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Short Communication

Freeze-Preservation of Cultured Flax Cells Utilizing Dimethyl Sulfoxide[1]

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Since the first use of glycerol (14) and dimethyl sulfoxide (DMSO) (8) as protective agents during freeze-preservation of cells, numerous tvpes of tissue from many different organisms have been successfully preserved for long periods of time (11). Cultured mammalian cell lines have been preserved in a defined medium in the presence of glycerol (5) or DMSO (2) and a culture bank of certified animal cell lines has been established. Similar techniques have not been described for the successful low temperature storage of cultured cells of higher plants Although Caplin (3) has reported that reduced growth of plant tissue cultures under mineral oil can increase the subculture period from weeks to months such tissue is still growing and capable of variations in differentiative ability. Well-documented studies have concluded that long-term subculturing of plant cells may lead to increased chromosome abnormalities which appear in some instances to be correlated with loss of differentiative capacities of the culture (12, 20). Attempts to preserve plant tissue cultures should, therefore, aim at a total arrest of growth

Cells of overwintering plants can naturally withstand low temperatures for several months, and the nature of survival properties possessed by cold hardy cells has recently been reviewed (7). However, relatively few studies have been concerned with the protective effects of various agents. Recently, ethylene glycol and DMSO were found to protect tissue sections of mulberry twigs from damage due to cooling in liquified nitrogen and subsequent warming (16, 17). These reports prompted me to examine several methods which combined chemical treatment and low temperature for the preservation of plant tissue cultures. This communication describes a freeze-thaw method using DMSO for low temperature storage of flax cell cultures for short periods of time without loss of characteristic growth and differentiative properties.

Liquid suspension cultures of *Linum usitatissimum* L. were started from agar-grown callus derived from hypocotyls of sterile germinated seeds. The light- and dark-grown suspensions were maintained on an inorganic salt medium (13) with the following organic constituents: sucrose, 40 g/liter; L-glutamine, 146 mg/liter; cysteine-HCl, 10 mg/liter; thiamine-HCl, 1 mg/liter; kinetin, 1 mg/liter; 2,4-D, 1 mg/liter. Cultures were kept at 25° ± 1° on a New Brunswick Gyrotory shaker at 250 rpm and transferred every 28 days. Light-grown cultures received 16 hr of cool white fluorescent light daily at an intensity of 400 to 450 ft-c at culture level. Cells were collected for growth determinations by centrifugation at 1000*g* for 3 min, homogenized in cold 80% (v/v) acetone, and stored at 2 to 4° in the dark overnight. The resulting precipitate was washed twice with 80% acetone and the acetone supernatants combined for spectrum analysis (Beckman DB spectrophotometer) and total chlorophyll determinations (1). The pellet was washed into a pre-weighed pan with acetone and dried at 100° for at least 24 hr for dry weight determinations.

Cells that were to be frozen were collected in screw-capped centrifuge tubes and resuspended in fresh medium containing 10% (v/v) of DMSO (Fisher). Five ml of such medium was added to approximately 10 ml of packed cells and placed at 2 to 4° for 30 min with occasional stirring. Tubes were then wrapped in 1 layer of heavy duty aluminum foil and placed in an ultra-low temperature freezer (Revco, Inc., Deerfield, Michigan) maintained at −50°. Under these conditions, the cooling rate of the medium was 5 to 10°/min. After the storage period (up to 1 month), tubes were removed from the freezer and immediately placed in a water bath at 40°. Within 30 to 45 sec, the liquid suspension of cells was diluted to 50 ml with fresh medium, centrifuged, and resuspended. This was repeated 3 times before cells were placed under culture conditions. All of the above procedures were carried out aseptically. Determination of cell viability was performed using the technique of Steponkus and Lanphear (18), which is based on the ability of living cells to reduce triphenyl tetrazolium chloride (TTC)

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to a red formazan derivative. Using this TTC test, the percent survival for equal wet weight samples was calculated as follows:

$$\% \text{ survival} = \frac{\text{absorbance at 530 m}\mu \text{ of frozen culture}}{\text{absorbance at 530 m}\mu \text{ of unfrozen control culture}} \times 100$$

Soluble proteins were extracted at 4° by passing 5 ml of packed cells through a French pressure cell at 20,000 psi in a medium (final volume, 10 ml) composed of 0.01 M tris-HCl buffer (pH 6.8), 0.001 M $CaCl_2$, 0.25 M sucrose, and 10% (v/v) glycerol. The resulting mixture was centrifuged at 30,000*g* for 15 min and samples were taken from the supernatant for protein determination (9) and for electrophoretic separation of proteins. The protein sample for electrophoresis (approximately 300 μg protein in 0.05–0.10 ml) was applied directly upon a spacer gel and separated in a 10% (w/v) acrylamide gel (4, 19).

Immediately upon thawing, cells exhibited their characteristic green color, but within the next 12 hr considerable loss of coloration was evident, and by 24 hr suspensions appeared bleached. Approximately 10 to 14 days after thawing, small nodules as well as localized areas in larger clumps of cells became pigmented. A similar delay in recovery from freezing has also been observed in the initial stages of development of thawed sporozoites from the parasite *Leucocytozoon simondi* (6). The TTC test at this time indicated that only 14% of the cells had survived the freezing and thawing cycle. When cells from a previously frozen culture were again frozen, thawed, and subcultured, the percent survival was not significantly increased. It also appeared that the percent survival was the same whether cells were thawed several hr, days, or weeks after freezing. Thus, the low survival is probably consequent on freezing and thawing injury rather than storage damage at −50°, at least for times up to 4 weeks. By 21 days after inoculation the pigmented nodules were larger and visibly greener than at 10 days but there had been no noticeable increase in their number. They could easily be removed from the flask and subcultured separately in fresh medium (see fig 1).

Since a relatively small percentage of *Linum* cells survived freezing in DMSO, it was essential to demonstrate that no changes in various growth characteristics or differentiative properties occurred if such cells were to be used for maintenance of stock cultures. The following comparisons between unfrozen and previously frozen cultures were made: amount chlorophyll/mg dry weight, spectrum of acetone-soluble pigments, light-induced chlorophyll synthesis, and soluble proteins.

During 1 subculture growth cycle of *Linum*, the greatest increase in acetone-insoluble dry weight occurs between 10 and 25 days, whereas increase in chlorophyll content of cells commences earlier. By plotting the amount of chlorophyll/mg dry weight, a characteristic and reproducible curve is obtained,

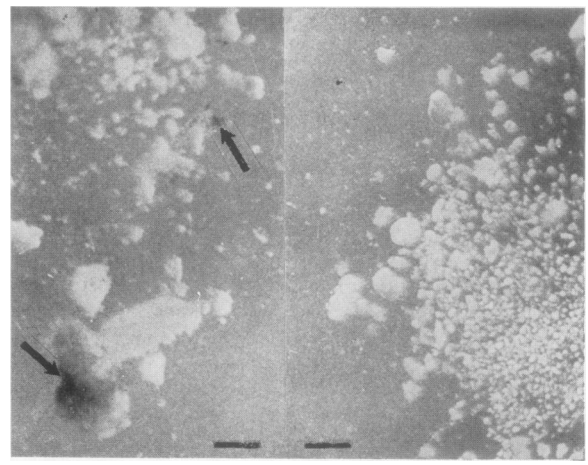

FIG. 1. Comparison of *Linum* cells frozen for 4 weeks with (left) and without (right) DMSO. In these photographs taken 21 days after thawing, chlorophyll containing areas appear dark and arrows in the DMSO treated suspension point to nodules which, when transferred, gave rise to normal suspension cultures. Note in the large nodule (lower left arrow), only certain portions and not the entire clump have dark chlorophyll areas. Cultures frozen in the absence of DMSO did not give rise to viable tissue despite repeated subculture. Each scale line represents 2 mm.

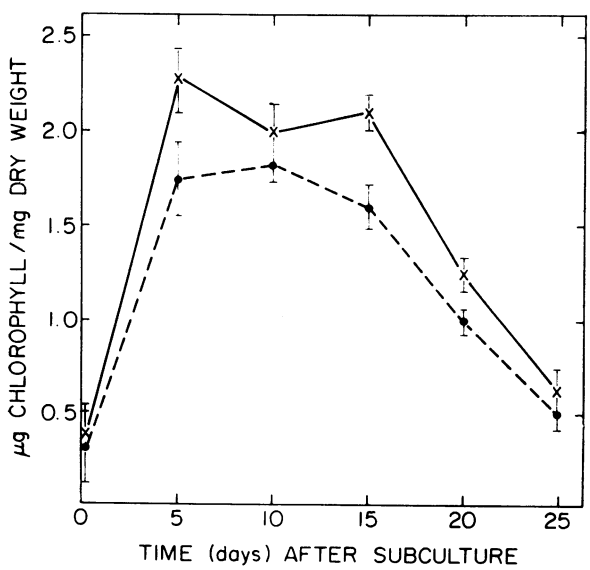

FIG. 2. Growth curves of *Linum*; (.----.) unfrozen control culture, (x———x) cultures derived from cells frozen in DMSO for 3 weeks. Total chlorophyll and dry weight determinations were carried out as described in the text.

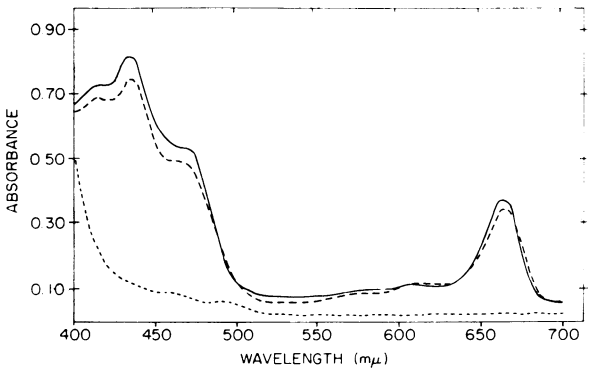

FIG. 3. Comparison of absorption spectra of 80 % acetone extracts from *Linum*; (———) unfrozen control culture; (— — — —) culture derived from cells frozen 2 weeks in DMSO; (-------) dark grown culture. All extracts were taken from equal fresh weight samples.

indicative of the different amounts of these 2 cell components during the growth cycle. A comparison of this ratio from cells derived from frozen cultures with unfrozen controls indicates no difference in shape of the curve although there is a slight increase in the amount of chlorophyll present in the former (fig 2). Acetone extracts of both cultures taken at different times in the subculture cycle did not show any significant qualitative or quantitative differences in their absorption spectra. A typical comparison of spectra from the 2 different cultures 15 days after subculture is shown in figure 3. Another characteristic property of normal cultures is their ability to grow as a colorless suspension in the dark, and, when exposed to light, to synthesize chlorophyll. Green cultures derived from freeze-preserved nodules were subcultured twice in the dark to obtain chlorophyll-less suspensions. These cells were then compared with dark-grown unfrozen controls with respect to their ability to synthesize chlorophyll when exposed to light. Results shown in table I demonstrate that colorless suspensions derived from frozen cells can synthesize chlorophyll when exposed to light at a rate similar to control suspensions. Finally, if changes in frozen cells had occurred, or if a specific cell type was selected, changes in the kinds of proteins found in such cells might be expected. Negatively charged soluble proteins from frozen (2 weeks) and unfrozen cultures were separated on acrylamide gels and no loss or gain in bands was observed. However, some clear quantitative differences were evident in certain bands (fig 4). Whether these increases in band densities are due to an increase in proteins already present or new proteins migrating at the same rate as preexisting proteins, cannot be determined from these data.

Table I. *Comparison of the Ability of Dark Grown Cells Derived From DMSO Frozen Cultures and Unfrozen Dark Controls to Synthesize Chlorophyll When Exposed to Light*

Log phase dark grown cell suspensions of frozen (3 weeks) and unfrozen *Linum* cultures were pipetted into flasks; one-half of each group remained in the dark, the other half being exposed to light. At 0, 5, and 10 days after subculture, cells from both groups were collected and analyzed for total chlorophyll and dry weight as described in text. Each value represents the average of 3 replicate flasks. Standard deviations of these ratios never exceeded ± 0.11.

Day of subculture	Light		Dark	
	Frozen	Unfrozen	Frozen	Unfrozen
	μg chlorophyll/mg dry wt			
0	0.18	0.20	0.18	0.20
5	1.20	1.00	0.42	0.48
10	2.10	2.64	0.56	0.62

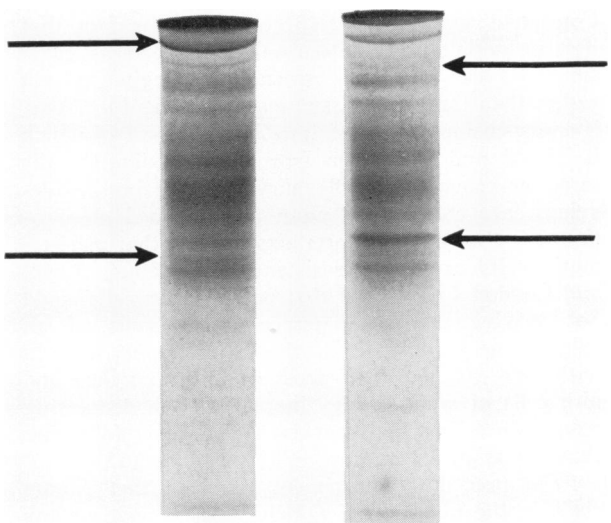

FIG. 4. Soluble proteins separated on acrylamide gels from unfrozen (left) and frozen (right), 21 day-old *Linum* cultures. There are no qualitative differences in banding pattern but a few clear quantitative differences are apparent (arrows). Densitometer tracings of gels substantiate these results. Protein extracts applied represented an equal volume of packed cells. Details of extraction and separation of proteins are given in the text.

The foregoing results indicate that nodules that survived freezing and began to grow 10 to 14 days after thawing were capable of continuing the culture line without any apparent loss in several characteristic growth and differentiative properties.

The fact that these properties are unaltered when only a small percentage of cells survives, and that the percent survival is not significantly increased by a second freezing cycle, suggests that cells giving rise to the population of cells during a normal subculture cycle are the ones most resistant to freezing and thawing. In large nodules of *Linum* grown in liquid culture, there are localized groups of meriste-

matic cells at intervals along the periphery (unpublished observations). These cells are non-vacuolate, green, and contain organelle-filled cytoplasm and prominent nucleoli. Inspection of that part of the suspension which passes 375 μ mesh reveals aggregates consisting of 25 to 75 densely packed, highly pigmented cells similar to those of the meristematic centers of the large nodules. These "meristematic nodules" in the suspension are presumably derived from fragmentation of large nodules, since single cells have not been observed to grow on several liquid or agar media. The macroscopic characteristics of these meristematic nodules giving rise to cells during the normal subculture cycle in liquid or plated on agar seem to be identical with those of the nodules which have the greatest resistance to freezing in the presence of DMSO. Thus, cells derived from such nodules that have survived freezing would exhibit characteristics similar to unfrozen cultures. Consistent with this interpretation is the fact that if large nodules are frozen, only certain areas (presumably meristematic) green and begin growth rather than the whole tissue mass (see fig 1). Also, if a suspension is divided into 2 fractions, 1 of which is plated and the other frozen, approximately the same percentage of cells survives freezing as develops into colonies on agar (unpublished observations). Two other reports also suggest that meristematic cells are more resistant to freezing. Luyet and Condon (10) found that potato tuber cells could not survive at 0.1°, whereas "small cells of an originating sprout within the tuber" were able to withstand −6.5°. Also, frost damage in xylem and pith cells of woody twigs increased with decreasing temperature, but buds survived and grew following thawing from storage at −70° for 6 hr (15).

The preceding experiments indicate that *Linum* cells, in the presence of DMSO, can survive at low temperature for periods up to a month. Similar results have been obtained with *Haplopappus gracilis* but preliminary attempts to freeze cultures of *Eucalyptus camaldulensis* have proved unsuccessful. To determine whether this can be developed as a routine preservation method for plant cell lines, experiments should now be aimed at; (i) storing cultures for longer periods of time at liquid nitrogen temperatures testing variations of this freeze-thaw method as well as other cryoprotective agents, (ii) observing the karyotypes and organ-forming ability of cultures after long-term storage at low temperature, to determine if these properties, which are altered during continued subculture, can be retained without variations, and (iii) extending such experimentation to a number of different plant culture systems.

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