Effects of Cryoprotectants in Combination on the Survival of Frozen Sugarcane Cells

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ABSTRACT
Cryoprotection of suspension cultures of sugarcane cells (Saccharum sp.) during freezing to various temperatures was tested using glucose, dimethylsulfoxide, and ethylene glycol at various concentrations, alone and in combinations. Viability of the thawed cells was assessed by triphenyltetrazolium chloride reduction, cell growth, and microscopic examination. Enhanced cryoprotection—as much as a doubling in viability value—was achieved by employing glucose and dimethylsulfoxide in mixtures, as compared with the lesser cryoprotective effect of either compound alone, at 1.9 molal total concentration in all cases; the mixture was most effective at a concentration of about 1.9 molar, with a molar ratio of the two components of about 1:3, respectively. Much of the increase in viability value arose from a decrease in toxic effect that came about through mixing the cryoprotective agents. Binary mixtures containing ethylene glycol and either glucose or dimethylsulfoxide were less effective and more toxic than comparable glucose-dimethylsulfoxide mixtures. Use of the optimized latter mixture allowed freezing of these tropical cells to −23 C with little decrease in survival, or to −40 C, still with the capability for delayed growth.

Many benefits, experimental and practical, are to be expected from the viable, low temperature frozen storage of plant tissues (1, 14, 26). In attempting to attain this goal, investigators have examined and attempted to understand or imitate the yearly adaptation undergone by temperate zone plants during their natural freeze-hardening process (8, 9).

During freeze-hardening, a large number of physical and chemical changes take place within the plant. These include tissue dehydration and the degradation and resynthesis of whole classes of chemical compounds of both low and high mol wt, whose cryoprotective roles are poorly understood (8, 15). In studying the protection of cultured plant cells against freezing damage in vitro, investigators have added chemical agents, singly or sometimes in arbitrary appearing combinations (4, 7, 12, 13, 17, 22). A drawback in the use of cryoprotective agents is a toxic effect on the treated cells, in itself a severe deterrent to their survival. In an attempt to rationalize, systematize, and comprehend the roles of such substances in freezing protection, we initiated an exploration of the effects of single additives at various concentrations, and of combinations of them to maximize the cryoprotective effect while, if possible, minimizing chemical toxicity. A better understanding of the effects of cryoprotective substances on the complex cellular reorientations involved in survival of cell freezing, and also of the origins of freezing damage itself and of natural freeze protection, could be derived from such information.

Cultures of sugarcane were used as the test material. As with many other vegetatively propagated crops (and in contrast to those plant species whose pure lines are best propagated through seeds) the seeds of the multiple polyploidy sugarcane cannot be relied upon for a straight line display of characteristics (16, 21). Finding a viable freezing procedure for maintaining identified clonal stocks would fill an immediate horticultural need.

Investigation was initiated with Glu, DMSO, and ethylene glycol, used earlier as a cryoprotective combination (4). The compounds are all low in mol wt but quite different in their chemical character and rate of cellular penetration. These substances, alone and in combination, were studied as cryoprotectants for cane cells grown in liquid suspension culture. While both positive and negative reports indicating synergistic (“complementary”) protective effects from the combining of cryoprotectants have appeared in the literature for various plant and animal tissues (2–4, 7, 9, 11–13, 17, 22), the results in this paper support the positive findings. They offer quantitative evidence of more than additive cryoprotection when sugarcane cells are treated with a combination of cryoprotective compounds.

MATERIALS AND METHODS
The cell cultures used were Saccharum cv. H50-7209 (trispecific hybrid) obtained from Dr. P. H. Moore, USDA, Honolulu. All suspensions were subcultured weekly and maintained on a rotary shaker (150 rpm) at 28 C. The medium for growing cane cells consisted of a modified Murashige-Skoog formula containing 2,4-D (3 mg/liter) and 10% coconut water (6).

Actively growing cells were usually harvested for freezing experiments 6 to 9 days after inoculation. The cell suspension was concentrated to a convenient cell density by decanting supernatant medium, after allowing the heterogeneous mixture of cell clump sizes to settle for 10 min. Aliquots (1–2 ml) from the gently stirred suspension were then distributed into graduated tubes in a routine randomized manner and chilled on ice. An equal volume of cold cryoprotective solution was added progressively in 0.3-ml increments, with stirring, over a period of 15 to 35 min. By the method of Sugawara and Sakai (22), modified, the sample suspensions were immersed in a −10 C bath for 2 min, then nucleated by chilling each sample with an external wedge of dry ice and placed in successive baths as follows: −10 C, −15 C, −23 C, −40 C, and −196 C, 4 min at each temperature, to the final temperatures indicated in individual experiments. After 4 min at the final temperature, samples were thawed by swirling in a 40 C bath just to the point of thawing, then diluted and centrifuged, read for packed cell volume, and washed, all at close to 0 C. The dilution and washing steps were carried out with a simplified medium containing only major inorganic salts and 3% sucrose, added stepwise to make 10 ml. Cell viability (survival) was determined

Abbreviations: TTC: triphenyltetrazolium chloride; Glu: glucose; DMSO: dimethylsulfoxide.
either after the addition of TTC to the cells or by transfer of the
cells to fresh culturing medium for assessing their growth capa-

city.

Loss of dye color in the TTC method described by Steponkus
and Lanphere (20) was used for routine assessment of the degree
of cell injury. Throughout the text, color value of the TTC
reduction product is expressed as $A_{560}/0.2$ ml packed cell volume.
Volumes of packed cells used were in the range 0.2 to 0.5 ml. The
average TTC value from aliquots of cell suspension, in duplicate
as a minimum number, was recorded for each experimental con-
dition. As reported by others (20, 23), differences in experimental
material (culture density, cluster inhomogeneities in the trans-
plants, and the number of serial transfers since culture isolation,
as examples in cell suspensions) may produce large variations in
characteristics, observed here between TTC color values of
different experimental batches of cells. The TTC value was used
for comparison of treatments within a given experiment and
should not be used for the direct comparison of cells from different
experiments. To establish a reliability figure for the reproducibility
of the TTC value, duplicate aliquots of cells (for each sample)
were taken from an arbitrary sequence of 114 samples from nine
consecutive experiments, from which the standard deviation in
measurements between duplicates was estimated. In the experiments,
injury to cells was caused by freezing and by the presence of
chemical agents used as cryoprotectants. The chemical and/or
cosmetic toxic effect (10) was evaluated from the decrease in the
TTC value of unfrozen cells held in an ice bath after additions of
cryoprotectants (while the other samples were frozen). Most of the
toxic effect was present just after the adding and washing out of
cryoprotectants but the TTC value also diminished with holding
time in the cryoprotective solution, e.g. by 13% after 45 min in
4%:12% Glu-DMSO. The interval of manipulating samples before
freezing (addition of cryoprotectants, etc.) was therefore kept to a
minimum, generally 15 to 35 min.

Each experiment with the same treatments were repeated several
times as needed to determine the pattern of results even when it
was not possible to have the treatment set complete for all repeti-
tions. Key results were handled statistically. In order to allow
the assembly of figure data and to permit the comparisons among
treatments to be made on a within-experiment basis, each set of
experiments was subjected to separate analyses of variance (18),
including the use of general least squares analysis of variance
when needed for data with unequal subclass numbers (25)—see
figure legends. These procedures allowed the removal of popula-
tion differences between experiments in assessing the experimental
effect (variance).

In order to ascertain the degree to which TTC reduction value
corresponds with the cells’ capability for growth, direct compar-
isons within an experiment were made. In determining growth,
duplicate aliquots of cells (0.05–0.2 ml packed volume) that had
been aseptically manipulated during the cryoprotective and freez-
ing treatments and washings were transferred to tubes containing
9 ml of medium and shaken on a reciprocal shaker. Growth of cells was recorded as an increase in turbidity of the sus-
ended cells, measured periodically in an Evelyn colorimeter with
a No. 620 color filter.

Concentrations of the cryoprotective compounds added to cell
suspensions are expressed as percent of each component in the
final solution (e.g. a 4%:6% Glu-DMSO solution contains 4% Glu
and 6% DMSO, w/v). Anhydrous Glu was from Fischer Scientific
Co., DMSO and ethylene glycol from J. T. Baker Chemical Co.,
and 2,3,5-TTC from Eastman Kodak. All other chemicals were
reagent grade when obtainable.

RESULTS

In early experiments an arbitrary combination of Glu (4%),
DMSO (3%), and ethylene glycol (2.5%) previously found to be
an effective cryoprotectant (4) was used, followed by changes in
concentrations, or elimination of compounds, toward the goal of
achieving increased cryoprotection at low freezing temperatures.

Variations in Glu and DMSO Concentrations to Establish a
Cryoprotective Range. Based on the preliminary results with Glu,
DMSO, and ethylene glycol, a wide range of concentrations of
Glu and DMSO, separately and in combinations, was explored
in order to establish an optimum range of cryoprotection by the
compounds. Glu and DMSO concentrations ranging up to 18%
each (1.0 m and 2.5 m, respectively) were tested. It was found
that combinations near the proportion 8%:12% Glu-DMSO, at about
2 m total solute concentration, offered improved protection at low
temperatures.

Limits to the benefit from increased cryoprotectant concentra-
tion were sought by adding Glu and DMSO in increments of 4%:
6%, respectively, total 1.07 m, to cell suspensions, then freezing
to various temperatures. A combination of results from these treat-
ments and from no added cryoprotectant is shown in Figure 1.
The figure demonstrates both the necessity for and the toxic effect
of cryoprotective additives. In the absence of cryoprotectant, freez-
ing the cells to low temperatures (−15 C and below) is lethal.
Addition of a 4%:6% concentration of Glu-DMSO produced a
lowered TTC value at 0 C (toxicity) but allowed the cells to be
frozen to −15 C and lower temperatures with considerable reten-
tion of TTC value, although values decreased as the temperature
was lowered. Glu-DMSO is more toxic at 8%:12% than at 4%:6%
but, at the same time, is cryoprotective to lower temperatures. It
gave approximately a plateau in viability index to −23 C, and
higher TTC values than those from the 4%:6% treatment at lower
temperatures. With the 12%:18% Glu-DMSO, a still greater toxic
effect more than counteracted any additional cryoprotective effect
so that the curve essentially paralleled the 8%:12% curve but at a
lower index of cryoprotection throughout the freezing range. Thus,
an optimized concentration of cryoprotectants—the maximum
cryoprotection compatible with a minimized toxicity—can main-
tain cell viability at lower freezing temperatures.

Variation of the Glu and DMSO Concentration Ratio to Estab-
lish Maximal Cryoprotection. Further trials of the proportions
of Glu and DMSO narrowed the optimum range of cryoprotection
to near 4%:12%, respectively (1.9 m total concentration) at −40 C.
The 1.9 m concentration of protective additives was used as the
basis for experiments to establish the optimum ratio of Glu and
DMSO as well as compare the results from solely Glu or DMSO
cryoprotectant, at 1.9 m. The results in Figure 2 indicate that at
all temperatures the maximum TTC value is obtained from a
mixture of the two additives and occurs as a relatively broad peak
near the Glu-DMSO ratio 8%:10.5%. This combination increased
the TTC values of frozen samples by 25 to 100%, compared with
the effect of Glu or DMSO alone at 1.9 m.

From the 0 C (unfrozen) curve, it also appears that the combi-
nation of agents contributes less chemical toxicity than do the
individual components. The increased freezing protection ob-
served when using the combination of cryoprotectants described
above was probably due, at least in part, to a decreased toxicity
that occurs when the cryoprotectants are used in combination.

The location of the curve maxima suggests that combining G

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2 Precision (standard deviation) was 0.026 for absorption readings of
diluted extracts of duplicate samples ($A_{560}/0.2$ ml packed cells). (Absorp-
tion value of measured extracts, times dilution factor, ranged from 0.07
to 4.3/0.2 ml packed cells.)

3 Reference to a company and/or product named by the Department is
only for purposes of information and does not imply approval or recom-
mandation of the product to the exclusion of others which may also be
suitable.
with a preponderant concentration of D (1:3 Glu-DMSO molar ratio) gives the best cryoprotection. A mixture near this maximum, 8%:10% Glu-DMSO, was selected for continuing cryoprotective experiments on the sugarcane cells at low temperatures.

Assessment of Ethylene Glycol Effect on Cryoprotection. Making use of the optimized cryoprotective combination 8%:10% Glu-DMSO arrived at above, the cryoprotective effect of ethylene glycol was again tested: (a) in replacement of each protective substance of the Glu-DMSO pair; or (b) as a supplement to Glu-DMSO combinations. Figure 3 shows the effect of replacing either the Glu or DMSO in the combination by ethylene glycol in an equimolar amount (E), or 0.5 molar equivalent (0.5 E), or 1.5 molar equivalent (1.5 E), of ethylene glycol when testing cryoprotection of the cells at -23 C. Results indicated that ethylene glycol, too, in a mixture of cryoprotectants, especially with Glu, offers greater protection than the single compounds at the same total molarity. They also indicated, however (not illustrated), that at several temperatures to -196 C, ethylene glycol alone or as a supplement (1, 2, or 4%) added to the Glu-DMSO combination is more toxic as well as less cryoprotective compared with the effect of Glu-DMSO.

Growth after Freezing: Comparison of Growth and TTC Assays. The convenient TTC reduction comparisons are a useful method for comparing the degree of cryoprotection received by cells; but use of this method would be justified only if the TTC values obtained represent a semiquantitative measure of the capability for growth of cells. The relationship between TTC values and capacity for growth of frozen cultured cells has seldom been quantitatively described in a direct comparison (e.g. 24).

Cell suspensions treated with cryoprotective mixtures were fro-
FIG. 2. Effects on TTC value brought about by varying Glu-DMSO ratios. Total sample concentration of Glu + DMSO = 1.9 M. Percentage composition of the mixtures (per cent of each component in the solution, w/v) and molar ratios of the cryoprotectants are both indicated in the abscissa. Error bar indicates the maximum ± se of the mean (from two to six sets of duplicated samples for each point) for each curve.

FIG. 3. Cryoprotective effects of combining ethylene glycol (E) with Glu (G) or DMSO (D). Sugarcane cells frozen to -23 C. Base line notations: concentrations of ethylene glycol represent a comparison relative to the total molar equivalence of 8% Glu + 10% DMSO (1.9 M total). (Concentrations of single compounds [1.9 M]: ethylene glycol alone = 11%; Glu alone = 32%; DMSO alone = 14%.) Error bar represents the ± se of the mean from three sets of duplicated samples.
zen and thawed. Two sets of samples from the same culture were treated in parallel, one (aseptically) for turbidometric measurements of cell growth, made over a several-week period, the other (without aseptic precautions) for the immediate determination of TTC reduction capability by the treated cells. The growth of cells after freezing, washing, and transferring to fresh medium, and the corresponding TTC values, were both recorded.

The addition of cryoprotectants was toxic to cells, expressed as both a decrease in TTC values at 0°C and a lengthened lag period during growth of unfrozen cells, with increasingly greater effects at greater concentrations. (See Fig. 1 for the effects on TTC values.) However, adding cryoprotectants also had the effect of decreasing the damage to frozen cells (increased TTC values).

Figure 4 illustrates that the toxic effect from a cryoprotectant can be more than compensated through an increased protection against low temperature freezing damage. In the figure, cryoprotection by 8%-12% Glu-ethylene glycol at -23°C was almost complete in terms of growth capability. A proportion of the cryoprotected cells survived freezing even to -40°C. In this case, growth was observed after a long lag period. By contrast with cryoprotected cells, unprotected cells showed no growth after being frozen to -23°C or even to -15°C. TTC values of unprotected cells, also shown in the figure, were likewise reduced almost to zero. Accumulated data have shown that, as illustrated in the figure, TTC values and growth of cell samples that are run in parallel agree with each other generally, even if not strictly proportionally, as has also been described for other tissues (20, 24).

Effects on Cell Structure. When sugarcane cells are frozen to -15°C or below without cryoprotective additions, irreversible plasmolysis is observed in all cells. The photomicrograph in Figure 5 illustrates that in the absence of freezing, permanent plasmolysis is produced in a proportion of the cells merely through adding (then washing out) cryoprotective agent (8%-12% Glu-DSMO). In parallel to this, adding of cryoprotectant to cells without freezing them also produces a loss in their TTC value (a "toxicity"; see Figs. 1 and 4). We have found that cells that are treated with adequate cryoprotectant (e.g. 8%-12% Glu-DSMO) and then frozen to -15°C or -23°C generally exhibit only a small additional proportion of permanently plasmolyzed cells as a result of the freezing step (not illustrated) and likewise only a small additional loss of TTC value (Fig. 1). It appears that most of those cells that were in a condition to survive the toxic effect from treatment with cryoprotectant (unplasmolyzed cells of Fig. 5) were also modified by that treatment to become resistant to subsequent damage from freezing to -15°C or -23°C.

**DISCUSSION**

When the described cryoprotective agents were used as a combination, the combination was more effective than were the single components in preventing freezing damage to sugarcane cells. Use of Glu and DMSO at about 1.9 M total concentration achieved maximum cryoprotection of these cells. Increases in cryoprotectant concentration of up to 1.9 M resulted in extending the survival plateau to lower temperatures; beyond this concentration toxic effects began to predominate (Fig. 1). At 1.9 M total concentration, a combination of Glu and DMSO at 8%-10%, respectively, chosen as near optimal, has a protective effect much greater than that of either Glu or DMSO alone at the same molar concentration. Using this combination results in a moderately high plateau of TTC values for cells frozen to -23°C and gives values often greater than 0.5/0.2 ml packed cells with cell suspensions frozen to -40°C. The A value 0.5 represents about 15% that of untreated, unfrozen cells. Cane cells that display this TTC value after freezing often survive the freezing treatments (Fig. 4). Combinations of ethylene glycol, especially with Glu, are also more effective than the single cryoadditives, as noted similarly in kale stem sections by Samygin and Matveeva (17); but pairs of compounds containing ethylene glycol fell short of the degree of protection by Glu-DSMO. Experiments with a cryoprotective combination such as Glu-ethylene glycol might be of special interest in a context where the presence of DMSO would be undesirable, such as during freezing experiments with a plant foodstuff.

A negative factor in the use of the cryoprotective compounds is
their large, irreversible toxic effects on the cells; but when cryoprotectants were added in the described combinations, a benefit appeared in the form of a decrease in the relative reagent toxicity (Fig. 2, 0 C curve). This diminished toxic effect—like the beneficial effect on over-all freezing protection already noted—is maximal near an 8%;10% ratio of Glu-DMSO. If the toxicity of the compounds in a combination of cryoprotectants is less than additive, through competition for combining sites, while cryoprotection is additive (for example, in decreasing the water activity), then the over-all protective effect observed on freezing the cells in a mixture of cryoprotectants would be more than additive—a threshold-like phenomenon, as appears from the figure.

From the experiments we could not determine whether the increase in low temperature protection from using mixtures of cryoprotectants might not, indeed, be due primarily to the decrease in toxicity from the combined reagent mixture.

TTC value, as an indicator of cell viability, was found here to be quite precise for replicate samples within a treatment; but yet, the TTC values resulting from experimental treatments (Fig. 4) offer only a semilinear indication of viability, as supported by the findings and interpretations of Steponkus (19). The approximate proportionality displayed here between TTC values and growth indicates that the TTC method is a useful preliminary tool for experimentally selecting favorable conditions of treatment of cultured cells during the freezing process.

A parallelism was observed between two indicators of cell damage, namely, permanent plasmolysis and loss of TTC reduction, when cells were treated with cryoprotectant or frozen (or both). The observed plasmolytic behavior served to authenticate, and also to explain, both the large decrease in TTC values resulting from use of additives at 0 C (Fig. 1), and the but small additional decrease sustained when these cells are then frozen to −15 C or −23 C. Since freezing the protected cells to these temperatures did not appear to increase the fraction of damaged cells greatly, it may be assumed that a major proportion of those cells that were not damaged by the chemical toxicity of the protectant were, instead, cryoprotected. It would be important to explore further both the structural and physiological differences that make particular cells more susceptible than others to injury by the cryoprotective compounds, and methods by which this injury might be avoided and thereby increase the proportion of freeze-viable cells.

Since a combination of two additives, particularly Glu and DMSO, of differing chemical properties demonstrated enhanced protection of the cane cells, it seems reasonable that other cryoprotective compounds of still other chemical properties might enhance the effect still further. In fact, this concept has been followed, using PEG in combination with both Glu and DMSO, with the result that protection of sugarcane cells to the temperature of liquid N₂ has been achieved (5). Further detailed observation of the interactions—cryoprotective and toxic—of such compounds...
with plant tissues could lead to a better comprehension of the mechanisms of both artificial and in vivo freezing protection. It seems likely, too, that through the use of combinations of cryoprotective agents, an enhanced cryoprotection of other species than sugarcane might be found, including many species whose tissues are less cold-sensitive than this tropical grass.

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