
Plant regeneration from rice (Oryza sativa L.)
embryogenic suspension cells cryopreserved by vitrification

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Summary. Rice cells were precultured for 10 d in medium containing 60 g/L sucrose and subsequently for 1 d in medium supplemented with 0.4 M sorbitol. After loading with 25%PVS2 at 22°C for 10 min and dehydration in 100%PVS2 at 0°C for 7.5 min, they were plunged into liquid nitrogen directly. Survival was 45.0 ± 5.1% (based on the reduction of triphenyl tetrazolium chloride) following warming and unloading. For re-growth, cells were plated on semi-solid medium replenished with 40% (w/v) starch for 2 d prior to reculture. Cell suspensions were re-established and plants were regenerated from recovered cells. Twenty eight plants set seeds in the greenhouse.

Key words: Cryopreservation — Vitrification — Cell suspension — Oryza sativa

Abbreviations: PVS: plant vitrification solution; P: preculture; LN: liquid nitrogen; TTC: triphenyl tetrazolium chloride; 2,4-D: 2,4-dichlorophenoxyacetic acid; DMSO: dimethyl sulfoxide; EG: ethylene glycol; BSA: bovine serum albumin.

Introduction

Vitrification refers to the physical process by which a concentrated aqueous solution solidifies into an amorphous glass (ice-free) state. It happens in two ways. Protocols of conventional cryopreservation generally involve a freeze-induced cell dehydration during cooling at an optimum rate in first step and vitrification of the dehydrated cells together with the unfrozen fraction of the suspending medium during rapid quenching in second step (Rall 1981). However, only the procedures that expose the cells to extremely concentrated cryoprotectant and vitrifying the solution as well as the cells are termed cryopreservation by vitrification (Rall and Fahy 1985). Such an approach would eliminate the need for controlled slow freezing. It might avoid injury due to ice formation and solution effects occurring under conventional processes. Some plant materials have been successfully cryopreserved by vitrification, for instance, shoot-tips or meristems (Towill 1990; Towill and Jarrr 1992; Niino et al. 1991, 1992; Yamada et al. 1991; Matsumoto et al. 1994), cell suspension cultures (Langis et al. 1989; Sakai et al. 1990, 1991; Nishizawa et al. 1993), protoplasts (Langis and Steponkus 1990, 1991), callus (Yamada et al. 1993) and somatic embryos (Uragami et al. 1989).

Embryogenic calli, regenerable cell suspension cultures and totipotent protoplasts are good sources for genetic manipulation of most cereal species (Vasil 1994). The establishment and maintenance of such cultures is difficult and time-consuming (Gnanapragasam and Vasil 1992). Cryopreservation is an attractive technique for storage of cultured cells of cereal crops.

There are some reports on cryopreservation of rice cell suspensions (Sala et al. 1979; Cella et al. 1982; Meijer et al. 1991; Yan et al. 1994) and calli (Finkle and Ulrich 1982; Kuriyama et al. 1989; Watanbe et al. 1992). In this paper we describe the procedures for cryopreservation of rice embryogenic cell suspensions by vitrification and regeneration of plants from recovered cells.

Materials and Methods

Cell suspension cultures

Mature embryos of rice variety 02428(Oryza sativa L. ssp. japonica) were cultured on LS medium (Linsmaier and Skoog 1965) with 2.5 mg/L 2,4-D, 1.0 mg/L thiamine HCl, 3% (w/v)sucrose and 0.7% (w/v) agar, pH5.8 (LS2.5) for callus initiation. Cultures were maintained in the dark at 26 ± 1°C. The embryogenic calli were placed into AA liquid medium (Müller and Grafe 1978) supplemented with 2 mg/L 2,4-D for
establishment of cell suspensions. The liquid cultures were maintained on a rotary shaker at 80–100 rpm in the dark at 26±1°C and were subcultured weekly until fast growing cell suspensions were established.

Preculture treatment

Exponentially growing cells were harvested and subcultured in preculture medium. The treatments were given as follows. P1 (preculture 1): cell suspensions were incubated in AA2 medium containing 60 g/L sucrose for 7 d and subcultured in a fresh medium for another 3 d. P2: incubated in AA2 medium supplemented with 0.4 M sorbitol for 1 d. P3: incubated in AA2 medium supplemented with 0.4 M sorbitol for 2 d. P4: incubated in AA2 medium supplemented with 0.8 M sorbitol for 1 d. P1+P2; immediately after P1 preculture, cells were subsequently subjected to P2 preculture. Other combinations of preculture (P1+P3, P1+P4, P2+P4, and P3+P4) were also tested.

Vitrification procedures

The complete vitrification procedure involves: (1) Loading 3 ml of stock cell suspension into a 10 ml centrifuge tube and allowing it to settle. 6 ml of 25% PVS2 (Sakai et al. 1990) replaced the liquid medium in tube and equillibrated the cells for different length of time (10 and 20 min) at 22°C. (II) Dehydration. Discarding the loading solution, 3 ml of cold vitrification solution 100% PVS2 consisting of 30% (w/v) glycerol, 15% (w/v) EG and 15% (w/v) DMSO in AA2 medium supplemented with 0.4 M sucrose was added and mixed with the cells. Before quenching into LN, 0.75 ml (at a final packed-cell-volume of 40%) of the suspension was transferred into a polypropylene straw. Usually 3–4 straws were obtained from one tube. The remainder was discarded due to low levels of viability. (III) Cooling. The straw was plunged into LN directly. The total time the cells were exposed to PVS2 before quenching in LN was precisely controlled (0.5, 1.5, 2.5, 5.0, 7.5, and 10.0 min). Samples were maintained in LN for at least 30 min. (IV) Warming. Rapid thawing was done by holding the straw in a water bath at 37°C. (V) Unloading. 1 ml of washing solution (containing 1.2 M sorbitol in AA2 medium) was introduced into the warmed straw. The supernatant was discarded within 2 min and another 1 ml of fresh washing solution was added. The suspension was then incubated at 25°C for 25 min and cells were drained at the end.

TTC assay

Survival of the cells was determined by TTC reduction using the method developed by Towill and Mazur (1975) which was slightly modified by Langis et al. (1989) in vitrification of Brassica campestris cell suspensions. Viability was expressed as % TTC reduction of the untreated control per unit dry weight of cells.

Regrowth culture

Cells subjected to vitrification procedures were plated on semi-solid (0.8% agar) AA2 medium supplemented with 1.2 M sorbitol, or 1.2 M sucrose or 40% soluble starch in petri dish for the first 2 d and then they were transferred to semi-solid (0.8% agar) AA2 medium for 12–14 d as the second step of reculture. Growth of the cells was judged by daily visual inspection.

Plant regeneration and cell suspension reestablishment

The revived cells were transferred to N6 medium (Chu et al. 1975) containing 0.5 mg/L 1-naphthalyeneacetic acid, 2 mg/L 6-benzylaminopurine and 2 mg/L kinetin to induce differentiation. Little shoots or small plantlets were transferred to N6 basic medium without plant growth regulators, from which they were transferred to pots and then to trough in the greenhouse. For reestablishment of cell suspensions, 1.0–1.5 g of recovered cells was incubated in liquid AA2 medium and subcultured under the condition mentioned above.

Results and Discussion

Dehydration

Successful vitrification requires the use of a highly concentrated yet effectively non-toxic solution of cryoprotectants. The plant vitrification solution PVS2 has been used in many studies (e.g., Sakai et al. 1990, 1991; Niino et al. 1991, 1992; Yamada et al. 1991; Ko-humura et al. 1992; Towill and Jarrrt 1992). Other plant vitrification solutions are also developed, for example, 40 wt% EG + 15 wt% sorbitol + 6 wt% BSA (Langis et al. 1989; Langis and Stepokkus 1990), 24 wt% EG + 30 wt% sorbitol + 6 wt% BSA (Langis and Stepokkus 1991), 35% (w/w) EG + 1 M DMSO + 10% polyethylene glycol-8000 (Towill 1990), PVS (Uragami et al. 1989) and PVS3 (Nishizawa et al. 1993). In this study, we found that PVS2 was suitable for vitrification of rice embryogenic suspension cells.