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CRYOGENIC PRESERVATION OF PLANT TISSUES

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ABSTRACT

CRYOGENIC PRESERVATION

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The increasing demand for new varieties resistant to diseases and new cultural constraints justifies the conservation of germplasm presently available in crop species. For some species, seed storage is the method of germplasm preservation. However, for crop species propagated vegetatively, an alternative means of conserving valuable genotypes is required.

The purpose of this work was to develop a cryogenic method for long term storage of plant tissues of vegetatively propagated species. Meristematic tips were chosen as the most suitable tissue for the advantages they represent compared to cell or callus cultures.

Both meristematic tips and small seedlings of four potato cultivars were subjected to three freezing methods:

i) Rapid cooling by direct immersion in liquid nitrogen;

ii) Slow cooling at different rates,, predominantly $1.7^{o}C$ per hour;

iii) Cooling by suspending vials containing tissue in

the vapor above the surface of liquid nitrogen. The vials were later immersed directly in the liquid nitrogen.

Previous to exposure to freezing temperatures, the plant material was subjected to various hardening pretreatments:

i) Plants from which tissue was removed were grown under short days;

ii) The isolated tissues were precultured in a nutritive medium under light regimes of either 16 hour days and 8 hour nights or continuous darkness;

iii) The pretreatment culture medium was supplemented in several experiments with dimethyl sulfoxide or mannitol;

iv) Explants were hardened by exposure to low temperatures, usually $4^{o}C$ and $0^{o}C$, in the dark.

Different presumptive cryoprotective agents were also tested. Glycerol and dimethylsulfoxide were chosen as penetrating cryoprotectants and sucrose as a non--penetrating one. All were used at several concentrations, individually or in combination.

Once the meristematic tips were subjected to the appropriate freezing treatments, they were stored in liquid nitrogen for approximately one hour. The frozen tissues were thawed by immersing the vials in a water bath of $37^{o}C$ or $40^{o}C$. For slower thawing rates, vials were wrapped in cotton wool and a plastic bag and placed in a refrigerator (at $5^{o}C$) overnight.

No tissue survived temperatures lower than $-23^{o}C$. Only

a chilling pretreatment protected tissues against freezing temperatures. Among the cryoprotectants tested, glycerol gave the best results when slow freezing procedures were followed. Survival at -23^{o}C was obtained when the concentration of glycerol was 30 percent (v/v).

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INTRODUCTION

I The Need for Plant Germplasm Preservation

The gene pools of many of the major crops are being depleted (Frankel and Hawkes, 1975). Improved crop varieties have been adopted by most farmers in the developed countries and are replacing local varieties and even occasionally the wild types themselves in their centers of origin. This trend may greatly reduce the natural reserves of variation in wild types and primitive varieties that abound in regions where modern agriculture has not supplanted them with "improved" varieties. In addition, man is altering the environment wherein occur the evolutionary events which generate genetic diversity with roads, buildings, altered drainage and other environmental perturbations. This decrease in genetic diversity is accompanied by an increase in genetic vulnerability, and an increased risk of economic loss caused by some new parasite, insect pest or unusual environmental stress. With new and menacing environmental problems facing us, the need to accumulate a broad base of genetic variability becomes increasingly important These wild types are the raw material that will be developed through breeding to produce crop varieties suited to the needs of the future farmer and consumer.

For most species, seed storage is the best method of germplast preservation. Seeds are relatively small, fairly abundant and can be stored over long periods of time with a minimal risk of genetic damage. However, aneuploids, odd-number polyploids and genotypes with various types of sterility (genetic, chromosomal or cytoplasmic) that produce few or no seeds can be stored only as vegetative propagules.

Among the crops most frequently propagated by vegetative means are bananas (Musa spp.), manioc (Manihot esculenta Grantz.), pineapples (Ananas comosus L., Merr.), potatoes (Solanum spp.), sugarcane (Saccharum officinarum L.), sweet potatoes (Ipomoea batatas L., Lam.), taro (Colocasia esculenta L., Schott.), cocoyam (Xanthosoma spp.), and yam (Dioscorea spp.). A vast array of deciduous and tropical fruit and nut crops, certain forage species and the perennial Arachis species are also maintained vegetatively. The long periods of time required to grow populations, the excessive cost of developing these collections and the lack of an adequate sexual cycle has limited the genetic advances in these crops to date.

Plant tissue culture techniques provide an alternative method of conserving plant genetic resources for extended periods of time. A great number of plant species can now be vegetatively propagated through cell, tissue and organ culture (Murashige, 1974). The plant material can be maintained

indefinitely either by regular medium replenishment (serial transfer) or by maintenance of cultures under minimal growth conditions. In some instances, preservation of cell cultures, calluses, adventitious asexual embryos or shoot apices has been achieved by storage of cultures at low -- but above freezing -- temperatures (Bannier and Steponkus, 1972; Meyer-Teuter and Reinert, 1973; Mullin and Schlegel, 1976). Morel (1975) was able to keep meristem derived plantlets of grape (Vitis vivipera) alive at $9^{o}C$ with only one transfer per year. These procedures present the following advantages:

1) The space requirement is relatively small in comparison with field cultivation.

2) The maintenance procedure is relatively simple and inexpensive.

3) The propagation potential of the cultures can be very high.

4) The problems of genetic erosion in stocks, which can be serious under field conditions, are completely avoided.

5) The techniques can also be used to produce and maintain pathogen-free stocks of plant material (Henshaw, 1975).

Unfortunately, each of these methods of storage exhibits serious practical disadvantages. Serial transfer incurs the risk of microorganism contamination, equipment failure, changes in chromosomal cytology (D'Amato, 1975; Sheridan, 1975) and

the loss of morphogenic potential. Maintenance of cultures on minimal medium does not completely arrest cellular processes and thus increases the time between successive transfers only a limited amount. Storage of cultures at low, though above freezing, temperatures requires the addition of liquid nutrient medium every few months to prevent desiccation. This takes time and also subjects the cultures to the possibility of contamination by microorganisms.

Two important requirements must be met in order for tissue culture to be of use in genome preservation: 1) the tissues must be genetically stable for long periods of time in vitro, and 2) they must retain the capabability to regenerate into whole plants. Thus, the central problem becomes inhibition of cellular change.

It is difficult to suppress cell division for any appreciable length of time in tissue cultures, using inhibitors or minimal media, without eventually causing cell death. Low temperature is certainly a more satisfactory way of suppressing cell division and DNA synthesis in tissue cultures for the purpose of long term genetic stabilization.

II Cryogenic Storage as an Alternative Means of Plant Germplasm Storage.

The freezing of biological materials has been investigated

for the last 200 years (Meryman, 1966); however, significant porgress has been made only since the middle of this century when the beneficial effects of cryoprotectant additives were first reported. In 1949, Polge and co-workers demonstrated the successful freezing of spermatozoa utilizing added glycerol. Cultured mammalian cells were similarly frozen by Scherer and Hoogasian in 1954. The freeze preservation of rat ovarian tissue has since been reported (Deansley, 1957), in addition to that of chick (Rey, 1957) and mammalian embryos (Wittingham et al., 1972).

Similar attempts with plant tissues and cell cultures by Sakai and co-workers have added greatly to our knowledge of frost hardiness, low temperature survival, cooling and freezing regimes and, more recently, freezing as a means of storage of calli and suspension cultures (Sakai and Otsuka, 1967; Sakai et al., 1968; Sakai and Sugawara, 1973; Sugawara and Sakai, 1974).

The general scheme for cryogenic plant germplasm preservation is diagrammed in Figure 1. The freeze-storage of plant tissues in vitro could be useful for the following additional purposes;

1) Preservation of haploids. Haploid cell cultures are highly unstable and have a tendency to revert to

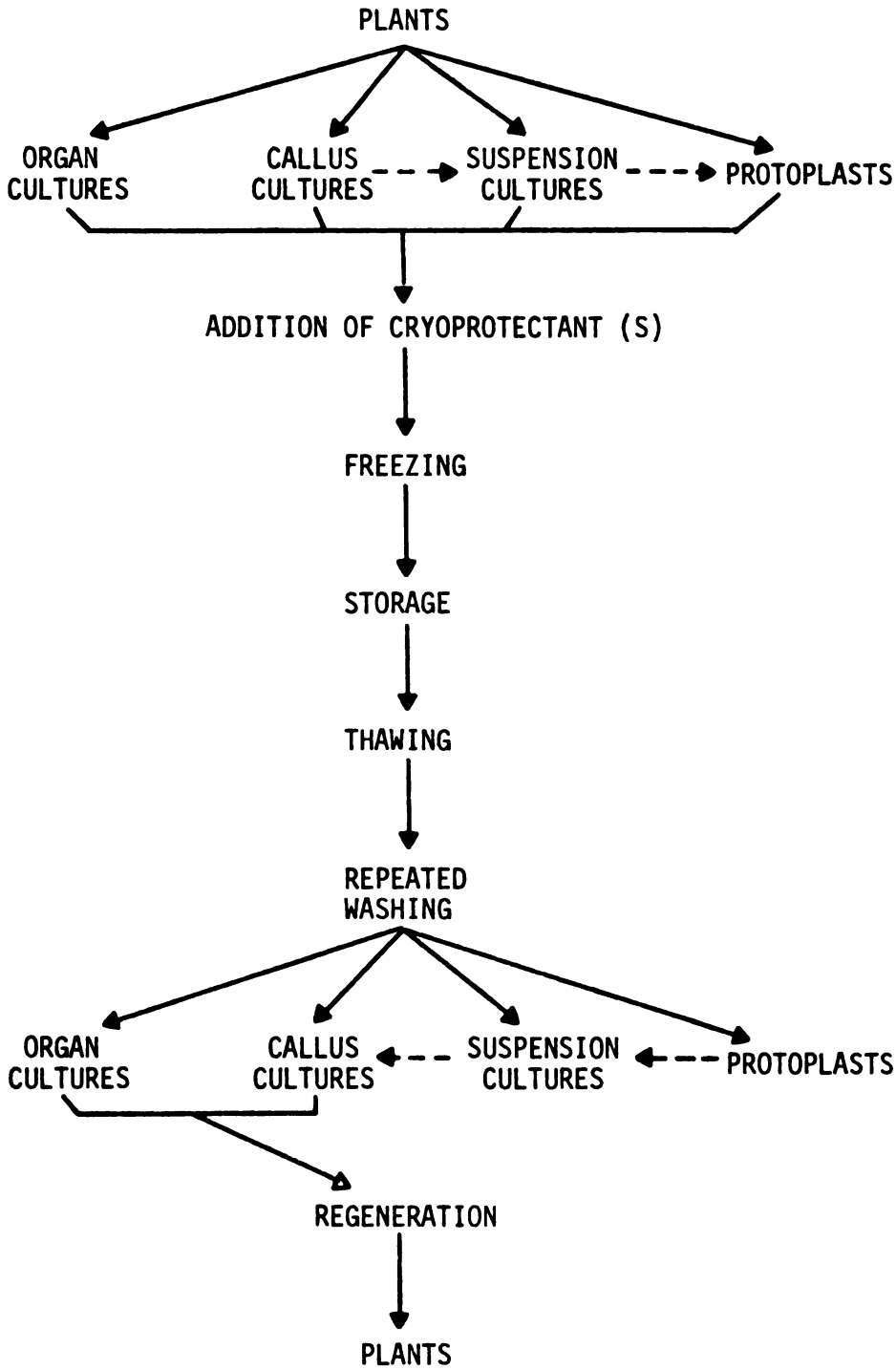


Figure 1: Possible scheme for cryogenic plant germplasm preservation

the diploid or polyploid level. In view of the importance of haploids for the induction of mutations and the rapid generation of homozygous diploid plants, the maintenance of haploids is highly desirable. Meristem tips, excised anthers, isolated pollen and pollen embryos would be suitable materials for freeze-preservation of haploids.

2) Preservation of rare genomes. Chromosome aberrations, mutations and ploidy changes, though undesirable for maintaining clonal integrity, can nevertheless provide genetic variation for breeding programs. For examples, in sugar cane, substantial variability occurs in callus-derived plant populations from single clonal selections (Heinz and Mee, 1971). Such variants could be maintained indefinitely in the frozen state.

3) Maintenance of disease-free material. Pathogen-free stocks of rare plant materials could be frozen, thawed and propagated when needed. This method would be ideal for the international exchange of vegetatively propagated material.

4) Cold acclimation and frost resistance studies. Cell and tissue cultures would provide a suitable material for the study of enhancing the hardiness of callus, and thus perhaps whole plants, to cold conditions (Steponkus and Bannier, 1971). Selection of cold-resistant mutant

cell lines which could then be differentiated into frost resistant plants might also be possible.

5) Obviating periodic subculture of stocks. Maintenance of a cell line requires transfer to fresh medium periodically. Freeze preservation could be used to suppress cell division and thus alleviate the need for regular nutrient replenishment.

6) Retention of morphogenetic potential. Tissue cultures of most species either show a decrease or complete loss of morphogenetic potential during long term culture (Torrey, 1967). By cryogenic storage, the capacity to undergo differentiation could be maintained.

7) Avoidance of senescence. At ultra low temperatures, the cells enter a non-metabolic state which arrests or delays the aging process.

III Examples of Low Temperature Storage of Plant Cell and Organ Cultures

In addition to Sakai and co-workers, many others have attempted to achieve the success attained by investigators working with cryogenic preservation of animal tissues. In Tables 1, 2 and 3 are compiled the past decade's most promising reports in cryogenic storage of plant tissue.

A. Cell suspension. In Table 1 are listed the

Table 1: Freezing of Plant Cell Suspension Cultures

[1]Cryoprotectants: DMSO = dimethyl sulfoxide

[2]Freezing regime and storage temperature: LN = liquid nitrogen

[3]Survival: EMB = embryogenesis from surviving material; FDA = fluorescein diacetate test; REG = regeneration of whole plants from surviving material; TTC = triphenyl tetrazolium chloride test; + = cell division and proliferation in surviving material

Species	Culture pretreatments	Cryoprotectants[1]	Freezing regime[2]	Temperature(°C)[2] Storage	Thawing	Survival[3]	Reference
Acer pseudoplatanus	none	none	prefrozen to -30 to -50°C, then to LN	LN	40	25% TTC +	Sugawara & Sakai, 1974
"	none	10% glycerol	2°C/min to -100°C, then to LN	LN	37	25% FDA	Nag & Street, 1975a
"	none	10% glycerol, 5% DMSO	2°C/min to -100°C, then to LN	LN	37	22% FDA	Nag & Street, 1975a
"	none	10% glycerol, 5% DMSO	1°C/min to -100°C, then to LN	LN	40	30% FDA	Withers & Street, 1977
"	pregrown in medium + 3.3% mannitol (w/v)	10% glycerol, 5% DMSO	1°C/min to -100°C, then to LN	LN	40	40% FDA +	Withers & Street, 1977
"	synchronized	10% glycerol, 5% DMSO	1°C/min to -100°C, then to LN	LN	40	25% FDA 45% TTC	Withers, 1978

Table 1: Freezing of Plant Cell Suspension Cultures (cont'd)

Species	Culture pretreatments	Cryoprotectants	Freezing regime	Temperature(°C) Storage	Thawing	Survival	Reference
Atropa belladona	none	10% glycerol, 5% DMSO	2°C/min to -100°C, then to LN	LN	37	15% FDA	Nag & Street, 1975a
"	none	5% glycerol	1°C/min to -100°C, then to LN	LN	37	25% FDA	Nag & Street, 1975b
"	pregrown in medium + 5% DMSO	5% DMSO	2°C/min to -100°C, then to LN	LN	37	30% FDA	Nag & Street, 1975b
Capsicum annuum	none	10% glycerol, 5% DMSO	1°C/min to -100°C, then to LN	LN	40	<1% FDA	Withers & Street, 1977
"	pregrown in medium + 5.2% mannitol	10% glycerol, 5% DMSO	chilled overnight, then 1°C/min to -100°C, then to LN	LN	40	25% FDA	Withers & Street, 1977
"	pregrown in medium + 5.2% mannitol	10% glycerol, 5% DMSO	chilled overnight, then 1°C/min to -30°C	LN	40	50% FDA +	Withers & Street, 1977
Daucus carota	none	5% glycerol or 10% DMSO	2-4°C/min to -40°C	-40	37	+	Latta, 1971
"	none	5% DMSO	2-4°C/min to -196°C (LN)	LN	37	+	Latta, 1971

Table 1: Freezing of Plant Cell Suspension Cultures (Cont'd)

Species	Culture pretreatments	Cryoprotectants	Freezing regime	Temperature(°C) Storage	Thawing	Survival	Reference
Daucus carota	none	5% DMSO	1.8°C/min to -196°C (LN)	LN	37	65% FDA + EMB	Nag & Street, 1973
"	none	5% glycerol, 5% DMSO	1.8°C/min to -196°C (LN)	LN	37	48% FDA EMB	Nag & Street, 1973
"	differentiated into pro-embryonic mass	5% or 10% DMSO	1-2°C/min to -70°C, then -196°C (LN)	LN	30 or air at room temp.	EMB +	Dougall & Wetherell, 1974
"	none	5-7% DMSO	prefrozen to -20 to -70°C, then to -100°C	LN	37	REG +	Bajaj & Reinert, 1975, Bajaj, 1976
"	none	5% DMSO	2°C/min to -100°C, then to LN	LN	40	65% FDA + EMB	Withers & Street, 1977
"	none	4% glucose, 3% DMSO, 2.5% ethylene glycol	"stepwise" to -15°C	not stored	"rapid"	40% TTC	Finkle et al., 1975
"	none	4% glucose, 3% DMSO, 2.5% ethylene glycol	"stepwise" to -23°C	not stored	"rapid"	20% TTC	Finkle et al., 1975

Table 1: Freezing of Plant Cell Suspension Cultures (cont'd)

Species	Culture pretreatment	Cryoprotectant	Freezing regime	Temperature(°C) Storage	Thawing	Survival	Reference
Glycine max	none	7% DMSO	prefrozen to -20 to -70°C or 2°C/min to -100°C	LN	37	52%	Bajaj, 1976
Haplopappus ravenii	none	10% DMSO	1.7°C/min to -20°C	not stored	not given	3 x control's TTC +	Hollen & Blakely, 1975
Ipomea sp.	none	2.5% glycerol+ 2.5% DMSO+ 6.5% sucrose	2-4°C/min to -40°C	not stored	37	+	Latta, 1971
Linum usitatissimum	none	3.3% DMSO	5-10°C/min to -50°C	-50	40	14% TTC	Quatrano, 1968
Nicotiana tabacum	none	4% glucose, 3% DMSO, 2.5% ethylene glycol	"stepwise" to -23°C	not stored	"rapid"	20% TTC	Finkle et al., 1975
Nicotiana tabacum (haploid)	none	5% DMSO	prefrozen to -20°C or 1-2°C/min	LN	37	+ REG	Bajaj & Reinert, 1975; Bajaj, 1976
Oryza sativa	none	5% DMSO	Prefrozen to -70°C	LN	30	65% TTC +	Sala et al., 1979

Table 2: Freezing of Plant Callus Cultures

[1,2,3]For abbreviations, see Table 1

Species	Culture pretreatments	Cryoprotectants[1]	Freezing regime[2]	Temperature(°C)[2] Storage	Thawing	Survival[3]	Reference
Chrysanthemum morifolium	pregrown on medium + 10% sucrose	none	2 weeks at 4.5°C, then to -3.5°C	-3.5°C	not given	8% TTC	Bannier & Steponkus, 1972
Populus euramericana	none	none	prefrozen to -30°C, then -70°C, then -120°C, then to LN	LN	air at room temp.	+	Sakai & Sugawara, 1973

Table 3: Freezing of Organized Plant Tissues

[1,2,3]For abbreviations, see Table 1

Species	Tissue	Cryoprotectants[1]	Freezing regime[2]	Temperature(°C)[2] Storage	Thawing	Survival[3]	Reference
Daucus carota	plantlets and embryos	2.5-20% DMSO	1-5°C/min to -100°C or 65°C/min	LN	40°C/min or 8°C/min	≤63%	Withers, 1979
Dianthus caryophyllus	shoot-tips	5% DMSO	direct immersion into LN, >1000°C/min	LN	37	15-33% callus regrowth, REG	Seibert, 1976
"	shoot-tips of plants grown for 3 days at 4°C	5% DMSO	direct immersion in LN, >1000°C/min	LN	37	30-66%	Seibert & Wetherbee, 1977
Lycopersicon esculentum	seedlings	2.5, 5.0, 10 and 15% DMSO at 20 min. intervals	direct immersion into LN or 2°C/min to -160°C or 20-25°C/min to -120°C	LN	40	≤40% REG	Grout et al., 1978
Solanum goniocalyx	shoot-tips	10% DMSO	direct immersion into LN, >1000°C/min	LN	35	25% green callus, REG	Grout & Henshaw, 1978

plant species and variations in freezing procedure used in cryogenic studies with cell suspensions. These studies have demonstrated that highly cytoplasmic, non-vacuolated thin-walled cells in small aggregates from actively growing young suspensions are able to withstand freezing much better than relatively older cultures containing large and thick-walled, vacuolated, free cells. The percentage of cell survival is strongly dependent on the nature of the cryoprotectant, rate of cooling, method of thawing and the storage temperature. In general, 5 to 10% dimethyl sulfoxide (DMSO), combined with slow cooling (1 to 3°C per minute or prefreezing) followed by rapid warming (at 35-40°C) and storage at -196°C have yielded optimal results.

B. Callus cultures. In a few instances, callus cultures exposed to freezing temperatures have survived (Table 2).

C. Meristem tip cultures. Shoot apices of carnation (Dianthus caryophyllus) exposed to liquid nitrogen have relatively high survival rates (Siebert, 1976; Table 3). Explanted apices from plants grown for 7 weeks under short photoperiods were cultured for 4 days on a synthetic medium, incubated in a solution of 5% DMSO, and then directly submerged in liquid nitrogen (-196°C).

Survival rates as high as 33% were reported, depending on cultivar. A cold pretreatment of the donor carnation plants at $4^{o}C$ for 3 days or more resulted in a doubling of the percentage of excised, frozen shoot apices which survived freezing and a 6 to 7 fold increase in the percentage which formed leaf primordia or shoots (Seibert and Wetherbee, 1977).

The survival of frozen meristems opens up new possibilities for cryogenic storage, as they have definite advantages over the cell and callus cultures:

1) Shoot apices are genetically more stable, and thus more equivalent to seeds, than callus or suspension cultured cells.

2) In many cases, it is difficult to regenerate an entire plant from callus, whereas shoot apices are relatively easy to manipulate.

3) Shoot meristems yield pathogen-free plants.

4) The cells in the meristematic tip are small, thin walled highly cytoplasmic and non-vacuolated, indicating that a higher percentage of cell survival might be expected once they have been frozen.

5) In most cases,the speed of multiplication of the stock <u>in vitro</u> is very high. Meristem culture affords an entirely new means of clonal multiplication and has

brought a real revolution in the cultivation of many horticultural plants (.e.g., orchids (Rao, 1976)).

Two important reports of successful preservation of meristematic tips in liquid nitrogen have appeared recently (Grout and Henshaw, 1978; Grout et al., 1978). Grout and Henshaw's (1978) procedure can be summarized as follows: Shoot tips removed from axillary buds of Solanum goniocalyx plants were incubated in petri dishes containing modified MS medium (Murashige and Skoog, 1962) for 72 hours. The dishes were then flooded with 10 ml of the same medium to which 10% (w/v) DMSO had been added, and incubated for an additional hour. Shoot-tips were then lifted individually on the tip of a sterile hypodermic needle and plunged to the bottom of a vessel containing a 6 cm depth of liquid nitrogen. After 3 weeks of storage in liquid nitrogen, the shoot-tips were thawed by plunging the needle directly into a vial containing modified MS medium at 35^{o}C. Once the tips floated free in the medium they were transferred to the same medium but at room temperature. The survival, assessed 4 weeks after thawing, was 20%. These investigators claimed that vitreous ice formation may have been the explanation for such good survival.

A more elaborate technique was that utilized by

the same investigators when freezing tomato shoot-meristems in liquid nitrogen. Tomato (Lycopersicon esculentum cv. Ailsa Craig) seedlings with 18 to 20 mm radicles were immersed in modified MS medium which had been cooled to 0^{o}. The seedlings were then transferred stepwise through similar media containing increasing concentrations of DMSO over a total pretreatment period of 2 hours. Pretreatment in 15% DMSO gave optimal survival. Three different freezing methods were then used on these tomato meristems: 1) rapid cooling by direct immersion in liquid nitrogen; b) slow cooling at $2^{o}C$ per minute to $-160^{o}C$ followed by direct immersion in liquid nitrogen; and, 3) cooling by suspending the vials above the surface of the liquid nitrogen. The vials were then immersed directly into the liquid nitrogen after 15 minutes in the vapor. A continuously changing cooling rate, from 20 to $55^{o}C$ per minute, between 0 and $-120^{o}C$ (i.e., in the vapor) gave the best survival. Thawing was accomplished by plunging the vials into water at $40^{o}C$ for 1.5 minutes. After rinsing, shoot meristems were dissected from the seedlings and cultured on filter paper moistened with modified MS medium. Under optimal conditions 40% of the seedlings survived.

Recently, freeze-preservation of somatic embryos

and clonal plantlets of carrot (Daucus carota L.) has been achieved (Withers, 1979) by modifying the procedure utilized for cryopreservation of carrot cell suspension cultures (Nag and Street, 1973; Withers and Street, 1977; Table 1). Superficial moisture was removed from cryoprotectant-treated embryos and plantlets which were enclosed in foil envelopes prior to freezing. The use of DMSO at levels between 2.5 and 20% (v/v) and freezing rates of 1 to 5°C per minute resulted in maximal survival under appropriate thawing conditions. In general, treatments which increased tissue dehydration before or during freezing were most successful when followed by relatively slow thawing (e.g., 8°C per minute). Conversely, where less dehydration was achieved, more rapid thawing was advantageous (e.g., about 40°C per minute between -100°C and 0°C). On semisolid regrowth medium, somatic embryos resumed normal development, whereas in plantlets, the root and shoot meristem regions gave rise to new growth. Percentage recoveries were greatly increased when the recovery medium was supplmented with activated charcoal. Using 5% (v/v) DMSO, a freezing rate of 10°C per minute, transfer to liquid nitrogen at -100°C and rapid thawing, up to 63% survival was achieve.

In conclusion, apical meristem cultures possess

great advantages for the long term storage of many economic plants. Conservation of large germplasm collection should be possible in a small space at low cost and maintenance. Furthermore, the material can be kept free of pathogens and be propagated quickly to provide large quantities of plants for evaluation or breeding purposes.

IV Theoretical Aspects of Cryobiology

A. Physiochemical events during freezing and thawing. The physiochemical events taking place when cells are exposed to freezing and thawing conditions have been described by Mazur (1969) in the following way:

(1) Both cells and their external medium supercool initially although ice soon forms in the medium. The cell membrane keeps the ice from seeding the cell interior at temperatures above -10°C, so the cell remains unfrozen and supercooled.

(2) As the temperature falls, and ice continues to form outside the cell, the concentration of solutes in the intracellular fluid rises and the aqueous vapor pressure falls. Since the cell is supercooled, its aqueous vapor pressure exceeds that of the extracellular water, and water flows out of the cell and freezes extracellularly. The resulting dehydration concentrates the intracellular solutes and decreases the spatial separation between

macromolecules. If the cell is sufficiently permeable to water or if cooling is sufficiently slow, the cell will dehydrate to the extent required to maintain equilibrium between the intra- and extracellular aqueous vapor pressures, and intracellular freezing will not occur. But if the cell is not sufficiently permeable to water, or if it is cooled too rapidly, it will not remain in equilibrium, but will continue to supercool until it eventually equilibrates by intracellular ice nucleation.

(3) As more and more water is converted to ice during cooling, the solubility of some electrolytes, whether intra- or extracellular, will be exceeded, and they will precipitate. If their solubilities differ, the differential precipitation may result in large changes in the cellular pH.

(4) Below a certain temperature (the eutectic point, defined as the lowest temperature at which a solution remains liquid), all free water will be converted to ice and all solutes precipitated. A cell held above these temperatures will be immersed in, and will contain, a highly concentrated solution, whose ionic composition and pH are likely to be far different from that prevailing under normal physiological conditions.

(5) Small or nonspherical crystals of ice have higher surface free energies than large spherical crystals. There will be a tendency, therefore, for the former to lose water to the latter, a process known as recrystallization or grain growth.

(6) During warming, the sequence of events essentially reverses. The progressive melting of the external solution will begin above the eutectic point or zone, and will cause the external aqueous vapor pressure to rise above that of cells that have dehydrated during cooling. As a result, water will flow into the cell at a rate dependent on both the warming velocity and the permeability of the cell to water.

The velocity with which cell cultures or tissues are cooled or thawed is therefore important for survival. The optimum cooling velocity varies for different kinds of cells, but depends mainly on the cell's surface/volume ratio and on its permeability to water. The critical rate should be lower for larger spherical cells and for those less permeable to water than for smaller or more permeable cells. Thus, the unprotected mammalian red cell, highly sensitive to high concentrations of electrolyte and extremely permeable to water, exhibits optimum survival at a cooling velocity of about 3000^{o}C per minute (Mazur, 1969) in contrast to 10^{o}C per minute for yeast (Mazur, 1969) and mulberry parenchyma cells (Sakai and Otsuka, 1967).

On the other hand, slow warming is more deleterious to rapidly cooled cells than to slowly cooled cells. The influence of warming velocity is believed to be through its effect on recrystallization. Slow warming of rapidly cooled cells containing small non-spherical crystals of ice maximizes recrystallization and thus maximizes

injury (Mazur, 1966).

Based upon all the observations described above, Mazur and co-workers (1972) postulated a "Two Factor Hypothesis" of freezing injury:

1) Cells cooled at a rate faster than optimal are killed by the formation of intracellular ice during cooling and its subsequent growth by recrystallization during warming (especially during slow warming).

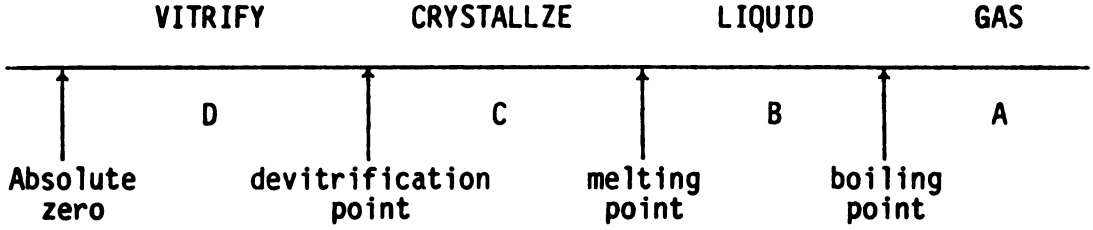
2) Cells cooled at a slower than optimal rate are killed by the relatively long exposure to the major alterations produced in extracellular and intracellular solutions by the conversion of extracellular water to ice. These alterations, called by Mazur, "solution effects", include the concentration of solutes, dehydration, changes in pH, and the precipitation of solutes.

There are microscopic and other forms of evidence that yeast cells and erythrocytes cooled at supraoptimal rates do in fact contain intracellular ice (Rapatz et al., 1966) and injury in erythrocytes at suboptimal rates is associated directly or indirectly with the high concentrations of electrolytes produced by ice formation (Meryman, 1970). Similar results have been obtained by Luyet and Keane (1955), Taylor (1960) and Sakai (1960 1965) using a slightly different cooling protocol. They noted that a variety of cells would survive rapid freezing to -70°C or below if freezing were carried out in two steps separated by a brief

holding period at an intermediate temperature. The cells included bull sperm, chick skin and plant parenchyma. The two-step freezing of mammalian cells has been investigated by other workers (Faurant, 1974, 1977; McGammetal, 1976; Walter et al., 1975). The intermediate holding temperature is critical. Sakai has proposed a partial explanation of the phenomenon: He suggests that the initial cooling to an intermediate temperature (e.g., $-30^{o}C$), in conjunction with the brief holding period, provides sufficient time for the cells to equilibrate by dehydration and extracellular freezing rather than by intracellular freezing. He proposed that $-30^{o}C$ is sufficiently low for nearly all freezable water to be removed from the cells by exosmosis. Thus, no intracellular freezing can occur during the subsequent rapid cooling to $-196^{o}C$. The cooling rate in the first cooling step may often be above the value that would have been required to produce intracellular ice if the tissue had been cooled to still lower temperatures. The other requirement for success may be that the intracellular holding temperature be above the intracellular nucleation temperature. According to this view, most of the dehydration of the cell would occur during the holding period at the intermediate temperature. If that period were long enough, the cells would have lost all freezable water prior to the second rapid cooling to less than $-70^{o}C$. Walter and co-workers (1975) and Farrant and co-workers (1977) have electron microscopic

evidence which directly supports this view.

A completely different picture of the events accompanying the cell's exposure to ultralow temperatures is that proposed by Luyet (1937). He demonstrated that protoplasts in leaves of Ligustrum vulgare enter the vitreous state if they are brought, without being previously crystalline, to the temperatures represented by zone D in the diagram below:



The only known method to vitrify a cell is to rapidly cool it so rapidly that it passes from the liquid into the vitreous state without being affected by the intermediate state. Two intrinsic factors control the attainment of the vitreous state: the velocity of crystallization and the size of the zone of crystallization temperatures. An additional, although extrinsic, factor is the cooling velocity.

The essential problem of the vitrification technique is therefore obtaining a cooling rate sufficient to prevent the formation of crystals. That is, the velocity of cooling must be greater than the velocity of crystallization. Due to slow transfer of heat through matter, the mass of the material must be minimal for rapid cooling. A medium of high viscosity is

essential to slow the diffusion of the molecules. Grout and co-workers (1978) invoked the vitrification hypothesis to explain the survival of potato shoot-tip cultures frozen at 1000°C per minute. Were Luyet's ideas supported by experimental evidence, vitrification of non-dehydrated protoplasm by rapid cooling might provide a useful approach for storing living matter at ultralow temperatures.

B. Mechanisms of freezing injury. Several current theories of the mechanism of freezing injury are referred to as "unitary" in that they ascribe all freezing injury to a simple physical or chemical event such as the attainment of a critical electrolyte concentration (Lovelock, 1953), the removal of "stabilizing water" (Meryman, 1967), the reduction of cell volume below a critical level (Meryman, 1968), the formation of disulfide bonds (Levitt, 1962), or ice adhesion (Olien, 1977). In essence, all of these theories relate freezing injury to dehydration. A detailed description of these theories is beyond the scope of the present work; however, a brief mention of their salient points and experimental support for them will reveal some of the problems in our understanding of freezing damage.

1. Theories of injury from intracellular freezing

As described previously, the death of cells cooled at supraoptimal rates is the result of the formation of intracellular ice crystals during cooling and their growth by recrystallization during warming.

The targets of injury in the cell are mainly enzymes, particularly those containing -SH groups (Levitt, 1966) and those localized in membranes. Consequently, processes that require membrane-localized enzymes will be harmed as freezing progresses. For example, phosphorylation in chloroplast membranes (Heber, 1964) and mitochondrial oxidative phosphorylation (Hausen, 1955) are inhibited by freezing. In addition to deactivation of the enzymes by conformational distortion, intracellular ice may kill the cell because, in an attempt to achieve its most stable state (large volume and spherical shape), sufficient forces may be produced to rupture organelle membranes (Mazur, 1966). This suggestion remains speculative. Farrant and co-workers (1977) have proposed an alternative mechanism. Here, injury from intracellular ice results from osmotic disruptions, especially when water appears during thawing.

2. Theories of injury from extracellular freezing

There is little agreement among the theories postulated to explain cellular damage by extracellular ice formation during cooling which is slow enough to allow dehydration. Most cryobiologists agree that extracellular ice does not injury by direct mechanical effects. Three major theories encompass this view by ascribing injury to the dehydration and concentration of solutes produced by freezing. Three other theories attribute injury to protein denaturation, disulfide bonds and ice adhesion.

a. Injury from dehydration. The dehydration that accompanies extracellular freezing results in: removal of liquid water, increased concentration of intra- and extracellular solutes, decreased volumes of cells, decreased spatial separation of macromolecules, precipitation of solutes and changes in pH. In no living cells have the six events been resolved to the point where their relative contributions to injury can be even approximately determined. However, instances of whole cells in which one or more causes can be excluded have been described, and there are cases of organelle injury where single causes can be identified (Mazur, 1969).

b. Injury from concentrated electrolytes. The most widely known and generally accepted theory of injury is that developed by Lovelock (1953) to account for freezing hemolysis of the red cell during freezing. It states that the injurious event is the concentration of intra- and extracellular electrolytes above a critical value. The theory accounts for many facts about the hemolysis of red cells by freezing. However, since the increase in salt concentration during freezing must be accompanied by a reduction in cell water content and cell volume, one cannot exclude the possibility that these are the factors actually responsible for hemolysis. Indeed, Meryman (1968) proposed that injury from solute concentration is not the result of toxic concentrations of any specific solute but is related to the osmotic

reduction of cell volume. His theory of "minimum cell volume" states that as the cell is osmotically dehydrated by extracellular ice formation, cell volume is reduced. Either because of forces induced by the volume reduction or from some direct osmotic effect on the cell membrane, a stress develops in the plasma membrane. Cell membranes can withstand such a stress only up to a distinct point, above which they collapse. Whether the collapse involves the development of a perforation through which cell contents escape or simply a loss of material from the membrane which itself remains intact is unknown. In plant cells, the latter appears to be the case. Garber and Steponkus (1976) have described the loss of a specific protein (coupling factor) from the chloroplast thylakoid membrane. Whatever the mechanism, this model proposes that injury during osmotic dehydration is basically physical damage to the plasma membrane.

c. Injury from thawing and rehydration. The effect of rehydration during thawing has not been clearly described. However, in a few cases, its role in freezing injury is clear. The bacteriophage T4B survives freezing much better when thawed slowly rather than rapidly. The basis of rapid thawing damage is osmotic shock (Leibo and Mazur, 1970).

d. Injury from low temperatures. This theory is based upon Brandt's hypothesis and demonstration (1967) that some proteins can be denatured at low temperature. Low temperature

denaturation is a consequence of the important role played by hydrophillic interactions in the stability of a protein's native tertiary structure.

e. The disulfide hypothesis of freezing injury. This theory (Levitt, 1962) ascribes injury to a combination of events during dehydration and rehydration. As water is removed from the cell during freezing, structural proteins are forced into closer proximity, and exposed sulfhydryl groups in adjoining proteins, or in adjoining strands of the same protein, may become linked by disulfide bonds. When water returns to the cell during thawing, competition between hydration forces and the newly formed disulfide bonds results in protein denaturation. Good evidence supports this theory despite a number of difficulties which will not be described here.

f. Injury from ice adhesion. According to this theory (Olien, 1974), extracellular ice may injure the cell by direct mechanical effects. During freezing, competition between ice and hydrophillic plant substances causes an energy of adhesion to develop through the interstitial liquid. As the amount of intercellular liquid diminishes, during the secondary growth of ice crystals, adhesion effects increase to the point of injuring tissues as the temperature decreases. Adhesions between ice, cell wall polymers and the plasmalemma form a complex interacting system in which the pattern of crystallization is a major factor

in the determination of stress and injury. Adhesions between ice and the plasmalemma prevent the cell from maintaining a minimum surface configuration as the skeletal crystals grow. The crystals deform the protoplast as they withdraw water and concentrate the solutes from the dehydrated cell on the crystal surface as an adhering layer. Distortion of the cell periphery between adjacent crystals is especially discernible and has been demonstrated in pure water as well as in solution of polymers which increase the ice crystals' interface. Effects of adhesion energy were demonstrated microscopically in conjunction with energies of crystallization and frost desiccation (Olien and Smith, 1977).

C. Protection against freezing.

1. Naturally occurring resistance to freezing.

Some animals and plants possess a property termed "hardness" which allows them to tolerate the cold temperatures of severe winters. In plants, environmental factors that reduce growth, such as low temperatures (0-5^{o}C), short photoperiods and water deficits, tend to induce hardiness.

Changes in hardiness have been correlated with changes in the levels of certain solutes, particularly sugars. The intracellular concentration of sugars usually increases during the hardening period, either because of hydrolysis of the starch accumulated over the summer in perennials, or an excess rate

of photosynthesis over respiration in winter annuals such as grains (Levitt, 1966). The opposite relation has also been reported (Levitt, 1966). That sugars can increase hardiness was shown by sugar feeding experiments (Chandler, 1913). These results could be explained if the protective effect of the sugars is osmotic in nature. Olien (1967) has also demonstrated a parallelism between the hardiness of cereal grains and the amount of soluble cell wall carbohydrate polymers extractable from crown tissues. These polymers interact with an advancing ice front as the polysaccharide solution flows across an ice interface.

In some insects, glycerol increases during hardening and acts as an antifreeze by favoring supercooling. Salt (1961) has suggested that perhaps all freezing-tolerant insects and possibly other invertebrates contain glycerol or some similar protective substance. The accumulation of glycerol during the hardening of insects and its disappearance during dehardening is apparently due to interconversions with glycogen (Takehara and Asahira, 1960a). Siminovitch and Briggs (1953, 1954, 1967) demonstrated a striking parallel between soluble protein content and frost hardiness in the cortical cells of trees both during hardening and dehardening. Similar data are available for grains (Johansson et al., 1955) and other plants (Li et al., 1966). Although these are the major components for which correlations between accumulation and hardiness exist, others that have been

studied include DNA (Jung et al., 1967), nicotinamide adenine dinucleotide (Kuraishi, et al., 1968), anthocyanins (Parker, 1962), certain organic acids (Li et al., 1966) and lipids (Siminovitch et al., 1967). Levitt and co-workers (1962) have reported a number of correlations, both positive and negative, between sulfhydryl and disulfide contents of tissues and their frost resistance. Usually sulfhydryl content increases during the early stages of hardening but sometimes decreases in later stages. In addition to these biochemical correlations, Levitt (1966) reported that hardier cells have a higher permeability to water than non-hardy cells.

Since a number of factors can produce injury during freezing, there may be a number of protective factors. Injury from high concentrations of electrolytes or of specific ion can be prevented by increased sucrose concentration during hardening, thus reducing the effective concentrations of the electrolytes. Proteins might also act by protecting sensitive sites on the membrane against the action of high levels of certain ions.

Meryman (1977) has reported several possible protective systems which are acceptable in terms of his theory of the "minimum cell volume" below which cells will be damaged. He proposed that hardening does not render the cell more resistant to osmotic dehydration but rather enables it to freeze to a lower temperature before achieving this same degree of cell dehydration. A number

of mechanisms could be responsible, including the synthesis of additional intracellular solutes, particularly carbohydrates, which increase the cell osmolarity. Thus, the extracellular osmolarity must be higher in order to achieve the same volume reduction. Johansson and co-workers (1970) showed this to be the case in wheat and Baust (1970) in insects. Another proposed mechanism involves the elaboration of bulky intracellular solutes which substantially increase the non-aqueous volume of the cell. When this occurs, more water must be removed from the cell in order to achieve a given reduction in cell volume (Meryman, 1974).

A third mechanism involves the "binding" of water. As the extracellular osmolality is inceased by the freezing out of water, the cell, in its osmotic response, behaves as though a portion of its intracellular water were no longer osmotically effective. The cell, at all plasmolytic osmolalities, is proportionately larger than would have been predicted and a lower freezing temperature is required to produce the same degree of osmotic plasmolysis as occurs in non-hardy cells (Williams et al., 1976). Apparent mechanical resistance represents a fourth mechanism. As freezing increases the extracellular osmolality, the cell initially behaves as an ideal osmometer, progressively plasmolzing as the extracellular osmolality increases. However, at some point above the killing temperature, volume reduction ceases despite a continuing increase in extracellular osmolality. The cell volume may remain unchanged despite a several-fold

increase in osmolality until the killing temperature is reached. This behavior has been reported both for plant cells (Williams et al., 1976) and for human blood platelets (Lindberg et al., 1972). A fifth mechanism that protects cells from freezing damage is membrane leakage. If the cell membrane becomes permeable to extracellular solutes, then extracellular solution can enter the cell during hyperosmotic exposure, preventing further volume reduction (Williams et al., 1970).

Olien (1965) has also explained how plants protect themselves from mechanical disruption caused by ice adhesion. He demonstrated that water soluble, cell wall carbohydrates of rye (Secale cereale L.) inhibit ice lattice formation during nonequilibrium freezing by interfering directly with ice at the ice-liquid interface. These carbohydrates compete with water molecules for sites on the advancing ice lattice. This affects the relationship between freezing velocity and crystallization energy, which, in turn, affects the size and destructiveness of ice crystals formed. Cell wall carbohydrates from barley (Hordeum vulgare L.) cultivars are less effective than those from rye cultivars.

2. Aritificially induced resistance to freezing

As mentioned above, Polge and co-workers (1949) first reported the protective effect of exogenous glycerol on animal cells subjected to low temperatures. Since then, many substances have been tested to protect cells from freezing damage. These

have been classified into two groups (Meryman, 1971). Penetrating cryoprotective agents are those which, at multimolar concentrations (2-4M), protect the living cell against injury from slow freezing. On the other hand, non-penetrating cryoprotective agents can protect in much lower concentrations and generally require a more rapid rate of freezing and thawing to confer protection.

a. Penetrating cryoprotective agents. Glycerol (Polge et al., 1949), dimethyl sulfoxide (DMSO; Lovelock and Bishop, 1959) and ethylene glycol (Sakai, 1960) are penetrating agents. The mechanism by which these compounds prevent injury is simply by reducing, on a purely colligative basis, the amount of ice formed, thereby reducing the concentration of cellular solutes. A compound's colligative characteristics make it more or less desirable as a cryoportective agent in two ways. First, its water-binding capacity can render it able to achieve cryoprotection with a lower concentration. Second, the elevated viscosity accompanying high concentration and reduced temperature can effectively reduce the final concentration achieved upon freezing, even though further concentration might theoretically be expected. Of course, were such a compound unable to penetrate the cell, it would dehydrate the cell osmotically and simulate freezing injury.

The rate at which various compounds penetrate cells varies not only with the agent but also with the type and origin of the cell. The movement of flycerol across an erythrocyte membrane

may occur by facilitated transport rather than simple diffusion (Hunter, 1970), which would explain the wide variability in penetration displayed by cells of different origins. This also helps to explain the temperature dependence (DeGrier et al., 1966) of glycerol flux.

One of the particularly attractive features of DMSO has been its rapid and apparently iniversal penetration into cells. With mouse ascites cells, equilibrium with 1M DMSO in isotonic Ringer's Solution is achieved within a few seconds at 37°C, in about 30 seconds at 22°C and in about 3 minutes at 5°C (Meryman, 1971). No reports are known in which DMSO failed to penetrate cells. Problems of penetrability are greatly magnified as one progresses from single cell suspensions to tissues and organs, where the agents must not only reach distant cells by diffusion through intervening cells, but must also be introduced initially by perfusion through a vascular system which may respond adversely to the osmotic gradients created (Meryman, 1968). In addition to their penetrability, these compounds must be non-toxic in the multimolar concentrations which are necessary to prevent excessive ice formation. When human erythrocytes were exposed for 60 minutes at room temperature to graded concentrations of cryoprotective agents which had been introduced in stepwise fashion to avoid osmotic damage. Methanol, ethanol, DMSO, dimethyl formamide and diethylene glycol all produced changes in cell

electrolyte distribution at concentrations comparable to those required for cryoprotection (Meryman 1971). Compounds which produced no changes in electrolyte content were glycerol (up to 10 M), ammonium acetate (6 M), trimethylamine acetate (3 M) and ethylene glycol (up to 8 M).

The non-toxicity of glycerol is supported by many observations of high concentrations of this compound elaborated as a natural cryoprotectant in insects. The most dramatic example is that reported by Baust (1969) of an Alaskan insect which accumulates glycerol to a whole body concentration of nearly 2.5 M and is uninjured by months of freezing at -40^{o}C.

Bajaj and Reinert (1977) reported that the viability of carrot cells exposed to DMSO concentrations of 5 to 10% was not significantly affected during a period of one hour at 3^{o}C. However, at 23^{o}C, survival declined with time. The nature of this toxicity has not been established.

b. Non-penetrating cryoprotective agents. The introduction into a cell suspension of an extracellular cryoprotective agent both reduces the maximum (optimum) tolerable cooling rate and increases the percentage recovery of cells. These effects were clearly demonstrated on addition of the non-penetrating agent polyvinylpyrrolidone (PVP) to a suspension of human erythrocytes prior to freezing (Rinfret et al., 1964).

Other non-penetrating compounds possessing cryoprotective activity include sugars and sugar alcohols such as sucrose,

lactose, glucose, mannitol and sorbitol. Some of the penetrating agents, such as glycerol and DMSO, may confer protection over and above their colligative function (Leibo et al., 1970).

The mechanism by which these agents confer protection has not been established. It is clearly dissimilar to that of the penetrating cryoprotective agents not only on the basis of non-penetrability but also because of the relatively low concentrations which are effective. PVP, for example, is almost fully protective for erythrocytes at a concentration of less than 3 mM. Lactose requires a higher concentration for effectiveness, approximately 450 mM, but still considerably short of the 2-4 M required for protection on a purely colligative basis (Meryman, 1977).

A possible mechanism for extracellular cryoprotection has been suggested by studies of the grana of spinach chloroplasts (Williams, 1970). The presence of extracellular cryoprotective agents including sucrose, sorbitol, a protein extracted from winter-hardened spinach, and PVP, permitted the grana to leak solute reversibly rather than to be dehydrated and reduced in volume below a minimum tolerated value. These data support the theory that the extracellular cryoprotective agents may confer protection by permitting a reversible influx and efflux of solute during freezing and thawing respectively, thus enabling the cells to avoid the otherwise irreversible effects of excessive osmotic gradients.

One is tempted to search for a biochemical mode of action for these cryoprotectants. However, Meryman and co-workers (1977) could find no evidence that biochemical mechanisms were involved in either freezing injury or cryoprotection. As mentioned above, they proposed a model which attributes freezing injury from extracellular ice to physical injury of the plasma membrane resulting from stresses produced by dehydration of the cell. They also suggested that cryoprotectants do not alter this endpoint, but confer protection in two other ways. They may act 1) colligatively, by reducing the amount of ice formed, and, 2) kinetically, by increasing the time required for water to leave the cell in response to a decreased vapor pressure of adjacent ice, probably by increasing the viscosity of the unfrozen solution outside the cell and, if the agent penetrates the cell, inside the cell as well.

The protective action of cell wall polymers on hardened plant cells led Olien and Smith (1977) to suggest that cryoprotectants should be evaluated with respect to the following specific activities:

a) control of crystal growth and redistribution;

b) prevention of injurious adhesions from forming between ice and cell structures especially the plasmalemma;

c) prevention of growth of ice from intercellular water into the protoplasm by stabilizing the plasmalemma, thereby

reducing the free energy of freezing across the plasmalemma. Such an evaluation might elucidate the mechanisms of action of these substances.

V Rationale for the Present Work

The objective of the work reported in this thesis was to develop a methodology for long term storage at supralow temperatures of meristematic tips from vegetatively propagated plants. Potato was chosen as appropriate experimental material since its cultured meristems freely regenerate whole plants and its germplasm is presently maintained at great cost and difficulty.

Many of the factors discussed in the foregoing literature review were considered in designing the experiments. Several rates of cooling and thawing were employed in combination with some of the factors that might confer protection against freezing injury. Three cryoprotective substances were tested at different concentrations and combinations. DMSO and glycerol were chosen as penetrating cryoprotectants, and sucrose as a non-penetrating one. In addition, hardening of the plant material prior to exposure to freezing temperatures was attempted by using short photoperiods. In other experiments, the meristematic explants themselves were subjected to hardening temperatures (0 to $4^{o}C$) for varying periods of time prior to freezing.

MATERIALS AND METHODS

I Meristem Tip Culture

Potato plants, Solanum tuberosum L. cvs. Russet Burbank and Katahdin were raised from tubers and maintained in a greenhouse under natural photoperiod with a day/night temperature regime of approximately $30^{o}C/15^{o}C$. The plants ranged in age from 10 to 75 days at the time of excision. The apical meristem was invariably floral in newly emergent plants. Therefore the apices were removed to release axillary buds from apical dominance. Vegetative meristematic apices were excised from axillary shoots and used as experimental material. The ontogenic age of these meristems was difficult to determine. Generally, the meristematic tip was assumed to be capable of regenerating a whole plant when the dome was fully hemispheric. The ability for regeneration decreased after this stage.

Apices of lateral shoots were stripped of their longer leaves, wrapped in small squares of cheesecloth and surface sterilized by soaking in a 0.25% sodium hypochlorite solution (laundry bleach diluted 20-fold and containing 0.1% sodium lauryl sulfate) for 5 minutes. The disinfected tissues were rinsed 3 times with sterile distilled water. Excess water was removed by blotting the tissues on sterilized paper in a clean plastic petri dish. Subsequently, the meristematic tip, composed of the dome and subjacent tissues including 2 or 3 leaf primordia

(approximately 0.8 mm in all), was excised with a sterile scalpel. The explants were placed in 60 x 15 mm plastic petri dishes containing 14 ml of semi-solid medium; the dishes were sealed with parafilm. Excision of the spices and transfer to petri dishes were carried out with the aid of a dissecting microscope under aseptic conditions in a laminar flow hood.

The cultures were placed at 23^{o}C with a light intensity of 1500 lux supplied by Sylvania Cool-White fluorescent lamps (Type FR96T12 CWVHO 135) and a photoperiod of 16 hours of light and 8 hours of darkness. In all future references to the growth room, this environmental regime is implied.

The basal culture medium (Tables 4 and 5) used was a modification of the medium of Murashige and Skoog (1962). Preliminary experiments indicated that gibberellic acid (GA) and naphthalene acetic acid (NAA) promoted development of cultured meristems. The medium was solidified with 0.9% (w/v) Difco Bacto agar. the pH of the salt solution was adjusted to 5.7 with KOH prior to autoclaving at 121^{o}C for 15 minutes. The salt solution plus agar was sterilized separately from the sucrose, were combined after autoclaving. The GA was filter-sterilized and added to the autoclaved medium while the NAA was autoclaved together with the salts.

II Hardening of the Plant Material

Prior to freezing plant material was subjected to several environmental regimes which are known to induce frost resistance

Table 4: Mineral salt composition of the Murashige and Skoog culture medium (1962)

Mineral Salt	Concentration	
	mg/l	Mm
NH_4NO_3	1650	20.0
KNO_3	1900	18.8
$CaCl_2 \cdot 2H_2O$	440	3.0
$MgSO_4 \cdot 7H_2O$	370	1.5
KH_2PO_4	170	1.2
H_3BO_3	6.2	0.1
$MnSO_4 \cdot H_2O$	16.8	0.1
$ZnSO_4 \cdot 7H_2O$	10.6	0.04
KI	0.83	0.005
$Na_2MoO_4 \cdot 2H_2O$	0.025	0.001
$CuSO_4 \cdot 5H_2O$	0.025	0.0001
$CoCl_2 \cdot 6H_2O$	0.025	0.0001
Na_2EDTA	37.3	0.1
$FeSO_4 \cdot 7H_2O$	27.8	0.1

Table 5: Organic constituents of the basal culture medium

Constituent	Concentration	
	mg/l	Mm
myo-inositol	100.0	0.56
Nicotinic acid	0.5	0.004
Thiamine-HCL	1.0	0.003
Pyridoxine-HCL	0.5	0.002
Naphthalene acetic acid	0.07	0.0004
Gibberellic acid	0.1	0.0003
Glycine	2.0	0.027
Sucrose	20,000	59.7

in certain plants. Except where otherwise indicated, controls were cultured as described in the preceding section.

A. Photoperiodic hardening of source plants. Potato plants were raised and maintained in the greenhouse under short day photoperiodic conditions of 8 hours of light and 16 hours of darkness. The 16 hour dark period was maintained by pulling a black cloth over the plants.

B. Hardening by exposure to chilling temperatures. Cultured shoot apices were held at $4^{o}C$ or $0^{o}C$ for 8 to 30 days.

C. Hardening by incubation in the dark. Petri dishes containing cultured shoot apices were wrapped with aluminum foil and placed in the growth room at $23^{o}C$ for 8 days prior to freezing.

D. Hardening by osmotic dehydration. Tissues were osmotically dehydrated in the presence of a non-metabolizable osmoticum. Mannitol was incorporated into the basal medium at 3.3 or 5.2% (w/v) and autoclaved with the salts and agar. Apices were cultured on mannitol medium for 9 to 10 days prior to freezing.

E. Acclimation to the in vitro environment. The length of the incubation period under controlled conditions was varied from 4 to 15 days prior to freezing.

III Freezing Procedure for Excised Shoot Tips

The technique for the freeze preservation of meristematic tip cultures and their subsequent regeneration into plants is

schematically represented in Figure 2.

A. Cryoprotectant application. After incubation under the conditions described in the previous section, shoot apices were transferred to screw-capped vials 16 x 72 mm containing 15 mm diameter filter paper disks impregnated with chilled liquid basal medium and cryoprotective substances.

Dimethyl sulfoxide (DMSO) and glycerol were chosen as penetrating cryoprotectants and sucrose as a non-penetrating one. These were tested alone at 5% (v/v) DMSO and 10, 15, 20, 25, and 30% (v/v) glycerol, and in combination as 2.5% (v/v) DMSO + 2.5% (v/v) glycerol + 6% (w/v) sucrose. This was the only cryoprotectant mixture employed and is designated the "mixture" henceforth. In all experiments, the cryoprotectants were incorporated into the liquid basal medium. These media were autoclaved at 121^{o}C for 15 minutes in batches of 50 ml each, in 125 ml Erlenmeyer flasks.

The distribution of cryoprotective solutions into vials as well as the transfer of the apices was carried out under aseptic conditions in a laminar flow hood.

For high concentrations, cryoprotectants were added in 4 steps over a period of 45 minutes to 1 hour, until the volume of cryoprotectant equalled that of the basal medium. This circumvented any damaging effects of osmotic shrinkage from exposure to a hypertonic solution.

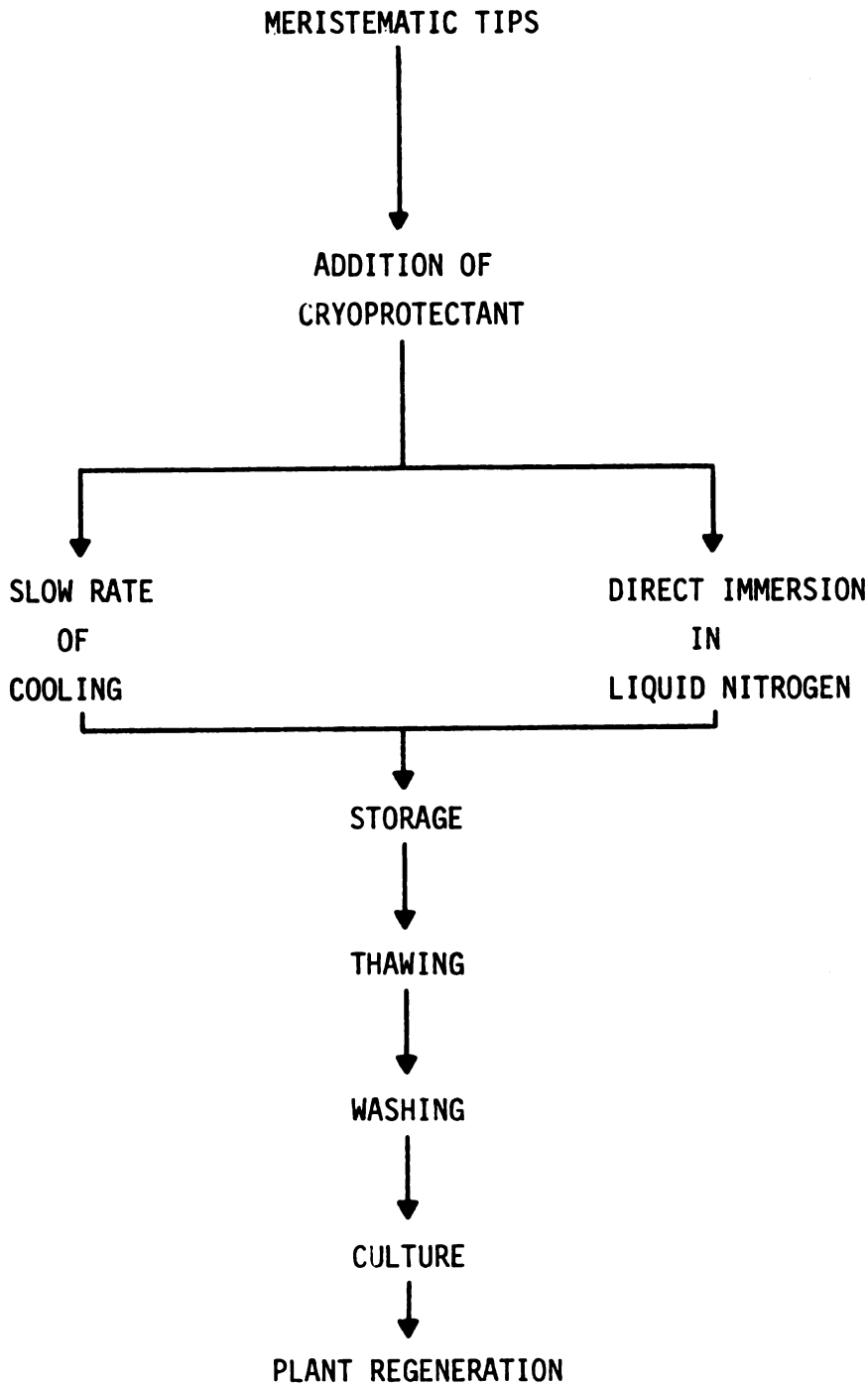


Figure 2: Plan of cryogenic freezing protocols

All non-frozen controls were inoculated with levels of cryoprotectant equal to those in the experimental vials.

Cultures containing cryoprotectants were kept in a cold room at $0^{o}C$ overnight (approximately 11 hours) because 1) low temperatures minimize injurious effects of cryoprotectants (Nag and Street, 1975a; Withers and Street, 1977), and, 2) the long exposure improves penetration of cryoprotectants particularly permeating ones such as glycerol. The incubation time was reduced to 2 hours for DMSO, which exhibits high toxicity and rapid penetration.

In one experiment, shoot tips were cultured in 60 x 15 mm petri dishes containing agar-solidified basal medium to which 5 or 10% (v/v) DMSO had been added. The explants were incubated for 48 hours in the growth room, then transferred to vials containing liquid basal medium with 5 or 10% (v/v) DMSO before freezing.

B. Freezing and thawing of the cultured apices. Both slow freezing at cooling rates from $0.8^{o}C$ to $21^{o}C$ per hour and rapid freezing by direct immersion in liquid nitrogen with rate approximately $60,000^{o}C$ per hour (Seibert, 1976) were used.

1. Slow rate of cooling

Vials containing shoot apices pretreated in cryoprotective solutions were transferred to a temperature-controlled freezer held at $0^{o}C$. Each vial contained a thin strip of filter paper against the wall and in direct contact with the filter paper

disk at the bottom, upon which the apices had been placed. The temperature was adjusted to 1 to 2°C below the estimated freezing point of the cryoprotective solution. At this point, vials were seeded by touching the outside of the vial with a sliver of dry ice,just at the end of the filter paper strip, until a white spot appeared inside. Ice nucleation thus occurred in the tissues without their being exposed to the dry ice. In this way, supercooling did not occur and ice crystal growth did not disrupt the tissue.

After seeding, the cooling rate was regulated by a timer connected to the freezer and temperature of the medium was monitored with a copper/constantan thermocouple. Samples were withdrawn from the freezer at 5°C intervals between -5°C and -30°C. When the freezer reached a temperature at which samples were to be removed, the appropriate vials were taken out and the temperature was permitted to fall to the next sampling temperature.

In some experiments, vials frozen to -30°C were plunged directly into liquid nitrogen (-196°C) for approximately 1 hour. Had greater survival been attained at -20°C to -30°C, then this final ultralow freezing step would have been included throughout. However, poor survival to -30°C precluded such a final step (see RESULTS AND DISCUSSION).

A total of 32 or 48 meristematic tips were cultured for each treatment. Two or 3 were placed in each vial and two replicate vials were prepared for each temperature at which vials

were to be removed from the freezer.

2. Direct immersion in liquid nitrogen

Vials containing the explants were transferred to an open Dewar flask filled with liquid nitrogen where they remained for 0.5 to 24 hours. In some instances, the tips were not placed on the filter paper disks in the vials, but were suspended in 0.5 ml of the cryoprotective solution as reported carnation shoot tips (Seibert, 1976).

3. Storage in the frozen state

Long term storage requires the use of temperatures sufficiently low to stop cell metabolic activity. In other systems, the temperature of liquid nitrogen has been sufficient in this regard.

For short-term storage, frozen meristematic tips were either transferred from the freezer to liquid nitrogen (for slow rate of cooling) or placed directly in liquid nitrogen (fast rate of cooling). Vials were suspended in the liquid nitrogen by a wire.

The period of time in liquid nitrogen was never shorter than 30 minutes, this being the assumed time for temperature equilibration.

4. Thawing of frozen tissues

Both slow and fast rates of thawing were tested. The fast rate was achieved by simply plunging the vials into an insulated

vessel containing water at 37 or 40°C. As soon as the ice covering the filter paper melted (1-2 minutes), the vials were transferred to air at room temperature.

For slow thawing the vials were transferred either from the liquid nitrogen or from the freezer at the selected temperature to air at room temperature. The time required for thawing of the ice on the filter paper was 15 to 30 minutes.

In a few cases, when tissues had been exposed to temperatures higher than -30°C, slower rates of thawing were tested. Vials were wrapped with cotton wool and a plastic bag, and held at 5°C before transferring to room temperature.

5. Rinsing of thawed tissues

The cryoprotective solution was washed from the experimental tissues by diluting it with the addition of 3 to 4 ml of fresh liquid basal medium to the vials after thawing. Following exposure to high concentrations, the cryoprotectant was diluted stepwise to avoid injury.

The period of time during which the explants were exposed to the rinsing solution was approximately 30 minutes. The shoot tips were then transferred to plastic petri dishes containing agar-solidified basal medium.

C. Assessment of freezing injury. The cultures were placed in the growth room and development of the tissues was observed. Shoot apices which showed signs of growth, differentiation or callus formation were designated as survivors. Differentiating apices were defined as those which formed new leaf primordia or shoots.

When regenerating plantlets were sufficiently large, they were removed to plastic pots containing sterile soil and placed under conditions identical to those in the growth room. Plantlets attaining a height of 5 cm (usually after about 3 months in culture) were deemed suitable for potting.

IV Freezing of Intact Seedlings

A set of experiments was carried out using a slight different protocol than that outlined above and in Figure 2.

F_1 seeds from the crosses Solanum tuberosum cv. Gineke (dihaploid 69-G-609) X Solanum phureja cv. IvP1 and S. tuberosum cv. Atlantic X S. tuberosum cv. Wischip-1 were surface sterilized in 3% sodium hpochlorite and germinated on moist filter paper under aseptic conditions in the dark at 20°C. The seedlings were selected for freezing when the emergent radicle had attained a length of 18 to 20 mm. Intact seedlings were then placed over filter paper in petri dishes containing liquid basal medium which had been cooled to the temperature of melting

ice. The seedlings were then transferred stepwise, at intervals of 30 minutes, through liquid basal medium containing 2.5, 5.0, 10 and 15% (v/v) DMSO and placed in small vials, made from single or double layers of domestic aluminum foil, containing 1 ml of the final medium. One of two freezing methods was then used: 1) rapid cooling by direct immersion of the vials into liquid nitrogen; or, 2) more gradual cooling by suspending the vials for 15 minutes in the vapor 7.0 cm above the surface of liquid nitrogen contained in a wide-necked Dewar flask, followed by immersion into the liquid nitrogen.

A total of 100 seedlings were frozen by each of the above methods. The vials were stored in liquid nitrogen for a minimum of 1 hour before thawing. The frozen seedlings were thawed by removing the vials from the liquid nitrogen and immediately plunging them into water at $40^{o}C$ for 1 to 2 minutes. The seedlings were then rinsed three times in fresh liquid basal medium.

After rinsing, whole seedlings and shoot apices dissected from the seedlings were cultured in 60 x 15 mm petri dishes over filter paper impregnated with basal liquid medium. The nonfrozen controls were rinsed and cultured on the same medium. Culture conditions were the same as those described in the previous section, except that liquid basal medium was added to petri dishes when needed in order to keep the filter paper

continuously moist. Survival was estimated as visible growth in culture.

A variant of this procedure was also tested, in an attempt to maximize the rate of cooling. Once the seedlings underwent the DMSO pretreatment, they were separated into groups of 10, and, by means of long forceps, were plunged directly into liquid nitrogen, unprotected by a vial or aluminum foil. For thawing, the seedlings were transferred from the liquid nitrogen to liquid basal medium at 37^{o}C for 1 minute, then rinsed in the same medium at room temperature. Culture of the tissues was the same as described above.

RESULTS AND DISCUSSION

I *In Vitro* Development of Unfrozen Meristematic Tips

A. *Development of untreated meristems in vitro*. When the culture medium used by Mellor and Stace-Smith (1969) for potato shoot tip regeneration was tested with the cultivars Katahdin and Russet Burbank, regeneration and rooting were limited and development was slow. Gibberellic acid (GA) and naphthalene acetic acid (NAA) were therefore incorporated into the basal medium. Morel (1964) found gibberellic acid to be active in promoting potato meristem development, suppressing the formation of callus and enabling the excised meristem tip to form a normal stem. Low concentrations of NAA stimulate early root formation and increase rooting in plantlets when potato meristem tips are cultured in GA-supplemented medium (Pennazio and Vecchiati, 1976). In addition, the sucrose concentration was reduced from 3 to 2% (w/v).

Sixty percent of the meristematic tips of approximately 0.8 mm in size developed into small plantlets on the modified medium (Tables 4 and 5). After three months *in vitro*, the meristem tip attained a large enough size (5 cm) to be transferred to soil. There was virtually no loss of plantlets following transfer.

No significant differences were observed between Katahdin and Russet Burbank cultivars in the ability of meristems to

regenerate whole plants or tolerate freezing temperatures. Figure 3 shows a Russet Burbank plant regenerated from a cultured meristematic tip, 8 months after transfer to soil.

B. Effects of hardening pretreatments on meristem tip regeneration.

1. Short-day photoperiod

The number of axillary buds developing following decapitation was lower under short days than under long days. No differences were observed between short and long day treated meristems in ability to develop shoots and roots.

2. Mannitol

Meristematic tips cultured on basal medium supplemented with mannitol at either 3.3 or 5.2% (w/v) did not develop normally. Cell divisions occurred, but resulted in the formation of globular masses 2 to 3 mm in diameter at the base of the tip. In a few cases, tiny rosetted leaves developed.

3. Darkness

Shoot tips cultured on basal medium and incubated in the dark at 23^{o}C for 8 days lacked chlorophyll and failed to grow. However, growth and greening occurred on exposure to light, and plantlets developed.

4. Chilling

Shoot tips cultured on basal medium in the dark at either

Figure 3: Russet Burbank plant regenerated from a cultured meristematic tip.

$0^{o}C$ or $4^{o}C$, remained dormant. Growth and regenerating capacity were restored upon transfer to $23^{o}C$ in the light. The ability to recover decreased with increasing time of exposure to chilling temperatures (Table 6, Figure 11). Almost no regeneration occurred after 30 days at $4^{o}C$.

C. Effects of cryoprotectants on meristem tip regeneration.

Cryoprotectants were never significantly detrimental to viability or to regeneration at any of the concentrations or incubation periods used (Table 7).

DMSO permeates cells very rapidly. The mechanism of its transport is unknown, but the compound's rapid penetration indicates that membrane integrity may be disturbed. The potentially toxic effect of DMSO was minimized by keeping the explants at chilling temperatures during incubation (2 hours).

Following exposure to 5% DMSO for 2 hours at $0^{o}C$, 50% of the explants regenerated, an insignificant decline from the control level of 60% (Table 7). Similar results were obtained (see below) when explants remained in the cryoportective solutions up to 12 hours.

Regeneration of shoottips was unaffected when donor seedlings were incubated in 2.5, 5.0, 10 or 15% DMSO for 2 to 4 hours. This high recovery rate is attributable to two factors. First, larger explants were taken from the seedlings than from the

Table 6: Effect of length of chilling period on recovery of meristematic tips

Days at 4°C	Meristematic tips (# regenerated/# cultured)	% Recovery
8	8/16	50
15	3/12	25
21	4/12	33
30	0/12	0
40	1/12	8

Table 7: Effect of cryoprotectants on recovery of unfrozen meristematic tips from different sources

Cryoprotectant	% Regeneration of meristems from:	
	Greenhouse-grown plants	Seedlings cultured *in vitro*
Untreated	60	100
5% DMSO	50	100
2.5% DMSO+ 2.5% glycerol+ 10% sucrose	60	---
10% Glycerol	15-50	---

greenhouse-grown plants because the meristems of the seedlings were extremely small. The supporting tissue which was inevitably explanted along with it contributed to regenerability. Secondly, the developmental state of the seedling meristems was unifrom and controllable, whereas that of greenhouse-grown plants was variable and unpredictable.

When the cryoprotectant mixture or 10% glycerol alone was used in freezing experiment, incubation time was increased to approximately 11 hours to allow for penetration. Only meristematic tips from greenhouse-grown plants were tested for sensitivity to the cryoprotectant mixture and glycerol. The mixture did not affect regeneration (Table 7, Figure 4), but glycerol alone reduced regeneration 50 to 85% (Table 7, Figure 5). Since the regenerative capacity was unaffected in at least one case, the low percentage of regeneration in other instances may have been due to inappropriate age of the donor plants or mechanical injury.

Experiments using 15, 20 and 30% (v/v) glycerol are currently in progress. Regeneration has not been observed, but all of the explants are alive and growing.

II Effects of Rate of Cooling

A. Direct immersion in liquid nitrogen. Based on Seibert's (1976) report of shoot initiation from frozen carnation shoot apices, my first attempt employed a fast rate of cooling by

Figure 4

Figure 5

direct immersion in liquid nitrogen.

Thirty meristem tips from plants grown under 8-hour days and 16-hour nights were incubated on solidified basal medium for 4 days in the dark at 23°C, then placed in vials containing 0.5 ml of 5% DMSO. Liquid nitrogen was poured into the vials, which were then immersed in liquid nitrogen. The rate of cooling is 1000°C per minute under these conditions (Seibert, 1976). Apices were thawed by plunging the vials into a water bath at 37°C. Storage, rinsing and viability assessment were carried out as described in the MATERIALS AND METHODS. This experiment was repeated 3 times but none of the treated tips showed any signs of life, even after 4 months in culture. The thawed apices were kept in culture for this period of time to ensure that a lag phase in recovery did not occur as reported by Bajaj (1976), Nag and Street (1973), and Withers and Street (1977).

The lack of success with Seibert's technique led to a number of modifications in the technique:

1) The apices were precultured on solidified basal medium containing 5% DMSO for 48 hours prior to freezing, a treatment indispensible for survival of pea meristems (O. L. Gamborg, personal communication).

2) The apices were placed on filter paper impregnated with the cryoprotective solution to reduce the probability

of ice puncturing the tissue from outside during freezing.

3) Other cryoprotectants including the mixture and 10% glycerol were tried.

4) Meristematic tips were subjected to hardening pretreatments such as chilling temperatures and mannitol, previous to the application of cryoprotectants.

5) Whole seedlings rather than isolated tips were frozen in liquid nitrogen either naked or enclosed in aluminum foil vials. Such meristems would be better protected by surrounding tissues and thus spared the stress of excision. Also the cooling rate was somewhat slower when the seedlings were enclosed in vials. The effect of the precipitous drop in temperature on the vapor pressure in the immediate vicinity of the tissues depends upon whether tissues are a) enclosed in a vial versus naked or b) bathed in liquid nitrogen versus only subjected to its vapor.

6) Theoretically slow thawing reduces the chance of survival in rapidly frozen tissues. Nevertheless, in a few instances the tissues were thawed at room temperature to test whether the thawing protocol was responsible for injury.

None of these changes in Seibert's protocol increased survival. The shoot tips remained green for 1 or 2 days but then turned brown. No sign of survival was evident even after 3 or 4 months. Because unfrozen controls included in each

of the modified experiments survived, the tissues were damaged by the freezing regime itself. Because the cooling rate exceeded the optimal rate (see INTRODUCTION), cells probably did not dehydrate to the extent required to maintain equilibrium between the intra- and extra-cellular aqueous vapor pressures and fatal intracellular freezing took place. The protoplast probably did not enter the vitreous state without being previously crystalline (after Grout et al., 1978).

B. Slow rates of cooling. To determine whether dehydration during the early stages of slow freezing would permit survival, the effects of slower rates of cooling and changes in other parameters were studied.

With subsequent experiments, I first attempted to freeze the tissues to a temperature (approximately -30°C; Sakai, 1965) at which all of the water remaining in the cells would be bound. Presumably no intracellular ice would form if the tissues were subsequently transferred to supralow temperature (-196°C).

1. Determination of the optimal rate of cooling

In preliminary experiments, the optimal rate of cooling for freezing potato shoot tips was determined. Temperature was manually controlled. Inadequacies in equipment caused large variances between experiments. Data for only the most reliable experiments are presented.

Three groups of 56 shoot tips each from plants grown under long days were incubated for 4 days on semisolid basal medium, then transferred to the cryoprotectant mixture where they remained overnight at $0^{o}C$. Freezing rates were $0.8^{o}C$, $1.7^{o}C$, and $4.7^{o}C$ per hour. Vials, each containing four tips, were seeded at $-5^{o}C$ and samples were removed from the freezer at $5^{o}C$ intervals and rapidly thawed. Rinsing and viability assessment were carried out as described in the MATERIALS AND METHODS.

Survival was maximum with slower rates of cooling (Figure 6), although there was no appreciable difference between 0.8 and $1.7^{o}C$ per hour. Tissues survived the presence of ice for a period of approximately 6 hours after seeding. Therefore, explants might tolerate further organized, however slow, growth of ice crystals if certain parameters, such as type and concentration of cryoprotectant, hardening pretreatment, or thawing rate, were changed.

2. Effect of pretreatments on freezing tolerance

The cooling rate chosen was approximately $1.7^{o}C$ per hour but fluctuated with ambient temperature changes. Room temperature was not constant, and frequent opening and closing of the freezer door was required.

The effects of several pretreatments, individually and in combination, were evaluated.

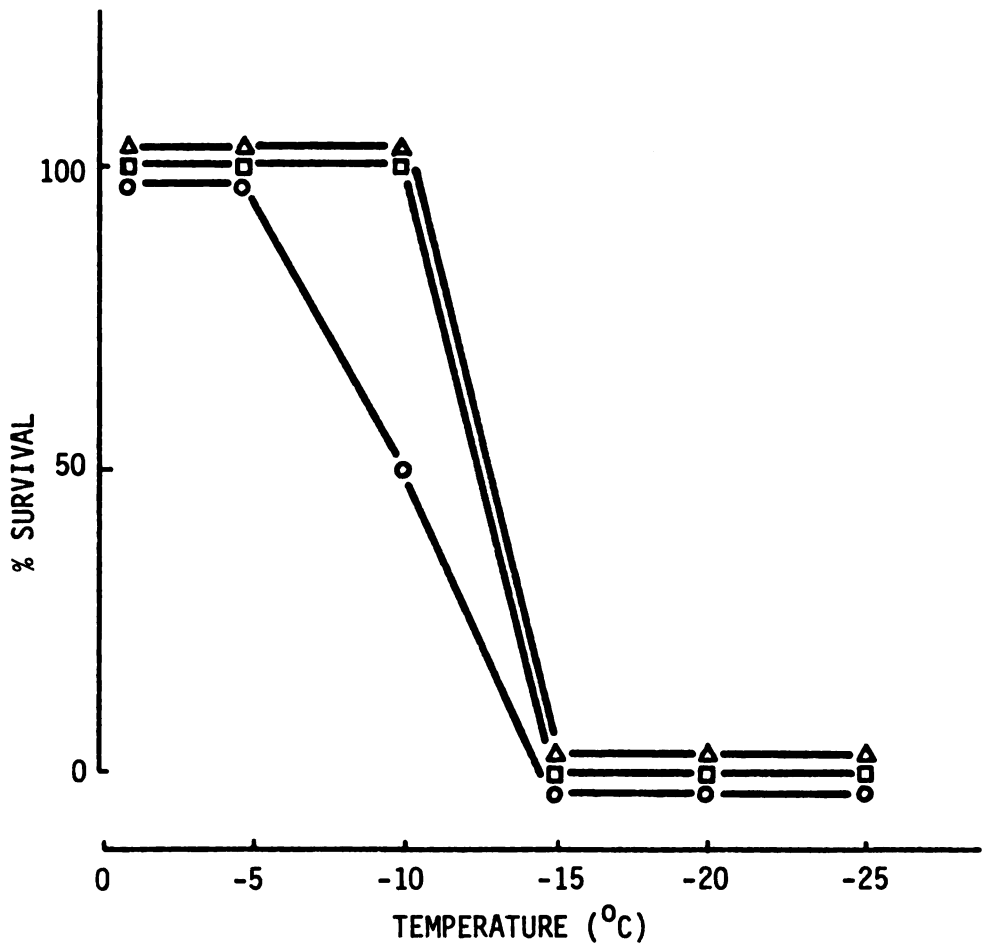


Figure 6: Effect of cooling rate on the percentage survival of meristems. □ : 0.8°C per hour; △ : 1.7°C per hour; ○ : 4.7°C per hour.

a. Effect of growing source plants under short days. Meristems from short and long day treated plants were frozen under identical conditions (Figure 7). In each experiment, the cryoprotectant was 10% glycerol, the cooling rate was 1.7°C per hour and the thawing rate was rapid. The experiments differed in the periods of preincubation of the cultures at 23°C and 4°C. In no case did survival of tips from short day treated plants exceed that of tips from long day treated plants. Indeed, short day treatment may have reduced hardiness. Prolonged incubation at 4°C appeared to reduce the weakening effect of the short day treatment (Figure 7c). This cold pretreatment effect is also seen in long day treated tissues (Figure 7c). Preincubation in the growth room seemed to reduce survival of explants exposed to -10°C (see below).

b. Effect of dark pretreatment. Half of the 84 shoot tips from plants grown under long days were precultured on semisolid basal medium in the dark for 8 days at 23°C whereas the other half were precultured in the light for 4 days at 23°C. They were then frozen in 10% glycerol at 1.7°C per hour and rapidly thawed.

In a first experiment, the dark pretreatment increased survival at -5°C, but not at -10°C (Figure 8a). Since the total preculture period was twice as long for the dark treatment as for the control, conclusions must be tentative. However,

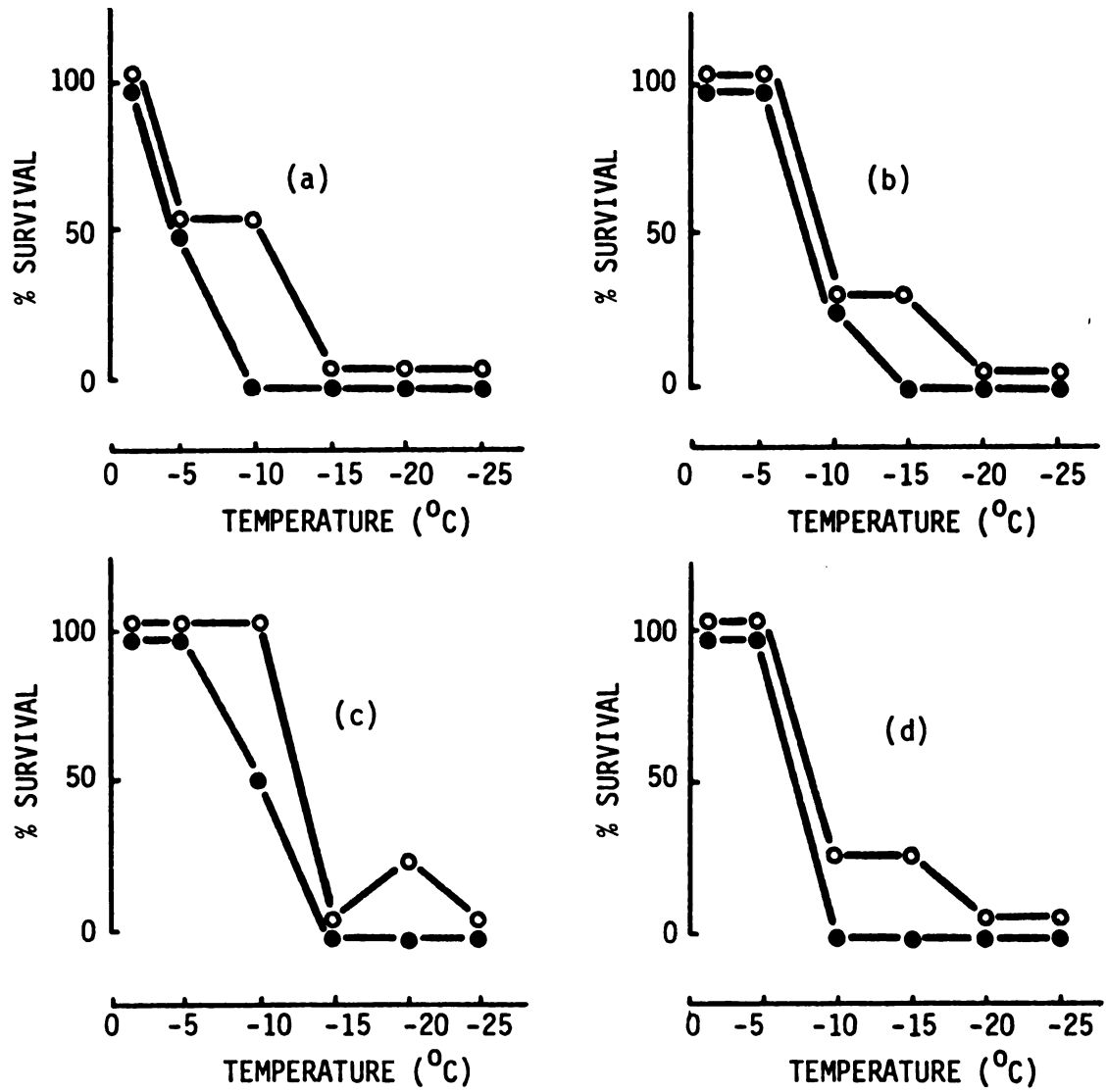


Figure 7: Effect of exposure of source plants to short (●) vs. long (○) photoperiods on survival of meristems following freezing. (a) 4 days at 23°C; (b) 8 days at 23°C; (c) 15 days at 4°C; (d) 4 days at 23°C + 15 days at 4°C.

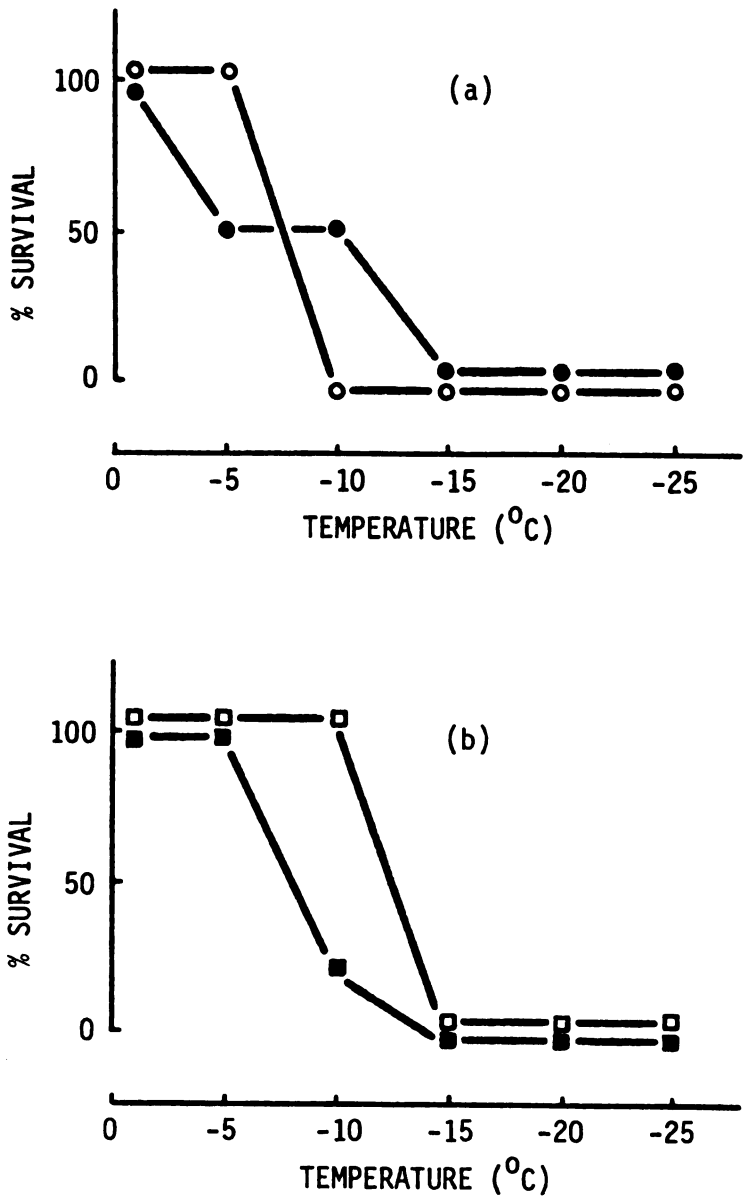


Figure 8: Effect of light and temperature during preincubation on survival of meristems following freezing. (a) o: 8 days dark (23°C); ●: 4 days light (23°C). (b) ■: 8 days cold (4°C); □: 8 days dark (23°C) + 8 days cold (4°C).

in subsequent experiments (see below), no effect of length of preculture was observed.

In a second experiment (Figure 8b), two groups of 42 shoot tips each from long day plants were precultured on semisolid basal medium. One group was precultured for 8 days in the dark (at 23°C) followed by 8 days in the cold room (at 4°C), the other for 8 days in the cold room only. Again, the cryoprotectant was 10% glycerol, the freezing rate was 1.7°C per hour and thawing was rapid. The dark pretreatment seemed to reduce hardiness, a reduction in survival occurring at -10°C. Preculture in the dark, therefore, does not increase hardiness.

c. Effect of pretreatment temperature. Shoot tips were precultured at 4°C for 8, 15, and 30 days prior to freezing. The cryoprotectant was 10% glycerol, the freezing rate was 1.7°C per hour and thawing was rapid. Control apices were precultured for 4 days at 23°C.

The 8- and 15-day cold pretreatment prevented injury at -10°C, although 25% of the cultures held at 4°C for 15 days survived -20°C (Figure 9). This was the best result obtained thus far. A meristematic tip that survived -21.5°C after being precultured in the cold room (4°C) for 15 days had developed roots but not shoots after 3 months (Figure 10). A 15-day cold pretreatment resulted in 100% and 25% survival at -10°C and -20°C, respectively, (Figure 7c), further

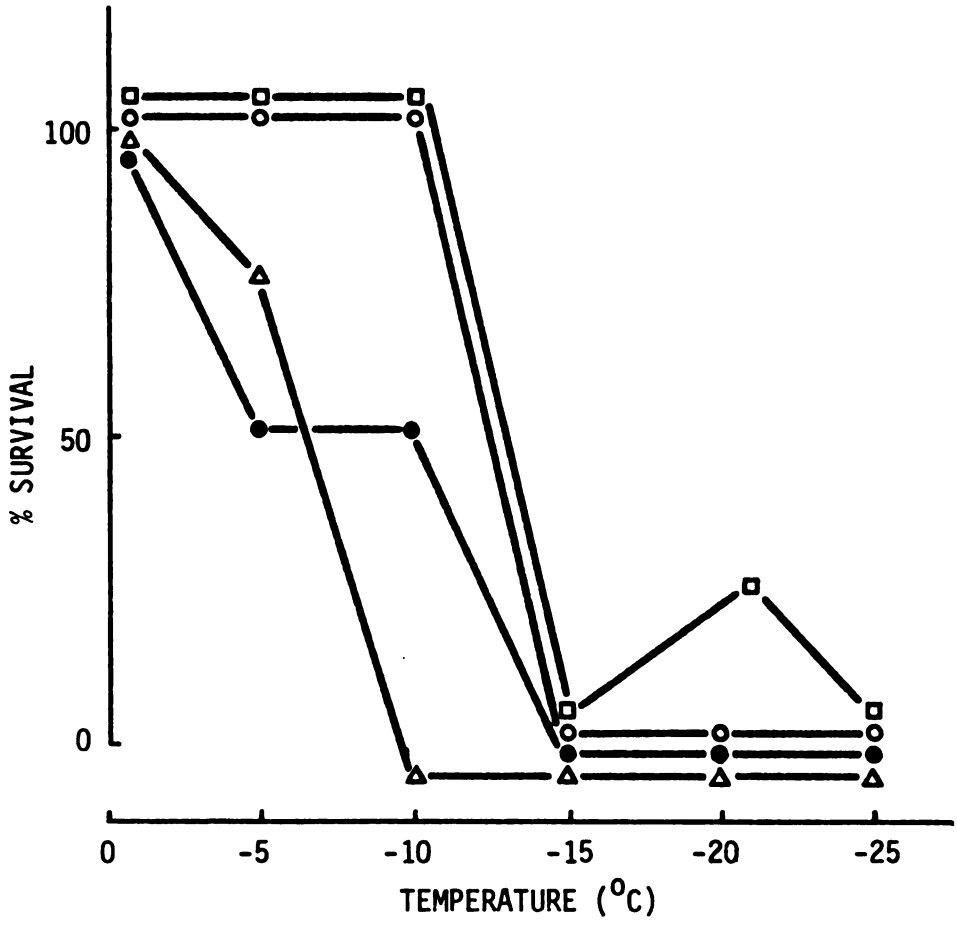


Figure 9: Effect of length of cold pretreatment on survival of meristems following freezing. ● : 0 days at 4°C; □ : 8 days at 4°C; ○ : 15 days at 4°C; △ : 30 days at 4°C.

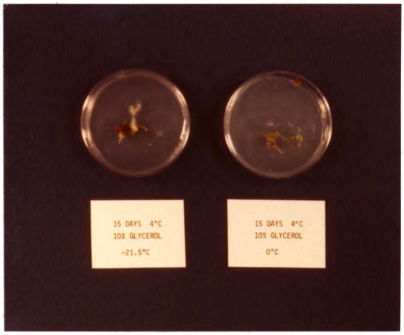


Figure 10: Survival of meristematic tips precultured for 15 days at $4^{o}C$.

indicating that cold pretreatment enhances hardiness.

The poor survival of 30-day cold-treated tissues supports previous results (Table 6). A significant loss of regenerability followed such a cold treatment, even in the absence of freezing. Chilling for a period of 15 days promotes hardiness. Viability then declines. Stress can thus improve one desirable characteristic while reducing another. This interaction is illustrated in Figure 11.

The increase in survival at -20°C indicates that the cold pretreatment hardened the cells by one or more of the mechanisms discussed in the INTRODUCTION: increase in the cell membrane permeability, accumulation of sugars within the protoplast, or cell dehydration.

d. Effect of preculture in the presence of mannitol. Because there is an inverse relationship between cell size and capacity to survive (Withers and Street, 1977), gentle plasmolysis of meristematic cells might enhance their survival. A medium containing a high concentration of an osmoticum such as mannitol could dehydrate and shrink cells placed in contact with it. Withers and Street (1977) reported higher survival of Acer pseudoplatanus and Capsicum annuum cells when 3.3 and 5.7% (w/v) mannitol, respectively, were incorporated into the culture medium. Therefore, these two concentrations of mannitol were tested. Although the meristematic tips so treated

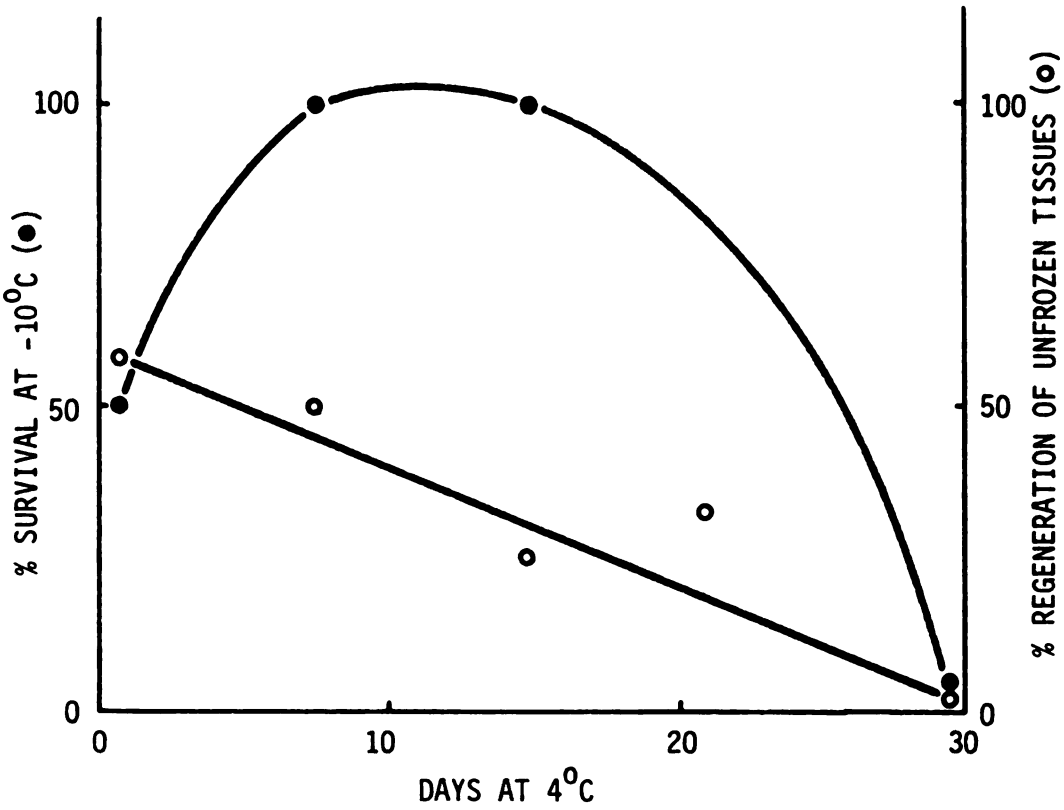


Figure 11: Effect of chilling pretreatments on freezing survival and regenerability of unfrozen shoot tips.

rapidly developed abnormal growths at their bases they were subjected to freezing protocols.

The effect of mannitol was tested individually and in combination with some of the other pretreatments discussed above. Shoot tips precultured for 10 days on basal medium containing 3.3% (w/v) mannitol and either 10% glycerol or the cryoprotectant mixture were frozen at a rate of 1.5°C per hour. Samples were removed from the freezer at 5°C intervals and rapidly thawed. Forty percent of the meristems sampled at -5°C survived when the cryoprotectant mixture was employed, but none survived lower temperatures than -5°C. No survival was observed when glycerol was the cryoprotectant. The experiment was repeated with shoot tips pregrown in medium containing 5.2% (w/v) mannitol. None survived after seeding the vials at -5°C.

e. Acclimation to the in vitro environment. As mentioned in the MATERIALS AND METHODS, the preincubation on basal medium could overcome one or more of the stresses which may be reducing ability to harden.

To test this hypothesis, shoot tips were cultured on basal medium in the growth room for 4, 7 and 15 days prior to freezing at a rate of -1.7°C per hour. The cryoprotectant used was 10% glycerol and thawing was rapid. Survival was not affected by prolonging the preincubation period, all results

being virtually the same as those shown for the long day tissues in Figure 7a. No survivors were recovered at temperatures lower than -10°C.

3. Comparison of cryoprotective compounds

Some slow freezing experiments were carried out to compare the protective effects of various cryoprotectants. In the first experiment, three groups of 42 shoot tips each were precultured on semisolid basal medium for 4 days in the growth room. Each group was then incubated in either 5% DMSO, 10% glycerol,or the cryoprotectant mixture for 2, 11 andll hours respectively. The cooling rate was 1.7°C per hour and thawing was rapid. Protection by 10% glycerol was clearly superior to that of 5% DMSO or the mixture (Figure 12a).

In a similar experiment, shoot tips were obtained from short day grown plants. Dissected apices were precultured for 8 days in the dark followed by 8 days in the cold room (4°C), and the rate of cooling was 0.8°C per hour. Both 10% glycerol and the cryoportectant mixture were more effective than 5% DMSO, but glycerol's advantage was not as clear as in the previous experiment.

To unequivocally establish the cryoprotective capacity of glycerol in this system, another experiment was designed. Meristematic tips from short and long day plants, not pretreated were placed in the vials containing 10% glycerol. Control

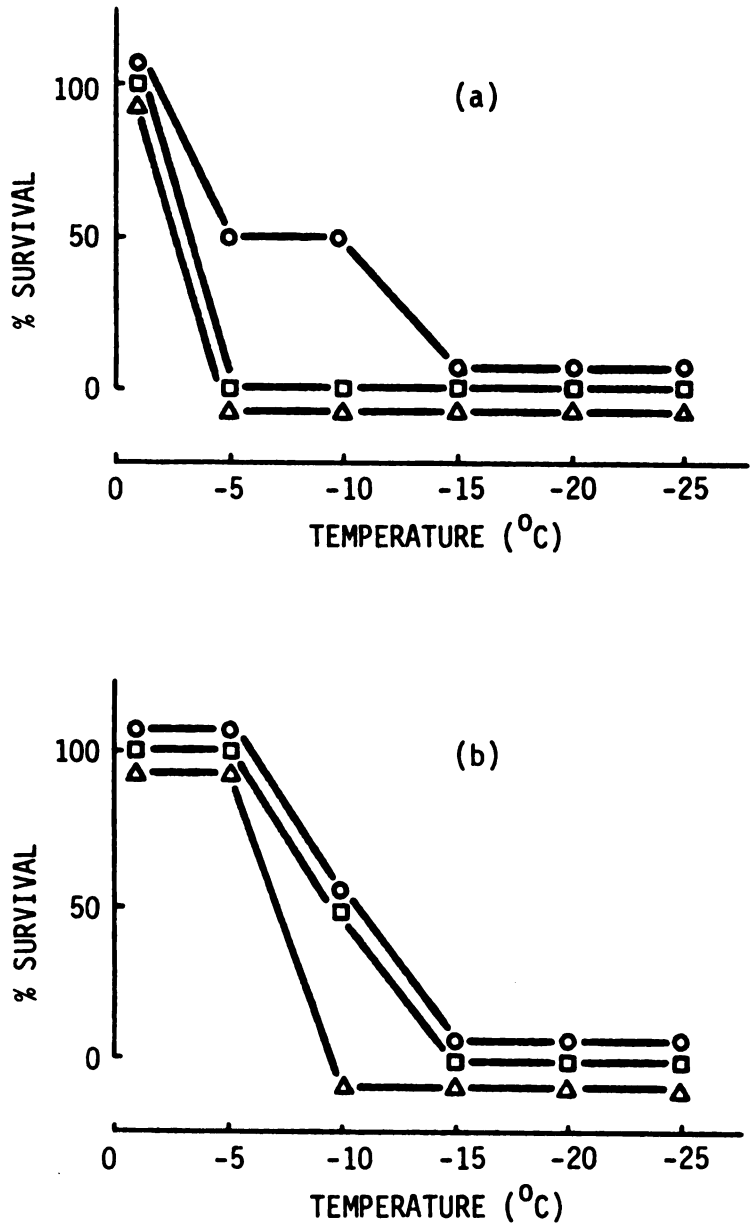


Figure 12: Effect of cryoprotective solutions on survival of meristematic tips: (a) 4 days preincubation at 23°C, frozen at 1.7°C per hour; (b) short day derived apices, preincubated for 8 days in the dark and 8 days at 4°C, frozen at 0.8°C per hour. o : 10% glycerol; □ : Mixture; Δ : 5% DMSO.

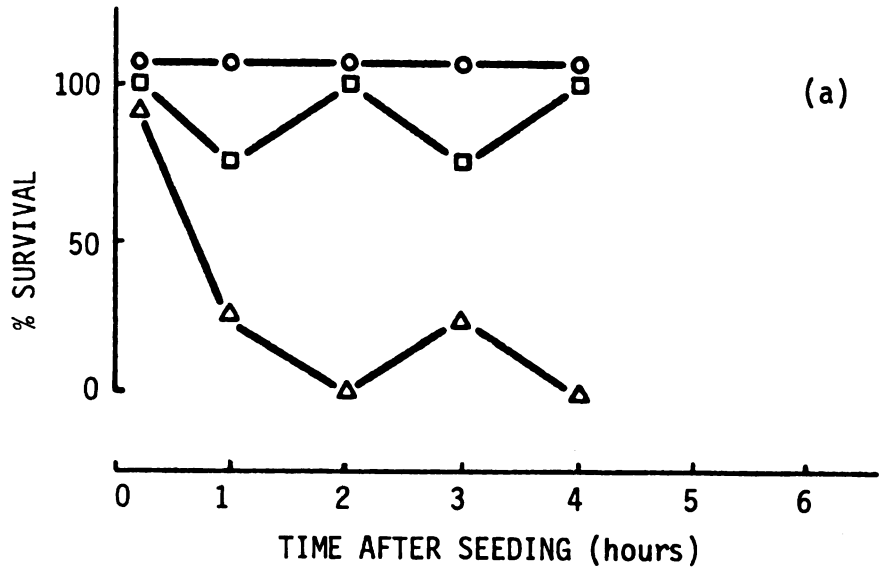
tips were placed in vials containing basal liquid medium without cryoprotectant. Freezing was carried out at 1.5°C per hour and thawing was rapid. The results of this experiment (Table 8) clearly indicate the protective effect of 10% glycerol. These data also confirm that long day treatment of source plants increases hardiness when cryoprotectants are used.

Although these experiments indicated the superior protective effect of glycerol, the range of temperatures through which comparisons could be made was limited to that bounded by the seeding temperature (-5°C) and that temperature which was invariably damaging, regardless of the treatment (-5 to -15°C). To overcome this limitation and to assure extracellular ice nucleation deep within the tissues, a new set of experiments was designed.

Shoot tips from long day grown plants cultured on basal medium for 4 days in the growth room were separated into three groups. Each group was incubated in 10% glycerol, the cryoprotectant mixture or 5% DMSO. After seeding the vials at -5°C, the temperature was kept constant and samples were removed at one hour intervals and thawed rapidly. Glycerol maintained the tissues' viability for up to 4 hours following seeding (Figure 13a). DMSO was not effective and the mixture's performance was variable. Figure 14 shows the growth of the meristematic tips one month after thawing.

Table 8: Effect of glycerol on hardiness of meristems excised from source plants grown under different day length regimes.

Treatment of source plants	Cryoprotectants	% Surviving meristems at 0°C	-5°C	-10°C
Long days	10% glycerol	100	100	50
	none	100	0	0
Short days	10% glycerol	100	50	0
	none	100	0	0



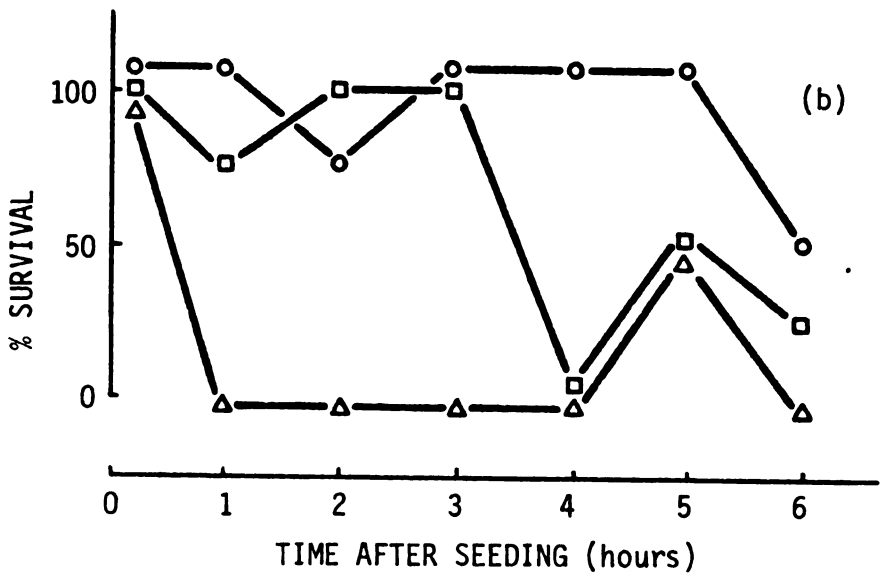


Figure 13: Relative protective effects of 3 cryoprotectants during a prolonged period at -5°C: (a) preculture for 4 days in growth room; (b) preculture for 4 days in the dark + 21 days at 4°C. o : 10% glycerol; □ : mixture; Δ : 5% DMSO.

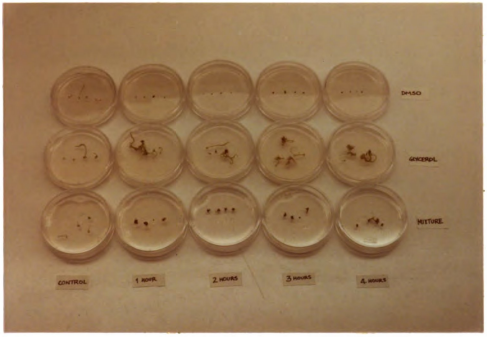


Figure 14: Effect of the cryoprotectants on shoot tip growth after 2, 3, and 4 hours at -5^{o}C.

In a similar experiment, shoot tips were precultured on basal medium for 4 days in the dark at 23°C, followed by 21 days in the dark at 4°C. A prolonged period at the seeding temperature in each of the 3 cryoprotectants yielded results consistent with those reported above (Figure 13b). In this case, however the protective action of the mixture and of glycerol appeared to diminish after 3 and 5 hours, respectively, at -5°C.

With the cryoprotective value of 10% glycerol thus clearly established, the logical next step was to evaluate its effects at higher concentrations. Shoot tips from long day grown plants, precultured for 6 days in the growth room, were divided into 5 groups. Each group was treated with a different concentration of glycerol, viz., 10, 15, 20, 25, and 30% (v/v). After an overnight preincubation, the vials were transferred to the freezer maintained at 0°C. The freezing procedure was carried out in three consecutive runs of two glycerol concentrations each, due to the lower freezing points of the more concentrated solutions. Consequently, seeding was concluded at -5, -6, -10, -13, and -16°C with the 10, 15, 20, 25 and 30% glycerol solutions respectively. Once the lower of the two seeding temperatures was attained and seeding accomplished, the temperature was lowered at a rate of -1.6°C per hour. Samples were removed from the freezer at 5 degree intervals and thawed slowly at

room temperature. In general, survival increased with increasing concentration of glycerol (Figure 15). When 30% glycerol was used, one meristem recovered from freezing to -23°C. The regenerating shoot tip (Figure 16) had not yet developed roots, but neither had the unfrozen controls.

The protective action of glycerol (a penetrating cryoprotectant) could be attributable to its colligative properties. By reducing the amount of ice formed during cooling, it may postpone the attainment of a damaging intracellular ionic strength. On the other hand, glycerol could also be acting as a nonpenetrating agent. Glycerol penetrates cells poorly at 0°C, and, under such conditions, acts as a nonpenetrating cryoprotectant (McGann, 1978). Although preincubation in glycerol lasted 11 hours at 0°C, a greater concentration of the compound may have remained outside the cell. If so, glycerol might osmotically remove water from the cells during the initial phases of freezing, between -10 and -20°C, since glycerol would have become concentrated in the extracellular region during this period. This could also protect the cells from adhesion injury between -10 and -20°C.

4. Thawing of the frozen tissues

The experiments reported above were carried out using an arbitrary fast rate of thawing, however; a few experiments compared fast and slow rates of thawing. The slow rate was

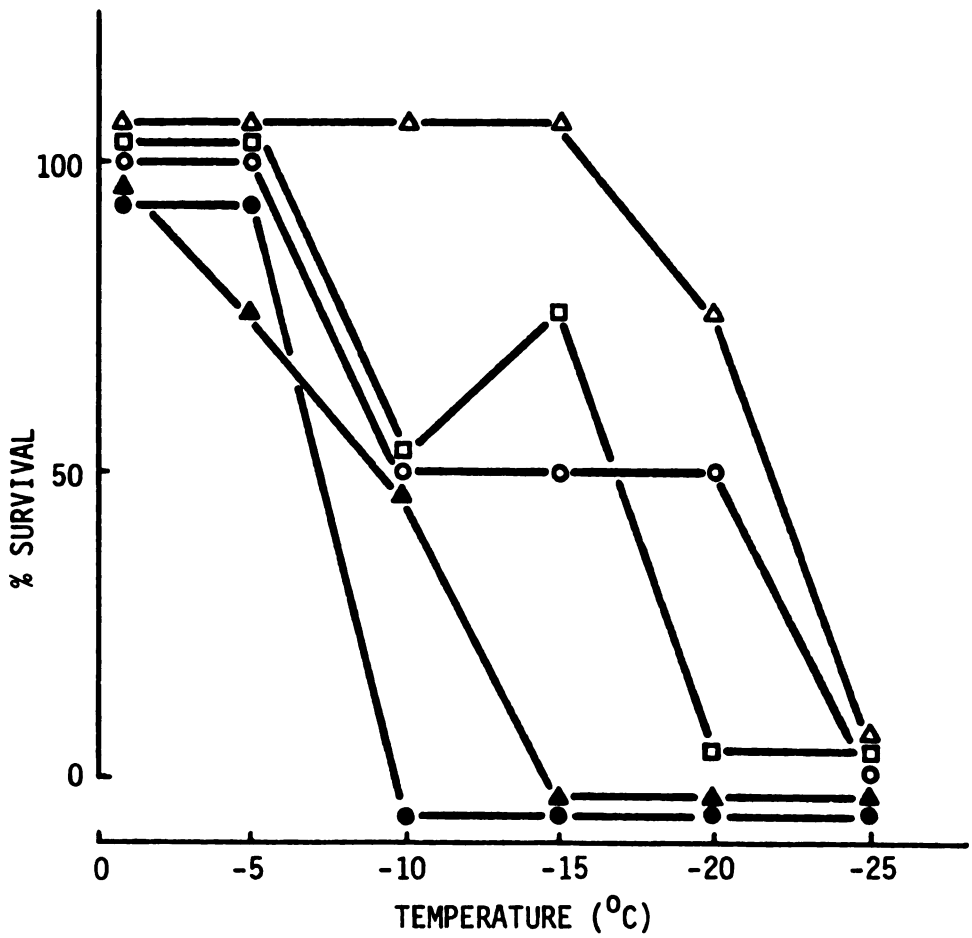


Figure 15: Effect of glycerol concentration on survival of meristematic tips during freezing. ● : 10% glycerol; ▲ : 15% glycerol; o : 20% glycerol; □ : 25% glycerol; △ : 30% glycerol.

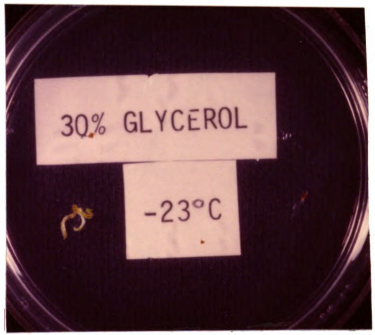


Figure 16: Surviving shoot tip after being frozen to -23°C in a cryoprotective solution containing 30% glycerol.

achieved in two ways: 1) by warming the vials in air at room temperature, and 2) by wrapping the vials in cotton wool and a plastic bag and allowing the tissues to thaw in a refrigerator at 5°C. Neither of these methods significantly affected survival of the tissues frozen at rates of 1.7°C per hour. Thus, under the freezing conditions used, the thawing procedure was not limiting recovery. The slow rate of cooling was therefore used in all subsequent experiments (e.g., the test of different glycerol concentrations).

III Freezing of Intact Seedlings

The freezing of potato seedlings followed the procedure reported by Grout and coworkers (1978), described in the MATERIALS AND METHODS. The results of freezing the seedlings by direct immersion in liquid nitrogen, either naked or protected by aluminum foil vials, has been reported above. The same negative results were obtained when seedlings contained in small aluminum foil vials were gradually cooled by suspending the vials in the vapor above the liquid nitrogen (Figure 17).

Successful freezing of plant cell suspensions has been achieved using rates of cooling of 1.0 to 3.0°C per minute (Table 1). In a final experiment, whole seedlings were frozen at a much faster rate than the usual 1.7°C per hour. The seedlings in glass vials were transferred to the freezer where they were seeded at -5°C in the usual manner. The temperature

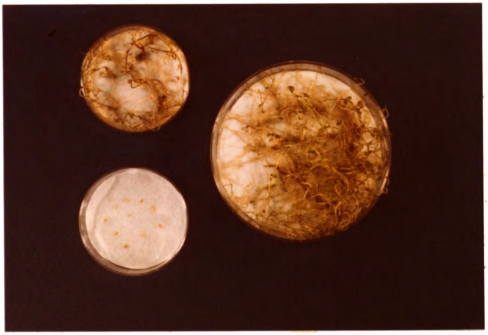


Figure 17: Results of freezing intact seedlings in the vapor of liquid nitrogen. Plantlets regenerated from unfrozen tips (upper left); dead tips from frozen seedlings (lower left); undissected control seedlings (right).

was lowered to -25°C at a rate of 0.35°C per minute (21°C per hour), the fastest controlled rate attainable with the equipment provided. Once -25°C was reached, the vials were immersed in liquid nitrogen. Frozen seedlings were rapidly thawed and cultured in the usual manner. None of the meristematic tips survived.

CONCLUSIONS

In our search for a methodology for the long term preservation of potato meristematic tips at supralow temperatures, none of the freezing protocols tested permitted survival and regeneration of a shoot tip after exposure to -196^{o}C. None of the freezing rates tested proved to be optimal and this parameter itself might have been the primary cause of failure. Limitations in the equipment prevented us from exploring cooling rates more thoroughly. Future experimentation should focus more on this parameter.

Among the hardening pretreatments tested, only preculture of tissues for approximately two weeks at 4^{o}C was effective.

Glycerol was the most effective cryoprotectant evaluated when freezing rates were approximately 1.7^{o}C per hour. Preincubation and freezing in 30% glycerol permitted a meristematic tip to survive to -23^{o}C after freezing at a rate of 1.6^{o}C per hour.

A logical experiment in continuing this work would be to test the effects of combining the two treatments that conferred the best protection. Shoot tips precultured at low temperatures (4^{o}C) for about 15 days should be frozen at a rate of 1.7^{o}C per hour after an overnight incubation in 30% glycerol. If the meristematic tissues survive exposure to -30^{o}C, then they

should be exposed to -196^{o}C and stored there for assessment of survival.

Even if meristematic tips can survive such treatment, the procedure remains tedious and unlikely to be accepted for practical application. If the procedure requires too many steps, a small change in temperature can irreversibly damage the tissues. Any increase in temperature after seeding can cause recrystallization and tissue injury. Therefore, future research should seek a simpler technique, possibly utilizing the more sophisticated instruments available for controlled freezing.

As for pretreatment of the tissues, desiccation is a stress that is frequently associated with tolerance to cold temperatures, but was omitted from this work in order to preserve the viability of the meristematic tissues. However, either moisture-stressing the source plants or drying-out the explants themselves and removing surface water from them after incubation in the cryoprotective solution should be tested (Withers, 1979).

The use of a slow cooling rate, an effective cryoprotectant, seeding and controlled thawing might yield a reliable method for preserving tissues and organs of plants cryogenically. A more reductionistic approach to the search for the appropriate parameters in successful cryopreservation might be a detailed

study of cell suspensions, using a cooling apparatus permitting microscopic observations during the course of cooling (Siminovitch et al., 1978). Nucleation and ice crystal growth could then be followed closely. If a protocol for effective cryoprotection of a cell suspension could be developed then progress might next be made with less finely divided cell suspensions. Finally, extrapolation from these results could provide optimal techniques for the freezing of tissues and organs.

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