PRELIMINARY EXPERIMENTS ON FREEZE PRESERVATION OF SUGARCANE CELLS

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Abstract: A preliminary investigation on freeze preservation of sugarcane cell cultures indicated that sugarcane cultures frozen at a rate of 1°C/min to −100°C after treating with 10% (v/v) glycerol +5% (v/v) dimethylsulfoxide (DMSO), and thawed in a water bath at 37°C, yielded up to 30% viable cells. 10% glycerol was found to be more effective cryoprotectant for sugarcane cells than 5% DMSO. Rapid thawing in a water bath at 37°C gave the highest viability of sugarcane cells recovered following freezing. Frozen sugarcane cells stored in the liquid nitrogen refrigerator, up to 4 months, indicate that no decrease in cell viability was observed after thawing as compared with control.

INTRODUCTION

The germplasm resources available to plant breeding are increasingly being eroded. High yielding varieties replace wild strains and the wild strains are lost. Extension of the area of the earth altered by man's building activities results in eliminating wild and ancestral varieties from the earth. However there is increased demand for genetic characteristics such as disease-resistance to minimize the need for chemical control measures and genetic resistance to enviromental contaminants. Conservation of germplasm is essential. For some species, seeds provide a method of storage. However there are many species which must be propagated vegetatively. For these, alternative methods of germplasm preservation are needed. The possibility of regenerating plants and rapid propagation from tissue cultures offers a possibility for genome preservation.

Various genetic variabilities, both at gene and chromosome level are a common trait in the serial propagation of callus and suspension cultures (Sunderland, 1973). A number of workers have attempted to control this instability by reducing the growth rate of tissues by maintence at low temperatures (Bannier and Steponkus, 1972), under mineral oil overlay (Caplin, 1959) and in minimal media (Jones, 1974). However only storage at super-low temperatures, producing total immobilization of metabolic activities can be use to minimize progressive change in the genome (Quatrano, 1968). Following the broad technical guide-lines available from the successful freeze preservation of animal cells and organs (Mazur, 1970) many advances have been made in the technology of freeze preservation of plant cell cultures which are expertly reviewed by Withers and Street (1977a), and Bajaj and Reinert (1977).

Sugarcane is a vegetatively propagated crop. The considerable expense of maintainning large numbers of stock varieties and the very real risk of loss through disease emphasis need for a more satisfactory method of preserving genetic materials. Sugarcane tissue and cell cultures have been successfuly grown in several institutes (Heinz, *et al.*, 1977). Under proper condition, many plantlets can be regenerated from callus of most sugarcane varieties (Liu, *et al.*, 1972). However because cells in culture are cytologically unstable, preservation of germplasm cannot be achieved by this method alone. Cryogenic storage of tissue cultures offers a solution to this problem in sugarcane as in other plants.

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MATERIALS AND METHODS

Sugarcane cell suspension cultures of F108 and F156 were grown under the conditions described by Chen (1978). Actively growing cell suspension cultures were filtered through nylon mesh (1 mm in diameter) to obtain a uniform cell suspension. The cell suspensions were allowed to settle in an ice-bath for 10–20 min. and concentrated to a known volume by removal of supernatant. An equal volume of chilled medium containing cryoprotectant at twice the chosen concentration was added in 4 qual aliquots over 20–60 min.

1.6 ml aliquots of the resulting suspension was dispensed into sterile plastic ampoules (1.2 × 4.5 cm), pre-chilled in an ice bath. The ampoules were removed into the specimen chamber of G. V. Planer R 201 Programmed Freezer (Planer Products Ltd.), frozen to −100°C at 1 or 2°C per min. and transferred to a liquid nitrogen refrigerator for storage. The cultures in ampoules were recovered after various lengths of storage time by thawing in an ice-bath at ca, 1°C; in air at ca. 20°C, or in a water bath at ca. 37°C. After thawing the cultures to the liquid state the ampoules were transferred to an ice-bath for cell viability estimation.

Cell viability test was assayed, using a modified Widholm (1972) technique described by Nag and Street (1975). A 0.5% (w/v) stock solution of fluorescein diacetate in acetone was stored at −20°C. Just before required, one volume of stock solution was diluted to 50 volumes with medium and chilled in ice box. One drop of this solution was mixed with one drop of cell suspension on a microscope slide and covered with a glass coverslip. After 2–20 min. the prepared slide was examined. The cellular units visible in tungsten light and then in the same field in ultraviolet light were counted in a number of separate fields. In small aggregates individual cells were scored by up and down focusing. Large clumps were however scored as single viable units if the whole outline of the aggregate was fluorescent. Viability was expressed as percent fluorescent units.

$$\frac{\text{No. of cellular units fluorescent}}{\text{No. of cellular units in tungsten light}} \times 100$$

Table 1. Effects of Cryoprotectant and Thawing Regime on Cell Viability**

Thawing method	F156			
	0 (Control)	10% Glycerol	5% DMSO	10% Glycerol + 5% DMSO
	(85)*	(71)	(77)	(66)
Ice bath about 1°C	<0.6	14	5	8
Air about 20°C	<0.1	10	9	9
Water bath at 37°C	<0.1	26	17	30

Thawing method	F108			
	0 (Control)	10% Glycerol	5% DMSO	10% Glycerol + 5% DMSO
	(70)	(58)	(68)	(60)
Ice bath about 1°C	<0.1	4	2	9
Air about 20°C	0	4	2	5
Water bath at 37°C	0	14	5	26

* Cell viability before "Freezing-thawing procedure" in parenthesis.

** Percentage of cell viability (fluorescein diacetate test) after 1 hour pretreatment with different cryoprotectant, cooling at 2°C/min to −100°C, storage overnight at −196°C followed by different thawing methods.

PLATE-1 500 μ

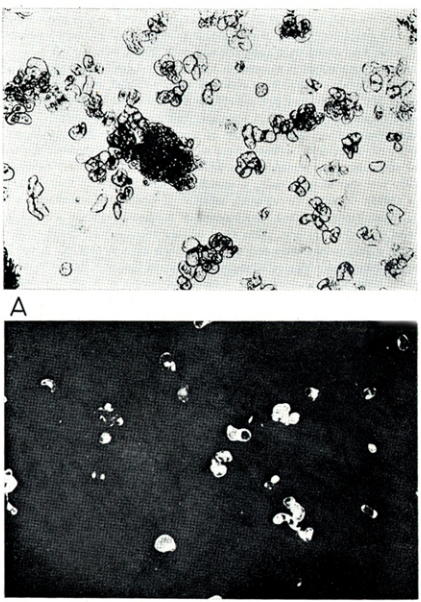

Plate 1. Viability test (fluorescin diaceate method) of sugarcane (F156) cell culture recovered from freezing-thawing procedure; 1 hour pretreatment of 10% glycerol+5% DMSO, cooling at 1°C/min. to −100°C, storing 4 months in liquid nitrogen refriginator, followed by thawing in water bath at 37°C.
A. Viewed in tungsten light.
B. Viewed in UV light.

RESULTS AND DISCUSSION

Effects of cryoprotectant and thawing regime on cell viability are summarized in Table 1. Two cryoprotectants, glycerol and dimethylsulphoxide (DMSO) were tested alone or in combination. 10% glycerol was found to be more effective for sugarcane cells than 5% DMSO. However 5% DMSO in addition to 10% glycerol gave the most effective protection. The studies with sycamore and capsicum cultures have also directed attention to the importance of choice of cryoprotectant. Survival of sycamore cells was greatly enhanced (up to 30% versus 2%) by using 10% glycerol instead of DMSO or DMSO+glycerol. Glycerol was the only cryoprotectant tested which gave survival of capsicum cells (Withers and Street, 1977b). However the pretreatment of cryoprotectant resulted in a considerable loss of cell viability (Table 1). This decline in percentage of cell viability increased with the extention of exposure in cryoprotectants. The loss of cell viability resulting from the use of glycerol was more than that resulting from the use of DMSO.

No appreciable difference in cell viability resulted from slow freezing at cooling rate in the range of 1°C, 2°C and 4°C per min. Frozen sugarcane cells stored in the liquid nitrogen refrigerator so far (up to 4 months) indicate that no decrease in cell viability was observed after thawing in water bath at 37°C as compared with control (Plate 1). Rapid thawing is a water bath at 37°C gave the highest viability in sugarcane cells recovered following freezing (Table 1). This particular benefit is interpreted as resulting from the prevention of ice recrystallization by a rapid transition through the critical temperature range (Withers and Street, 1977a).

Regarding future work it must be emphasised that it is essential to be able to preserve a high percentage of the sugarcane cells, to regrow the cells, and to regenerate plantlets. However, from the literature accumulated so far a low proportion of the plant cells are involved in regrowth of the recovered cultures. The possibility of selection from within the cell population must be considered. It is conceiveable however that cells which survive cryogenic storage might have characteristics valuable in the development of cold tolerant varieties.

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