snedecor and Cochran, 1989); a probability of $P < 0.05$ was considered significant.

Results

The mean absorbance, as an indicator of cell viability, of cryopreserved Taipei 309 cells following recovery in the presence of oxygenated perfluorodecalin (0.45 ± 0.07; n = 20) was significantly ($P < 0.05$) greater than for the mean of the control treatment which lacked perfluorodecalin (0.35 ± 0.08; n = 20). The recovery of cells with oxygenated perfluorodecalin promoted sustained mitotic division, since biomass, measured as mean fresh weight at 30 d post-thaw, was significantly ($P < 0.05$) greater (0.9 ± 0.03 g; n = 20), compared to untreated controls (0.65 ± 0.03; n = 20).

Supplementation of culture medium for rice cv. Taipei 309 with Pluronic® F-68 at 0.01% (w/v) increased significantly the mean post-thaw cell absorbance following TTC reduction to more than 2-fold ($P < 0.001$) that of untreated controls (Table 1). A similar, but less pronounced effect also occurred with 0.1% (w/v) of surfactant (Table 1). In contrast, there was no corresponding increase in absorbance in these rice cells when 0.2% (w/v) Pluronic® F-68 was incorporated into the culture medium.

In the case of the rice cv. Tarom, supplementation of medium with Pluronic® F-68 had a consistently greater stimulatory effect on the mean post-thaw cell absorbance compared to that of untreated controls (Table 1). The most pronounced increase occurred with 0.1% (w/v) of the surfactant, which promoted a 4-fold increase ($P < 0.001$) in absorbance over the control. Supplementation of medium for Tarom cells with 0.01% (w/v) or 0.2% (w/v) Pluronic® F-68 increased the mean cell absorbances by 2-fold ($P < 0.001$) and 3-fold ($P < 0.001$),

respectively (Table 1).

Table 1. : Mean (± s.e.m., n = 20) absorbance (490 nm), following TTC reduction, by rice cells post-thawed in the presence of Pluronic® F-68. **P < 0.01, ***P < 0.001 compared to mean control (0% Pluronic).

Pluronic® F-68 (% w/v)	*O. sativa* cv. Taipei 309	*O. sativa* cv. Tarom
0 (Control)	0.40 ± 0.06	0.19 ± 0.02
0.01	0.98 ± 0.10 **	0.36 ± 0.06 ***
0.1	0.75 ± 0.13	0.76 ± 0.05 ***
0.2	0.59 ± 0.09	0.48 ± 0.05 ***

Addition of Pluronic® F-68 to culture medium also fostered an increase in biomass, as measured by cell fresh weight following 30 d of post-thaw culture. Rice cells of cv. Taipei 309 in medium supplemented with 0.01% (w/v) of Pluronic® F-68, exhibited a mean fresh weight which was 32% greater than the corresponding mean control value (Table 2).

Table 2. : Mean (± s.e.m., n = 20) fresh weight (g) of cryopreserved cells of Oryza sativa cv. Taipei 309 after 30 d with 0.01-0.2% (w/v) Pluronic® F-68 in the culture medium. In all treatments, ca. 0.2 g of cells was used as starting material (day 0). *P < 0.05 compared to control (0% Pluronic).

Pluronic® F-68 (% w/v)	Mean fresh weight (g)
0% Control	2.07 ± 0.28
0.01	2.74 ± 0.13*
0.1	2.46 ± 0.11
0.2	1.98 ± 0.16

Supplementation of culture medium with Erythrogen™ (1:100 v:v) significantly (P < 0.05) increased the mean post-thaw cell absorbance, as measured 8 d after thawing (1.30 ± 0.10; n = 10 throughout), compared with the absorbance of frozen cells recovered in the absence of Erythrogen™ (0.81 ± 0.06; Table 3). A similar, but less pronounced, increase in absorbance also occurred with 1:50 (v:v) Erythrogen™ (1.22 ± 0.08; Table 3). In contrast, there was a small, but not significant, increase in mean absorbance (1.06 ± 0.11) in response to the addition of 1:500 (v:v) Erythrogen™ to the culture medium. Interestingly, there were no significant differences in cells exposed to all Hb treatments, compared to control at day 4, in terms of mean absorbance (Table 3).

Table 3. : Mean (± s.e.m., n = 10) absorbance (490 nm), following TTC reduction, of rice cells recovered from cryopreservation in the presence of Hb (Erythrogen™). *P < 0.05 compared to control (0% Erythrogen™) mean value.

Concentration of *Erythrogen*™ (% v:v)	Absorbance (490 nm) 4 d	Absorbance (490 nm) 8 d
0 (control)	1.03 ± 0.09	0.81 ± 0.06
1:50	1.16 ± 0.07	1.22 ± 0.08*
1:100	1.14 ± 0.06	1.30 ± 0.10*
1:500	1.14 ± 0.07	1.06 ± 0.11

Supplementation of culture medium with Hb also increased biomass, as measured by cell fresh weight following 24 d of post-thaw culture, with significant (P < 0.05) increases at all concentrations used. The maximum increase in biomass occurred with 1:100 (v:v) of Erythrogen™ (1.45 ± 0.01; n = 10) compared to control (1.16 ± 0.02; n = 10), while less pronounced increases in biomass were also recorded with 1:50 (v:v) (1.42 ± 0.04; n = 10) and 1:500 (v:v) (1.30 ± 0.03; n = 10) Hb.

Cell suspensions (n = 5), reinitiated simultaneously from cells recovered from all treatments, ultimately exhibited growth rates after 35 d of culture comparable to those of unfrozen suspensions maintained by regular sub-culture every 7 d. There were no significant differences, in terms of protoplast yields, viabilities, plating efficiencies and plant regeneration frequencies (data not shown), between each experimental treatment and unfrozen controls. Plants regenerated from cryopreserved cells were morphologically normal,

with expected diploid chromosome complements (2n = 2x = 24).

Discussion

The results of these experiments demonstrate that supplementation of the culture medium with oxygenated perfluorodecalin, Pluronic® F-68 and Erythrogen™ increases the post-thaw viability and growth of cryopreserved rice cells. Whilst it is generally accepted that reduction of TTC can be used as an indicator of cell viability and metabolism (Benson et al., 1995), the present study also includes data on biomass increases to support the conclusion of the growth-enhancing effects of these media supplements.

Earlier studies demonstrated that culture of rice protoplasts and protoplast-derived cells at the interface between oxygen-gassed PFC overlaid with liquid or agarose-solidified culture medium also enhances mitotic division and, in totipotent systems, stimulates shoot regeneration (Anthony et al., 1994; Wardrop et al., 1996). This confirms a genuine growth enhancement of this treatment, rather than a marginal effect on cells recovered from cryopreservation. PFC is thought to act as a reservoir for oxygen, which diffuses into the aqueous medium/cell phase during initial culture. This is supported by changes in oxygen tension in the medium (Anthony et al., 1994). It is probable, therefore, that the increased and sustainable post-thaw growth of rice cells was also due to an enhanced oxygen supply provided by PFC. Indirect evidence for the diffusion of oxygen from the PFC to aqueous culture medium, comes from related observations with suspension-derived protoplasts of Salpiglossis sinuata, in which an increase in intracellular superoxide dismutase (SOD) occurred after 3 d of culture (Lowe et al., 1997). This, in turn, was consistent with studies using Mycobacterium spp., where SOD was elevated during culture of the bacterium in perfluorodecalin-supplemented medium (Popkova et al., 1988). Lipid peroxidation and protein degradation can occur in the early stages of post-thaw recovery (Fuller et al., 1988; Benson et al., 1995). Increased SOD biosynthesis associated with culture of protoplasts and protoplast-derived cells with oxygenated PFC may protect cells not only against a supplemented oxygen supply, but also against oxygen radicals generated by impaired oxygen flux during thawing. Future studies will evaluate the early time-course of changes in SOD and other oxygen-sensitive enzymes during cell recovery.

Studies using animal cells have shown that Pluronic® F-68 adsorbs onto cytoplasmic membranes, conferring increased resistance to mechanical damage (Handa-Corrigan et al., 1992; Lowe et al., 1993). The Pluronic polyols have hydrophobic POP cores, which are believed to become embedded in the phospholipid membranes of cells, leaving their hydrophilic, POE tails outside. Adsorption of Pluronic molecules onto post-thawed plant cells may also reduce cellular damage, which can occur during rehydration when the dimethylsulphoxide cryoprotectant is removed progressively from the system (Benson and Withers, 1987). The surfactant may thus help to preserve, in the short term, a stable cell:medium density equilibrium crucial to the re-establishment of maximal mitotic activity.

An important finding from this investigation was that the optimum concentration of surfactant, which increased cell growth, differed between the two rice cultivars Taipei 309 and Tarom. The present observations, showing that there are cultivar-specific responses to Pluronic® F-68, are consistent with previous observations using Chrysanthemum morifolium, in which the optimum concentration of surfactant which stimulated adventive shoot regeneration from cultured leaf explants differed by an order of magnitude between cultivars (Khehra et al., 1995). Pluronic® F-68 may also promote the increased uptake of nutrients, growth regulators or oxygen into cells during the post-thaw period. Indeed, related experiments using animal cells cultured under static conditions have shown that concentrations of Pluronic® F-68, comparable to those used in the present investigation, stimulated both 2-deoxyglucose uptake and cellular amino acid incorporation (Cawrse et al., 1991). Changes in nutrient uptake, promoted by Pluronic, would be expected to alter metabolic flux, allowing biochemical pathways to operate more efficiently, especially under the stress of initial post-thaw recovery.

Further studies are required to determine the mechanism(s) by which surfactants, such as Pluronic® F-68, and Hb solutions, such as Erythrogen™, can facilitate post-thaw survival and growth of plant cells. Previous studies with animal hybridoma cells have demonstrated that Erythrogen™ not only enhanced cell division but, additionally, stimulated antibody production (Goffe et al. 1994). Erythrogen™ is believed to act by "trapping" oxygen from air-medium interfaces, thus facilitating delivery of the gas to cells. Future investigations with plant cells should assess the effects of these media supplements on respiratory gas dynamics, since related work has provided evidence that Pluronic® F-68 can alter oxygen transport in agitated, sparged bioreactors (Murhammer et al., 1992). Earlier assessments have also demonstrated that mitochondria isolated from freeze-thawed rice cells exhibited the same degree of coupling mitochondrial electron transport with ATP

synthesis as unfrozen cells (Cella et al., 1982). Erythrogen™ may enhance mitochondrial oxygen consumption leading to increases in cellular ATP and related metabolites.

The present results indicate that oxygenated PFC, Pluronic® F-68 and Erythrogen™ could be incorporated routinely into post-thaw culture media and handling strategies, in order to maximise cell recovery and to promote growth during the post-thaw handling procedures. Additional studies, with cells of other species, will determine the applicability of these simple procedures to cells of monocotyledons and dicotyledons, including those of other major crops. A further advantage of using PFCs in such systems is that they are easily recoverable and recyclable, thereby providing a cost effective underpin to germplasm storage technologies (Lowe et al., 1997).

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