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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 5(6): 679-682, 2002

Initiation and Cryopreservation of Cell Suspension of Rice Basmati Varieties

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Abstract: Efforts were made to optimize tissue culture conditions for quick establishment of cell suspension and simple procedure for the cryopreservation of embryogenic cells suspension cultures of three rice varieties. Suspension cultures were initiated from friable, globular embryogenic calli in MS/R_2 media supplemented with 2mg/l 2,4-D. These suspension cultures were regenerated and cryopreserved. Both MS and R_2 media supplemented with 2mg/l 2,4-D were quiet suitable for developing cell suspension. All the three varieties gave compact light yellow calli on MMS medium. The regeneration frequency was 55% on average for 10-14 weeks old cell suspension. Vigorous regeneration was observed on MS containing sorbitol. Post thaw cell viability varied from variety to variety. No significant difference in viability was found in varieties cryopreserved for different periods. In conclusion cryopreservation can be used to preserve cell suspension line for a reasonable time. It can easily be used on culture medium or liquid R_2 medium.

Key words: Rice, *Oryza sativa* L., cell suspension, callus, cryopreservation, TTC

Introduction

Cell suspension is considered of prime importance for preparing protoplast. Direct DNA uptake into protoplast has been widely applied method for inserting genes into cereals (Potrykus, 1990). Techniques have been established for callus induction and protoplast preparation for various cereals like pearl millet (Vasil and Vasil, 1981), guinea grass (Lu and Vasil, 1981), sugar cane (Ho and Vasil, 1983). The genotype or variety of the original plant is critical for the success of plant regeneration from callus and cell suspension (Tamura, 1981; Yamada and Loh, 1984). Establishment and maintenance of embryogenic cell suspension of Indica rice is difficult. Once established, cell suspension lines lose their regeneration ability. They lose their morphogenetic capability with prolong tissue culture and acquire undesirable somaclonal variation (Abdullah *et al.*, 1986) and initiation of new cell suspension is laborious and time consuming.

Cryopreservation of embryogenic cell suspension culture provides a source of constant supply of competent cells (Shillito *et al.*, 1989). Several research groups have reported the cryopreservation of rice cell suspension mainly of Japonica varieties and their subsequent regeneration (Lynch *et al.*, 1994, Meijer *et al.*, 1991). These research groups have mostly employed a slow rate programmable thermo freezer for freezing cells to -25 or -40°C before cryopreserving cells at -70°C or in liquid nitrogen. Efforts were made in the present study to optimize tissue culture conditions for quick establishment of cell suspension and a simple procedure for the cryopreservation of embryogenic cell suspension cultures of Indica rice varieties Basmati 370, Basmati 385 & Super Basmati.

In this study the strategy of cryopreserving asparagus and navel orange was followed for rice cell suspension, avoiding the use of slow rate thermo freezer, osmotically dehydrating cells before cooling in an ordinary freezer at -20°C to -30°C (Saki *et al.*, 1991; Nistrizawa *et al.*, 1993).

Materials and Methods

Establishment of homogenous cell suspension culture: Embryogenic calli were initiated from scutella of mature seeds of three varieties Basmati 370, Super Basmati and Basmati 385 on Murashige and Skoog (MS) nutrient medium (Murashige and Skoog, 1962) supplemented with 9µM (2mg/l) 2,4-D, 30gm/l sucrose, 500mg/l proline and 300mg/l casein enzymatic hydrolysate (CHL) under dark conditions. Four to six weeks old embryogenic calli (2.0g fresh weight) were transferred to 25ml liquid MS medium and R_2 medium (Ohira *et al.*, 1973) supplemented with 2 mg/l 2,4-D. Initially, 100ml Erlenmeyer flasks were used and cultures were agitated at 120 rpm on a gyratory shaker. The cultures were maintained in the dark at 26± 2°C. The suspensions were routinely subcultured after every 5-days by adding 2.0g of packed cells collected by passing the cell suspension through nylon sieves of 450µm to a flask containing 25ml of the respective liquid medium.

Regeneration studies: For regeneration cell suspensions were incubated on MS medium supplemented with 2mg/l 2,4-D and 0.5mg/l kinetin for ten days in the dark and for the last three days in the light. The embryogenic calli thus obtained were transferred to RM regeneration medium (Jan *et al.*, 2001). The regenerated plants were shifted to magenta containing simple MS medium.

Cryopreservation of cell suspension: Exponentially growing embryogenic suspensions of the three varieties were used for cryopreservation purpose. The cryoprotectant was prepared by adding 4.6g glycerol, 3.9g DMSO, 34.23g sucrose, 0.5g proline in 100ml of AA_2 medium (Lynch *et al.*, 1994). The cryoprotectant was filter sterilized. Cell suspensions were subcultured into A_2 medium for high osmotic treatment as described by Lynch *et al.* (1994). Cells were passed through a 45µm nylon sieve into 1.5ml cryogenic tubes, each tube containing 0.3ml packed cell volume. Each tube was filled with 1ml cryoprotectant solution. The packed cell volume (PCV) was determined by pipetting both cells and medium in 10 or 25ml wide bored disposable pipet and then slowly releasing the medium, holding the pipet against the wall of the flask. Cells settled in the lower part of the pipet were measured as packed cell volume. Tubes were fixed on a stand and kept on ice for 45 minutes. The experiment was conducted in four batches, each batch containing 27 tubes, (9 tubes of each variety). These tubes were fixed in a Styrofoam stand, sealed with tap and kept at -20°C for one hour, then the Styrofoam rack was immediately kept at -70 °C in cold refrigerator or in liquid nitrogen can. Tubes were kept at this low temperature for a period of one week to three months.

Thawing and cell viability testing: Stored in liquid nitrogen cane for a period of one week, two weeks, one month and three months, cells in cryogenic tubes were thawed in a water bath at 40°C for about 45 minutes. Excess cryoprotectant was removed. Thawed cells were either plated on a double layer of Whatman No.1 filter paper overlying the surface of MS medium in a petri plate or transferred to 100ml flasks, containing liquid R_2 medium, incubated on a gyratory shaker (120rpm). All cultures were kept in the dark at 26°C. After thawing, cell clumps were used for their viability after 12 hours and 12 days of incubation on medium. The post thaw viability of cells was tested by measuring cellular respiration using 2,3,5-triphenyltetrazolium (TTC) assay (Jain *et al.*, 1996). TTC assay was performed, using cell samples of 100mg and 1.0% (w/v) TTC in a 2:1 v/v solutions of 0.02M

sodium phosphate buffer (pH 7.5) and R_2 medium. The red fermozan formed after 16 hours of incubation was repeatedly extracted from the cells in 20ml of 95% ethanol for 90min at 60°C. Absorbance of the extract was recorded at 530nm.

Results

Establishment of homogenous cell suspension culture: Embryogenic callus was obtained from scutellum of mature embryos. All the three varieties gave compact light yellow calli on modified MS medium. Granular calli, when transferred to MS/ R_2 liquid medium, gave rise to homogenous cell suspension in about 10 to 14 weeks. Cell suspensions became rich in elongated and highly vacuolated cells (Fig. 2). These cells were able to divide and grow to a cluster of 100-250 cells and even fine 0.1mm homogenous callus suspension when left unsubcultured for 15-30 days. These homogenous small granular calli regenerated more efficiently than fine cell suspension, as fine cells were observed to first form small colonies or calli and then they regenerated on regeneration medium (Fig. 1). The effect of time was observed on embryogenesis and plantlets formation from rice cell suspension (Fig. 3). It was observed that the frequency of regeneration was 55% on average for 10-14 weeks old cell suspension. This frequency decreased significantly for old cultures such as 18-20 weeks old cell suspensions.

Regeneration of cell cultures: The homogenous cell suspension and cell clumps transferred to MS regeneration medium containing sorbitol did not regenerate and browning of suspension and clumps was observed. While homogenous cell suspensions transferred to MS regeneration medium without sorbitol, produced embryogenic regenerating calli and when these embryogenic regenerating calli were transferred onto MS regeneration medium containing sorbitol, vigorous regeneration was observed.

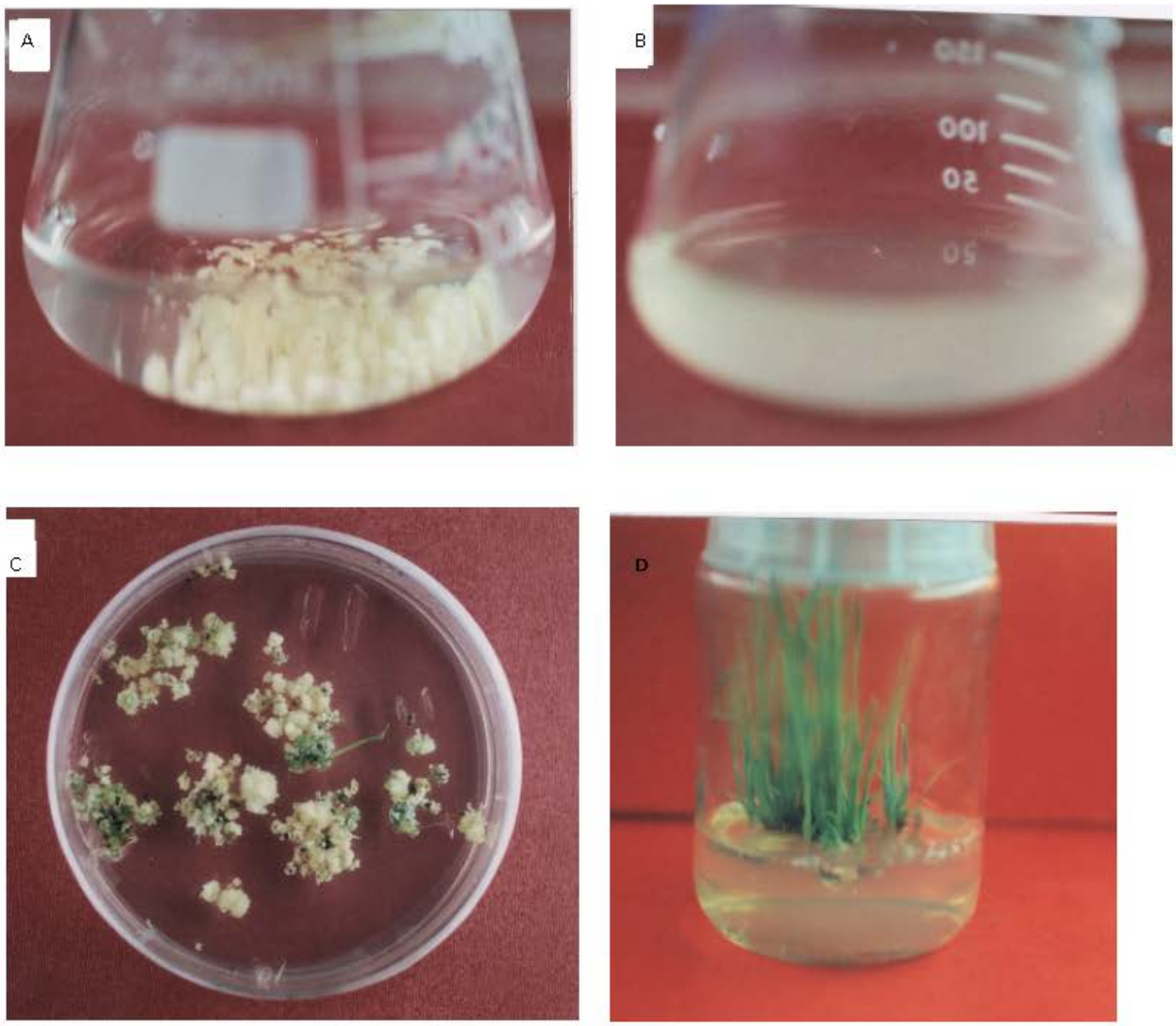

Fig. 1: Different stages of regeneration of rice cell suspension. A. Initial stage of establishment of cell suspension B. Fine cell suspension. C. Regenerated cell suspension D. Plants regenerated from cell suspension

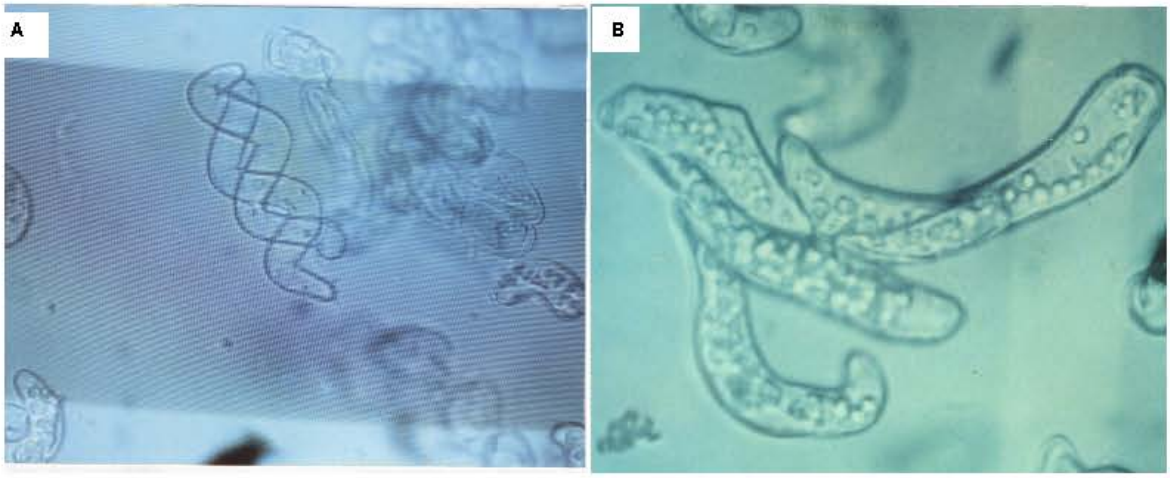

Fig. 2: Cells of rice cell suspension. A. Twelve weeks old cells B. Eighteen weeks old cells

Table 1: Values of Optical Density (OD) recorded for cell suspensions of different varieties of indica Basmati rice cryopreserved for different period of time.

Variety	One week	Two weeks	One month	Three months
Super Kernal(S.K)	0.232± 0.01	0.208± 0.01	0.203± 0.02	0.185± 0.02
Basmati 385(B-385)	0.103± 0.01	0.100± 0.01	0.90± 0.01	0.083± 0.01
Basmati 370(B-370)	0.328± 0.01	0.411± 0.01	0.308± 0.015	0.246± 0.02
Control (S.K)	1.243± 0.01	1.240± 0.01	1.240± 0.022	1,100± 0.013
Control (B-385)	1.107± 0.02	1.100± 0.014	1.200± 0.014	1.080± 0.021
Control (B-370)	1.300± 0.013	1.400± 0.02	1.300± 0.02	1.200± 0.015

Table 2: Post thaw recovery of viable cells & their growth from cryopreserved cell suspensions of different rice varieties cryopreserved for different period of time.

Variety	%Cell viability (Post thaw period; 12hr)	%Cell viability (Post thaw period; 12days)	%Callus formation (proliferation after 20days of culture on paper disc)
Super Basmati	24.9± 0.12	60± 0.2	54± 0.09
Basmati 385	9.36± 0.13	29± 0.012	16.7± 0.15
Basmati 370	24.4± 0.09	54± 0.15	47± 0.22

Values are the means ± SE, of three replicates per experiment.

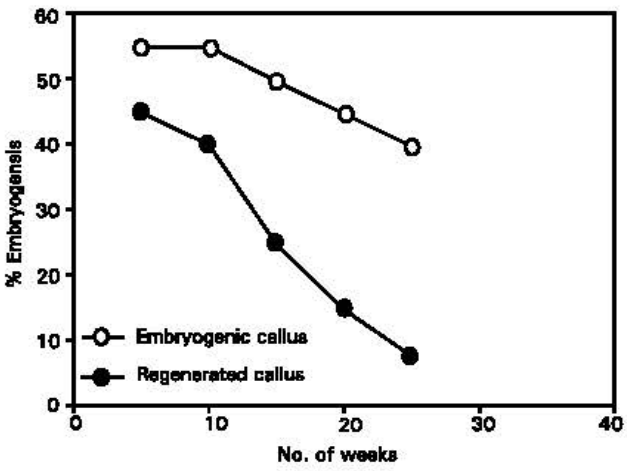

Fig. 3: Effect of prolong culturing on percent callus differentiation and regeneration

Cryopreservation of embryogenic cell suspension: Three varieties of indica Basmati rice were used for cryopreservation. It was observed that post thaw cell viability varied from variety to variety. The initial cell viability in case of Basmati 385, after 12 hr, was minimum compared to Super Basmati (25%) when one week old cryopreserved suspension was tested for viability as clear from the low values of optical density recorded at 530nm (Table 1). Cells after thawing gave bleached appearance, which is a probable symptom of cryoinjury, deplasmolysis and oxidation stress (Lynch *et al.*, 1994). Thawed cell suspension plated onto filter paper discs overlying phytagel solidified MS medium supplemented with 2mg/l 2,4-D and 1g/l casein enzymatic hydrolysate resulted in rapid cell proliferation. The percent viability determined after 12 days of culture on MS medium varied from 29 to 60%, depending on the initial cell viability values taken after 12 hrs of post thaw culture (Table 2). Similarly, Super Basmati variety gave the highest proliferation rate compared to the other two varieties (Table 2). Cell viabilities of cryopreserved rice cell suspensions were recorded for different periods of cryopreservation. For a given variety no significant difference was found in viability, cryopreserved for different periods (Table 1).

Discussion

Aim of the present study was to develop an efficient and convenient explant, which may prove to be an ideal tissue for biolistic transformation and for high yield of totipotent protoplasts. It was found that both MS and R_2 media supplemented with 2-mg/l 2,4-D are quite suitable for developing cell suspension (8-12 weeks). However, cell suspensions regenerability is not maintained for longer period. It may be speculated that longer *in vitro* condition of rice cell suspension block the pathways responsible for regeneration of cells. Hence

either other different auxin for the initiation of cell suspension should be tried or such regeneration media, having compounds or additives that could help in the initiation of regeneration, should be used.

Plant regeneration was observed from fine cell suspension, but first embryogenic calli were produced on regeneration medium lacking 2,4-D and thus plantlets regenerated via somatic embryogenesis. Plantlets apparently germinated from somatic embryos consisted of a coleoptile and a root bilaterally in the initial development stage.

Cryopreservation was used to maintain the embryogenicity of cell suspensions. Varieties differed in their viability after cryopreservation. Basmati 385 was found less viable (9.36%) than other two varieties. Microscopic observation of Basmati 385 cells showed that this suspension was consisted of large and more vacuolated cells than the cells of the other two varieties. It has been reported that large vacuolated cells of cell suspension generally do not survive freezing and thawing processes (Withers and Street, 1977). Viability decreased after cryopreservation. This suggests that some selection does take place in favour of cells or varieties that withstand cryopreservation as in maize (Shillito *et al.*, 1989).

Embryogenic cells which are small and contains dense cytoplasm with fewer vacuoles can better tolerate or withstand cryopreservation (Jain *et al.*, 1996). Shillito *et al.* (1989) is of the opinion that cryopreservation can provide a selection process for embryogenic cells.

Cells were found to be bleached in appearance in case of Basmati 385. This is supposed to be symptomatic of cryoinjury, deplasmolysis and oxidation stress (Lynch *et al.*, 1994). It was also observed that viability of different varieties after different interval of cryopreservation did not differ significantly. Our study shows that cryopreservation can be used as a tool to preserve an important cell suspension line for quite reasonable time without using expensive programmable thermo freezer and which can easily be used either for subsequent callus formation on culture medium or for the establishment of cell suspension in liquid R_2 medium.

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