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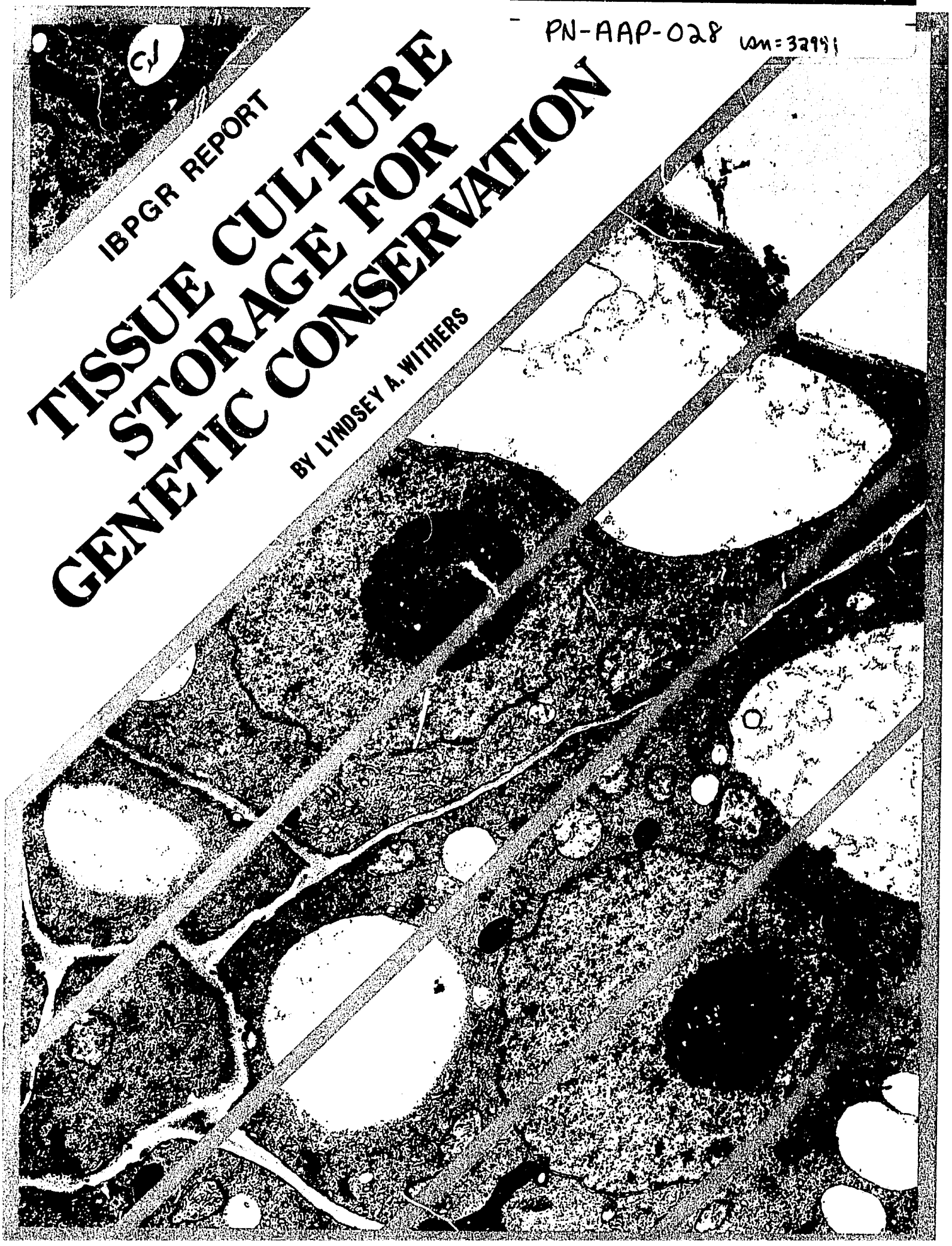
IBPGR REPORT

TISSUE CULTURE STORAGE FOR GENETIC CONSERVATION

BY LYNDSEY A. WITHERS



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TISSUE CULTURE STORAGE FOR GENETIC CONSERVATION

by

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The International Board for Plant Genetic Resources (IBPGR) is an autonomous, international, scientific organization under the aegis of the Consultative Group on International Agricultural Research (CGIAR). The IBPGR, which was established by the CGIAR in 1974, is composed of 15 members from 13 countries; its Executive Secretariat is provided by the Food and Agriculture Organization of the United Nations. The basic function of the IBPGR, as defined by the Consultative Group, is to promote an international nework of genetic resources centres to further the collection, conservation, documentation, evaluation and use of plant germplasm and thereby contribute to raising the standard of living and welfare of people throughout the world. The Consultative Group mobilizes financial support from its members to meet the budgetary requirements of the Board.

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ABSTRACT

The requirement for a novel in vitro germplasm conservation method can be identified in several plant groups. In particular, vegetatively propagated staple food crops and plants with recalcitrant seed present an especially serious problem. The possibility can be entertained of introducing the plants into tissue culture and storing their germplasm either in a growing state or in 'suspended animation' - freeze-preservation. A drawback to storage in culture is the incidence of genetic variability deriving from mixoploidy in the original explant, and from events occurring during culture initiation and maintenance. There is evidence that cultures may be stabilized by careful choice of inoculum and care in maintenance. Genetic variation may increase with time in growing cultures but in the frozen state, background radiation is the only source of instability. The latter is unlikely to be of any consequence except in very long term storage. Two basic approaches to preservation can be considered: minimal growth or ultra-low temperature storage. In the case of the former, only temperature reduction and medium modification can realistically be adopted. Other 'fringe' methods would require extensive developmental research. Minimal growth is particularly suitable for meristems but unsuitable for suspension cultures although callus may survive at low growth temperatures. Several species have successfully been stored by minimal growth but the continued possibility of genetic variation and selection must be drawbacks to the method. Only freeze-preservation can avoid the requirement for periodic storage renewal and testing for variation. The specimen can be conveyed to ultralow temperatures by one of two basic methods. Rapid freezing causes the formation of intracellular ice; provided that the rate of freezing and subsequent thawing is adequately rapid, ice damage can be minimised. Slow (step-wise or pre-) freezing causes extracellular ice formation and protective dehydration. The majority of tissues require chemical cryoprotection prior to freezing. Storage must be carried out at below ca. -80°C to avoid progressive deterioration in storage. Pregrowth treatments such as cold hardening for plants from which meristems are to be excised, or culture in medium with osmotic supplements for suspension cultures can greatly increase survival potential. Recovery depends both upon an adequate initial viability and the provision of supportive post-thaw conditions. Protoplasts and pollen may be preserved by a rapid or slow method and may not require cryoprotection. Pollen embryos are less responsive. Suspension cultures of a wide range of species have been preserved by a slow type of method and always after cryoprotectant treatment. The use of cryoprotectant mixtures and amino acids has improved the response of some species. Recovery ensues after a lag-period in the case of those species which will regenerate a culture after thawing. However, the control rate of growth is usually resumed within a short time. Callus is relatively difficult to freeze-preserve. Clonal plantlets can be preserved by the 'dry-freezing' method in which the specimen is drained dry of suspensing medium before slow freezing. Desiccation prior to freezing may improve survival. Meristems may be preserved by a rapid or slow freezing method, although the latter alone is appropriate for meristems which have arisen in vitro. Meristems always require cryoprotection, respond to appropriate pregrowth treatments and can be recovered by several post-thaw culture methods. Callusing in the recovered material is a problem and outgrowth of the original meristem may not occur. Seed, excised embryos, seedlings and twigs may also be preserved using freezing. In evaluating the choice of tissue culture systems available, and the range of storage methods which can be used for their preservation, the following conclusions are drawn: Minimal growth has a place as the most appropriate storage method for some species and is particularly suitable for meristems. However, the apparent superiority of meristems for germplasm storage based

upon their genetic stability is questioned, in view of the fact that propagation of plants frequently involves adventitious budding and, in commercial programmes, the number of multiplication stages is limited to avoid 'sports'. Propagation through maintenance of organization and development of pre-existing buds is rare and may be impossible after freeze-preservation. The bad reputation of suspension cultures may be exaggerated. They do lose morphogenic potential with time in culture, but freeze-preservation is designed to reduce the culture period. Care in culture maintenance and screening for abnormalities should produce a suspension type culture which is morphogenic and which embodies the ideal characteristics of a specimen for freeze-preservation. A storage programme in which both freeze-preservation and minimal growth storage are included, can be proposed. The former may be more appropriate for long term stock maintenance and for international exchange. The latter may be more appropriate for specimens which are unresponsive to freeze-preservation at the time of their acquisition, and for shorter term stocks. In the case of some staple food crops this programme could be adopted now but for many others the necessary groundwork in tissue culture must still be carried out. For recalcitrant seed species, there is the potential for developing tissue cultures from the seed embryo and freeze-preserving them, or freezing the seed itself. This group of plants and others which lack a good vegetative propagation method could benefit from the development of in vitro propagation and storage of cultures derived from somatic plant tissues. This area is largely unexplored but in the case of the Palms, progress in tissue culture suggests that it may be an appropriate time to attempt either minimal growth storage or freeze-preservation. If the full potential of in vitro storage is to be realised, research must be carried out on basic physiological aspects of pregrowth, preservation and recovery, an attempt must be made to bring about closer liaison between tissue culturists and those working in conservation, and research into species which are identified as presenting a particularly urgent conservation problem must be encouraged and supported. In the future those working with tissue cultures per se may turn their attention away from morphogenic cultures, leaving only those involved with mass propagation and those involved with conservation, to tackle problems in storage of 'in vivo' germplasm.

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I. INTRODUCTION

1.1 The case for seeking new conservation methods

Several categories of economic plants present serious conservation problems because their germplasm cannot be preserved in storage using conventional techniques. Vegetatively propagated plants which have a high degree of heterozygosity or which do not produced seed must be stored in a vegetative form as tubers, roots, cuttings, etc. This involves relatively costly field collections and/or storage of propagules for short periods (Martin, 1975). By either method, the material is open to several threats such as attack by pests or pathogens, loss by climatic perturbations or other natural disasters and human error.

Among the vegetatively propagated crops of this type are Dioscorea spp. (yams), Ipomoea batatas (sweet potato), Manihot spp. (cassava), Solanum spp. (potatoes), Colocasia esculenta (taro), Xanthosoma sagittifolium (cocoyam) and Musa spp. (bananas). The scale of this problem can be illustrated by considering potato germplasm. The germplasm collection of Solanum spp. acquired at the Centro Internacional de la Papa, Peru, through exploration in South America, grew from 4000 accessions in 1975 to an anticipated and realised 12000 in 1979-80 (Ochoa, 1975; Schilde, pers. com.). Among the accessions are some infertile triploid and pentaploid cultivars for which seed storage would be impossible even if desirable (Schilde, pers. com.).

Another important group of plants includes those which can be propagated by seed but which would profit from a vegetative means of propagation in order to increase stocks of valuable F_1 hybrids or high yielding and disease resistant genotypes. For palms, including Cocos nucifera (coconut), Elaeis guineensis (oil palm) and Phoenix dactylifera (date palm), there is no useful method of vegetative reproduction. It would be useful if there could be developed methods for clonal propagation and for propagule storage (see Eeuwens and Blake, 1977). Many long-lived forest trees including Angiosperms and Gymnosperms do not produce seed until a certain age, and must be propagated vegetatively when it is desired to propagate parent rather than progeny. Where propagation through cuttings is expensive or problematic, alternative methods must be sought (see Webb and Street, 1977; Winton, 1978).

A further conservation problem (which compounds the storage difficulties in some species in the above categories) is that of seed recalcitrance. Seeds which lose viability when dried below a certain water content or when exposed to low temperatures (occasionally well above zero degrees) are described as 'recalcitrant' distinguishing them from 'orthodox' seeds which can be stored at a low temperature (ca. -20°C or lower) and low moisture content. This subject is covered in detail elsewhere (King and Roberts, 1979; Roberts, 1975; Villiers, 1975). Among the plants with seeds showing this type of behaviour are many timber, food and other commercial crops e.g. Juglans spp., Quercus spp.; Citrus spp.; and Coffea spp., Hevea brasiliensis and Elaeis guineensis.

In order to resolve these problems of germplasm conservation attention has been given to the possibility of using in vitro methods for long-term storage. Thus, if explants from plants of vegetatively propagated, mature perennials and plants with recalcitrant seeds (or from the seeds) could be introduced into sterile culture, perhaps they could be maintained in a secure, pathogen-free environment, available for future use.

Furthermore, if the sterile culture could be conveyed to ultralow temperatures, the germplasm could exist in 'suspended animation' until required. This simplistic scenario provides the framework around which the technical report presented here is structured. In vitro culture (or 'tissue culture' as it is more popularly known) is not without problems and many of these are relevant to the question of the feasibility of in vitro culture for genetic conservation. Nonetheless, although it may be inappropriate to state a conclusion at the outset, it is clear that tissue culture methods are going to provide an answer to some problems in genetic conservation and the important matter for the present is to realistically appreciate the present state of knowledge and its limitations, and ensure that in the future its potential is fully exploited.

Now that vegetative propagation in vitro is becoming increasingly common as a means of propagating cheaply important horticultural and agricultural plants (see Murashige, 1974, 1977, 1978), with the benefit of in vitro pathogen elimination (Walkey, 1978), storage of clean stock propagules for future utilization would be useful. This line of thought takes us to a further category of plants - those which are products of in vitro selection or genetic manipulation. It would be beneficial to store novel genomes in a vegetative form for future evaluation and utilization in agriculture and secondary product synthesis (see Alferman and Reinhard, 1978). However, it must be stated emphatically that the latter two areas in which new conservation methods have a potential use are not central to the main issue of genetic conservation: but any spin-off could be beneficial.

1.2 General remarks on plant tissue culture and cryopreservation

The term 'tissue culture' covers a wide range of techniques including the in vitro culture of cells, organs, (meristems, roots, florets, anthers etc.) ovules, pollen, protoplasts and embryos as well as tissues per se. Pioneering studies by Haberlandt date back to the beginning of this century, although predated by relevant dissection and transplanting experiments by Vöchting and Carriere. In the 1930s, several workers turned their attention to the culture of excised root tips and cambial tissues, the latter (White, Gautheret and Nobecourt, working independently) marking the first example of the prolonged culture of unorganised cell masses - true tissue culture. During the subsequent 20 years, further investigations by workers throughout the world elucidated the following: the control of vascular differentiation; the role of vitamins; coconut milk and nursing tissues in supporting growth; the phenomenon of somatic embryogenesis; and, the quantitative analysis of culture growth and the induction of tissue fragmentation to yield suspension cultures leading to the refinement of plating and cloning techniques. In the 1960s and 1970s, important developments were: the induction of haploid tissues and plants from anther culture; the culture of apical meristems; the refinement of clonal propagation techniques; the isolation and culture of protoplasts; and, most recently, the potential for modifying plant genomes through genetic manipulation in vitro. (For references and further discussion of the history of plant tissue culture see Street, 1977a, and White, 1954)

The progressive development of tissue culture has not, however, resolved all problems at all stages. Thus there are many plant tissues which are difficult to establish and maintain in culture, many which do not yield the desired type of culture and many which will not yield regenerating plants under inductive conditions. Unfortunately, many of the deficiencies in our knowledge of plant tissue culture lie in areas of great relevance to the consideration of its use in genetic conservation.

Although a more recent subject for consideration, a similar history could be expounded for the development of cryopreservation. Critical landmarks are the demonstration of the successful freeze-preservation of spermatozoa using glycerol as a protective additive (Polge et al. 1949); the preservation of cultured animal cells in 1954 (Scherer and Hoogasian); the establishment of a frozen animal cell bank in the USA (Registry, 1964); the preservation of ovarian and embryonic tissues; and, whole animal embryos (Deansley, 1954; Rey, 1959; Whittingham et al, 1972, respectively); and the demonstration of the cryoprotective properties of dimethylsulphoxide (Lovelock and Bishop, 1959).

The cryopreservation of cultured plant cells (Quatrano, 1968), meristems (Seibert, 1977), and somatic embryos (Withers, 1979) give parallel botanical examples. Living material across the phylogenetic spectrum can now be preserved (see Meryman, 1966b; Morris, 1980; Takeuchi, 1978; and references in Ashwood-Smith and Farrant, 1980). Again there are many deficiencies in our knowledge, especially in the area of botanical cryopreservation. Thus the exploitation of cryogenic methods for the storage of plant tissues still has a highly empirical element - but its potential is vast. An overriding consideration in the bringing together of plant tissue culture and cryogenic methodology is the advantage that plant cells have over the majority of animal cells - and that is that they are, or rather can be, totipotent. This gives a much greater flexibility to the approaches which can be made in searching for ways of storing plant germplasm.

1.3 The present report

In this report the author attempts to give the broadest possible examination of the present level of expertise in the use of tissue culture methods for genetic conservation. It is felt that through looking at the full scope of in vitro culture methodology, there is the greatest potential for identifying, developing and exploiting novel methods for conservation. It is important to bear in mind the applied aspect of conservation, namely utilization of genetic resources. Thus the approach taken to the storage of material must be appropriate to future utilization. Although outside the strict brief of this report, it is worthwhile considering that the vegetative propagative potential of plant tissue culture may offer a natural and valuable adjunct to tissue culture storage, providing multiple cloned specimens for storage, rapid dissemination and field growth.

In addition to tackling the broadest possible range of specimens, a similar approach has been taken to the possible range of storage systems and experimental protocols. This is not to suggest that all of the techniques should be adopted, but to indicate that we are in the infancy of the subject and that it is far to soon to develop prejudices which may limit the future exploitation of the approach. It is entirely conceivable that present shortcomings in conservation technology may be resolved by progress in areas of tissue culture technology per se. By way of explanation: many of the experimental findings described here relate to favourite model systems of tissue culturists. This derives from two factors: firstly that in such systems the expertise in handling and understanding the biological material is most highly refined; and secondly, that there is a very strong case for developing a cheap and reliable storage method for plant tissue cultures themselves. Consequently, a large proportion of the effort in developing, in particular, freeze-preservation technology has been made with a physiological, tissue cultural approach rather than from a conservation motive. This explains the strong emphasis in the literature on suspension cultures. Such cultures

may not, at present, be appropriate for genetic conservation but may, through exploration of their responses to freezing and thawing, tell us a great deal of relevance to other tissue culture systems and eventually, with progress in tissue culture technology itself, become entirely suitable for genome preservation.

This introduction has provided the reasons for the need to develop an in vitro storage system for germplasm. Aspects of stability relating to tissue culture initiation and maintenance, and to storage are discussed in Section 2 of this report. The technical approaches to storage fall basically into two categories - those dependent upon normal or suppressed growth and those dependent upon suppression of all metabolic activity i.e. freeze-preservation. The former are considered in Section 3 and the latter in Sections 4 and 5, with theoretical points and basic methodology being confined to Section 4 and empirical findings in Section 5. The approaches to storage are critically evaluated in Section 6 and on the basis of this evaluation a prognosis is given in Section 7 for the adoption of in vitro techniques for the storage of plants with recommendations for the direction of effort, interest and support in the future.

2. STABILITY

SYNOPSIS: Euploid and aneuploid abnormalities may be found in plant tissue cultures and plants regenerated from culture. The abnormalities may relate to heterogeneity in the parent plant but are more likely to have developed during culture initiation and maintenance. Organised cultures such as meristems are more likely to maintain genetic stability but with progress in tissue culture technology, it is feasible that other types of culture may be stabilized. Natural or induced variant genotypes may be valuable and should not be ignored. Little evidence can be presented to support or detract from the maintenance of genetic stability in storage but indications are that there need be little cause for concern provided that suitable storage conditions are maintained. The only serious threat to genetic stability is the effect, in the long-term, of background radiation. Further work is needed to characterise specimens before and after storage to settle questions in this area.

2.1 Genetic stability in the induction and maintenance of tissue cultures

From the outset, it should be stated that the consideration of the growing, whole plant as a paragon of genetic stability is fallacious. Some plants are well known as chromosome mosaics, for example Saccharum officinalis and Ribes nigrum. In these cases, the causal genetic instability may be related to hybridity or recently developed tetraploidy (see D'Amato, 1978). The maintenance of a consistent 'germ-line' through generations only requires an ordered process of chromosome replication in the cells of the apical and other meristem regions. Endoreduplication and the generation of aneuploidy in other tissues will not threaten the genetic consistency of sexual progeny of the plant. Indeed, histological differentiation in the whole plant by cells in a diploid state is characteristic only of a minority of Angiosperms (ca. 20%; D'Amato, 1978). The consequences of genetic variation can readily be appreciated. Thus where propagation in vitro proceeds from other than certifiably diploid (G_1 = 2C or other accepted value) cells and tissues, there is the risk of introducing genetic variation into culture.

For reasons given above, the choice of apical meristem tissues for the production of clones and as storage specimens is favoured. This is also a technically satisfactory choice since procedures for meristem tip isolation and introduction into culture are well documented and can readily be adopted with a little practice (see Morel, 1975; Walkey, 1978 and references therein).

In some cases, however, meristems may not be available and other sources of inoculum have to be considered. Of cambial tissues and differentiated organs, the former must be favoured from the point of view of stability but it should be noted that many other tissues are commonly used for culture initiation (see e.g. Bragadø-Aas, 1977; Geier, 1977; Hedtrich, 1977; Lavee, 1977; Sunderland and Dunwell, 1977; Tran Than Van and Trinh, 1978; Yeoman and MacLeod, 1977).

Where continued organised growth of the meristem in culture follows culture initiation, genetic stability is likely to be maintained. Conversely, when culture initiation involves the alteration of the pattern of growth in a tissue or organ, or the reintroduction of cell division in a differentiated specimen, there is the likelihood that genetic changes will ensue. This possibility is often overlooked, there being a more general awareness of progressive genetic changes occurring with time in culture. In addition to mixoploidy in a parent polysomatic plant being perpetuated as mixoploidy in culture, this condition can become established during the first cell divisions (D'Amato, 1977).

In cases where the traumatic process of protoplast isolation marks the initiation of the culture, there is further scope for variation. Multinucleate protoplasts may be generated by spontaneous fusing during isolation (Pearce et al., 1974; Withers, 1973; Withers and Cocking, 1972). Further, evidence is accumulating to suggest that the first mitosis, especially in haploid protoplasts, may be preceeded by endoreduplication (Furner et al., 1978; Gleba and Gleba, 1978). Electron microscopical examination of such material indicates that the first cell division may be abortive or involve incomplete cytokinesis (Batchelor and Withers, 1980). Nuclear fragmentation within a heterogeneous population of nuclei may lead to euploid and aneuploid products which are separated by subsequent cellularization (Bennici et al., 1976; D'Amato, 1978). There is some evidence that the induction of genetic changes at the time of culture initiation may relate to the hormonal content of the induction medium and the ratio of component hormones (D'Amato, 1978).

Turning now to maintenance of cultures: there are further opportunities for the introduction of variation especially in suspension and callus cultures. Polyploidization, (in particular the shift from haploid to diploid, but also diploid to tetraploid, octoploid and higher) may result from spindle failure and chromosome lagging during mitosis. Odd-ploidies may result from nuclear and spindle fusion and genome segregation during multipolar anaphases (D'Amato, 1978; Sunderland, 1977). Although increases in chromosome number are most common during culture, reductions leading to lower aneuploidies and even haploidy or hypohaploidy may occur through nuclear fragmentation, homologous chromosome segregation and progressive loss of chromosomes (Huskins, 1948; D'Amato, 1977, 1978; Novak, 1974; Sunderland, 1977). The frequencies and variety of aneuploidy are likely to increase with the age of the culture. There would appear to be a pronounced tolerance of variation in many cultures. Sokolov et al., (1974) suggest that as long as there is one nucleolar organizer present, then mitosis will persist in a culture. Sunderland (1977) gives a comprehensive review of the mechanisms for chromosome complement alteration in a cultured cell. Many deductions relating to the origin of abnormal genotypes depend upon careful cytological examination but it must be pointed out that other changes will also occur below the level which can be detected by optical techniques.

There is some evidence that the composition of the culture medium may influence the net genetic constitution of the culture, through increasing the probability of change or presenting a selective advantage to a certain genotype. Bayliss (1977a, b) has examined the competition between lines within a mixed diploid and tetraploid suspension culture of Daucus carota: phosphate limitation presents an advantage to the tetraploid line (which is better able to utilize low levels of phosphate), and growth under a rapid subculturing regime presents an advantage to the diploid line (which has a more rapid rate of growth when free of phosphate limitation). A similar phenomenon has been

reported in animal cells growing in vitro (Earley, 1973). Singh et al. (1975) found that alteration of the subculture passage length could alter the tetraploid frequency in suspension cultures of **Haplopappus gracilis** (see also Singh et al., 1972) suggesting, with the evidence of Bayliss, that the simple matter of culture routine may help to determine the genetic stability or otherwise of a culture. Corduan and Spix (1974) reported an effect upon the ploidy of organised cultures developing on a callus, of the imposed lighting.

The hormonal content of the culture medium is widely considered to be instrumental in determining the net genetic constitution of a culture. 2,4-D (2,4-dichlorophenoxyacetic acid) is thought to induce polyploidy (Shamina, 1966) and, with kinetin, to selectively stimulate the division of polyploid cells (Torrey, 1961, 1967). NAA (napthaleneacetic acid) and kinetin concentrations are reported to influence the ploidy complement of cultures and differentiating structures (Bennici et al., 1971; Brossard, 1976).

Several factors, including effects upon cell cycle kinetics (Bayliss, 1977c), the enhanced production of albino plants from anthers (Wang et al., 1977) and herbicide effects (see Gressel et al., 1978), which all involve higher than necessary levels, contribute to a general caution towards use of 2,4-D. (See also Novak, 1980). Although it is difficult to put a convincing case for abandoning the use of this or any other growth substance, there is a strong case for gathering data which are not circumstantial on the detrimental effects of hormones by the careful determination of the minimum effective levels and the screening of cultures for genetic abnormalities at all stages.

Until there is a clearer understanding of the role of medium constituents, the length of time in culture and the subculture routines on constitution of any given culture, no clear recommendations can be made for controlling relevant processes, except to: a) reiterate the caution relating to the use of hormones, b) suggest that the original explant be carefully karyotyped, and c) suggest that the period in culture be minimised, well documented and designed to avoid selective conditions.

2.2 Plant production

Eventual plant regeneration will be the aim in the majority of cases of tissue storage. Thus it is important that morphogenic potential be retained. The simplest way of doing this is to ensure that the culture remains in an organised form throughout. Thus meristem tips can be cultured to produce clonal plantlets from nodal/axillary meristems (e.g. Mantell et al., 1978; Murashige, 1977). This process is largely 'accident-proof' although in commercial programmes, the total number of divisions to which a cultured meristem is subjected is restricted in order to reduce the incidence of abnormalities due to unspecified causes (Anon, 1976). The possibility of local mutations or abnormal mitoses producing variant genotypes must be entertained along with the more serious consideration of adventitious bud formation on deformed or callused explants. Once these possibilities are considered, the meristem culture is as vulnerable to genetic change as is a callus or suspension culture, with resultant genetic variability in the progeny (Murashige, 1977).

A study by Denton et al., (1977) into phenotypic variation in plants of Solanum tuberosum regenerated directly from shoot-tip culture indicates that whilst there may be significant variation, this need not have an underlying biochemical basis. Environmental factors are implicated in the variation but cytological analysis was not carried out to confirm genetic stability.

In the cases of callus and suspension cultures, loss of morphogenic potential is a relatively common occurrence, normally being related to the age of the culture (Meyer-Teuter and Reinert, 1973; Novak, 1980; Reinert, 1959; Skirvin, 1978; Smith and Street, 1974). Genetic changes alone are not thought to be completely responsible for the loss of morphogenic potential, although ploidy shifts, aneuploidy and the possibility of non-morphogenic cell lines having a selective advantage in culture have been proposed (Murashige and Nakano, 1967; Smith and Street, 1974). However, other reports suggest that careful adjustments of culture conditions and medium constituents may be required to 'reveal' morphogenic potential which had been thought to have been lost during prolonged culture (e.g. Asuwa, 1972; Drew, 1979; Koblitz and Schumann, 1976). The idea that all that is required to induce differentiation is a transfer to a proven morphogenesis supporting medium is clearly wrong. Cultures of differing origin and history may require markedly different conditions (Chaturvedi and Mitra, 1975; Reinert, 1959; Steward et al., 1967; Syono, 1965; Wu and Li, 1971). Meyer-Teuter and Reinert (1973) suggested that a loss of morphogenic potential in cultures may be epigenetic and therefore potentialy reversible, analogous to the suggestion that habituation (loss of an auxin requirement) may be epigenetic rather than mutational (Meins, 1974). Thus the trigger for organised growth may be more complex than simply the removal of an auxin from the medium (see Kohlenbach, 1978). The inference is that we need to know more about the factors controlling morphogenesis before making any broad conclusions. It would appear to be entirely possible that growth as a suspension of callus culture need not automatically lead to the risk of losing morphogenic potential. This is a question to be answered in the future by careful research and not at present by assumption and pessimism.

Although some genetic changes in culture will lead to the loss of morphogenic potential this will not always be the case. Regenerant plants may embody genetic changes produced in the preceeding culture period. Although some morphological differences between parent and progeny plants may be attributable e.g. to virus eradication (Kartha and Gamborg, 1979; Skirvin, 1978) chimeral segregation (Skirvin, 1978; Skirvin and Janick, 1976a) or exposure to certain hormones during the culture period (Ibrahim, 1969), there are many cases where a novel genetic change is involved (see Skirvin, 1978). A clear example is the regeneration of sterile aneuploid plants with morphological abnormalities from 'old' cultures of Nicotiana tabacum (Sacristán and Melchers, 1969). A recent study by Shepard (see Day, 1980) demonstrates the great potential for producing variant plants with desirable characteristics, through the process of protoplast isolation, callus regeneration and plant production in Solanum tuberosum. Variations in the progeny relate to tuber maturation date, photoperiodic requirements for flowering, pathogen and toxin resistance, tuber yield and morphology. These 'spontaneous' variations may represent the inherent variation in the leaf mesophyll tissue from which the protoplasts were isolated; a situation somewhat analogous to the isolation of clones from chimeral tissues of Saccharum sp. (see Heinz et al., 1977). Both examples show the potential for isolating desirable genotypes.

The examples quoted above introduce an interesting philosophical point. Taken to its logical conclusion, the desire to conserve genomes - through meristem culture or other methods which putatively preclude genetic change - will indeed conserve the 'germ-line' but may lose in the process many instances of naturally occurring genetic change which may be considered to be 'valid' expressions of the plant's genome (is not meiosis such a process?). In the event, a combined puristic and opportune approach may be the most pragmatic.

In addition to changes which occur with time in culture,, it is possible to induce genetic variations at ploidy and more subtle levels. Irradiation may induce mutations through direct effects on the cell or through effects on the culture medium. Chemical mutagens may alternatively be used. Further consideration of this source of genetic variation is not appropriate here. The reader is directed to Maliga, 1978; Scheider, 1978; Skirvin, 1978, and Street, 1974, for further discussion and access to the literature.

2.3 Stability in storage

Several questions are relevant in relation to the quantitative and qualitative security of storage and the possibility of the stresses involved in transfer to storage exerting selective effects upon heterogeneous cell populations. A serious impediment to answering these questions is the lack of information, due in part to the relative youth of the subject, such that long-term studies are only now beginning.

Quantitative loss

A certain basic percentage loss is inevitable in freeze-preservation, for reasons given in Section 4. This loss may be represented as a percentage of the total number of cells present in a suspension, as regions of tissue in individual stored specimens or as a percentage of the total number of individuals. The decision as to whether this loss is tolerable will depend upon the uniqueness and availabiilty of the specimen.

More important than basic loss is the possibility of progressive loss with time. The storage temperature is critical here. A number of reports of survival potential declining with the time in storage at temperatures such as -20 and -78°C indicate that deterioration can occur at temperatures well below the freezing point of water. This deterioration may be due to the fact that some enzyme reactions may proceed more rapidly at sub-zero temperatures but is most probably due to recrystallization of water within the cell (see Section 4; examples of deterioration at intermediate storage temperatures: Bajaj, 1976b; Nag and Street, 1973; see references in Withers 1980a). One report quoted in support of the possibility of deterioration at -196°C, the temperature of liquid nitrogen (Peterson and Stulberg, 1964) does not in fact clearly state the storage temperature involved in the experiment in question. 1/

To date, there is no convincing evidence that, provided the storage temperature is sufficiently low, freeze-preservation need involve any progressive loss of viability. Storage periods are not often quoted but successful storage for 100 days (Nag and Street, 1973) and one year (Dougall and Wetherell, 1974) should promote optimism. Only one report documents a deterioration in storage. Kartha et al. (1980) observed a survival rate of 95% in specimens (meristems of Fragaria) stored in a liquid nitrogen refrigerator for 1 week, but the survival rate declined within a further week to stabilize at 50 - 65% throughout an 8 week storage period. No explanation is offered and it is tempting to suggest that some perturbation in storage conditions may have occurred - e.g. a

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1/ Vagueness about actual storage temperatures is not restricted to this report. A number of publications describe the transfer of various plant tissues to various temperatures and it often requires very close reading to determine, if then, to which temperature successfully 'preserved' specimens had in fact been exposed.

transient increase in the storage temperature due to loss of coolant. Such an event would give the observation recorded, rather than the progressive decline with time which would be expected if a continuous process of deterioration ensued. Clearly this calls for close examination and suggests that extrapolation from short-term storage data should be done with caution.

Comparable data on quantitative loss with time are not available for storage methods other than freeze-preservation, largely since the studies have not been so extensive and since a periodical transfer/renewal is a feature of the storage methods. Some loss similar to that expected in a routine culture transfer must be allowed for at each transfer stage, and the time between transfers will be determined by a balance between the threat of irreversible loss of viability and technical convenience. No situation can be envisaged where a single-stage long-term storage process could be developed from the alternative storage methods described in Section 3.

Qualitative changes

Nag and Street (1973) described karyotypic consistency between original and recovered cultures of **Daucus carota**, with an unimpaired capacity to undergo somatic embryogenesis and plant regeneration. Similar confirmation in this (Dougall and Whetherell, 1974; Withers, 1979; Withers and Street, 1977a) and other morphogenic species preserved as cell cultures (**Atropa belladonna**; Nag and Street, 1975; **Hyoscyamus muticus**, Withers and King, 1980a; **Nicotiana tabacum**, Bajaj and Reinert, 1977) can be given. However, bearing in mind the observations made earlier, there is no confirmation that the genome is completely unaltered, only that morphogenic potential has not been lost. In a study under way at present, it is attempted to évaluate control and variant lines of **Acer pseudoplatanus** in terms of auxin and cytokinin independence (see Everett, 1980), and **Hyoscyamus muticus** and **Daucus carota** in terms of toxin and/or antimetabolite resistance (see Strauss et al., 1980) before and after freezing. Preliminary findings with **D. carota** indicate that resistance is maintained (Withers and King, 1980b).

Where growth is occurring during storage then the sources of variation related to growth and cell division are likely to persist at a rate related to the rate of growth. Meyer-Teuter and Reinert (1973) reported that cultures of **Daucus carota** lose their morphogenic potential more slowly at 5°C than at 13°C and more slowly at the latter temperature than at the normal growth temperature of 28°C. Little other evidence can be presented on this point, but data of Roca et al. (1978) relating to evaluation of clonally propagated plantlets of **Solanum** spp. in comparison with parent plants are relevant, since the propagation stage is intended as part of a germplasm collection, multiplication and dissemination programme (Roca et al., 1978; 1979). It would appear that clonal multiplication through the 'multi-meristem' technique does not introduce any variation detectable by electrophoresis of tuber proteins and esterase, or by evaluation of a number of gross morphological characteristics. It would be heartening to see similar data relating to plantlets regenerated from material maintained in culture for a period of time under normal or suppressed growth (see Section 3).

In storage where no growth occurs - i.e. under freeze-preservation, there is only one possible source of genetic variation, namely mutation induced by background radiation. At ultra-low temperatures, healing processes will not occur and therefore mutations will accumulate. Appropriate screening and the use of glass rather than polypropylene specimen containers will reduce the risk (but see safety note in Section 4.3). Additionally, dimethylsulphoxide is reported to have radioprotectant properties

(see Withers, 1980a). Cells frozen slowly and subject to protective dehydration (see Section 4.3.1) will contain less intracellular water than rapidly frozen cells. This will reduce the incidence of free radical formation by irradiation within the cell and will reduce the level of damage accordingly (Wood and Taylor, 1957). The problem of the level of mutation becoming unacceptable is unlikely to be of any real consequence for decades at least (Whittingham et al., 1977) as evidenced by experimentation with animal embryos. Nonetheless, it has to be seen in the context of a very long-term storage programme within which several factors all contribute to the security of the storage. Net acceptable levels of induced variation may have to be laid-down.

Selection as a result of storage

There can be no doubt that phenotypic selection will follow in any procedure which imposes physical stress upon the specimen. Whether phenotypic differences reflect genotypic ones is a different matter. Freeze-preservation will selectively preserve small cells, small aggregates and early developmental stages in, for instance, embryogenesis (Nag and Street, 1975b, Withers, 1979; Withers and Street, 1977b). Normally this is of little consequence beyond imposing the requirement that the culture should not be of a morphology which renders it entirely unsuitable for freeze-preservation, e.g. senescent (see Section 4.3.5). However, in cases such as that of a mixed haploid and diploid culture within which the ploidy difference is reflected in the mean cell size, selection could occur; a potential ploidy-screen perhaps ? (Shillito, 1978). It is relevant that long-term survival and regeneration of plants has only been reported in haploid cell cultures of **Nicotiana tabacum** after exposure to ultra-low temperatures (Bajaj and Reinert, 1977).

Selection during, for example, cold hardening prior to freeze-preservation or cold-storage, or cold storage itself is a potentially more serious problem. Heritable tolerance of low temperatures has been shown in cultures of **Capsicum annuum** (Dix and Street, 1976). The levels of recovery from minimal growth storage (see Section 3.2), ranging from ca. 40% to more than 80%, suggest that selection for stress-tolerant genotypes is unlikely to be occurring. However, this can only be clarified when storage has been carried out for many more years with specific testing for selection at each successive storage renewal stage. Retesting of preserved specimens is rarely carried out. In the author's experience, freeze-preserved cell cultures may show a transient increase in freeze-tolerance upon thawing and retesting, but this can be attributed to changes in the culture morphology consequent upon the trauma of freezing, thawing and recovery (Withers, 1980f). In conclusion, it must be said that there is no solid evidence either way regarding a connection between preservation and selection; this matter deserves attention.

3. GERMPLASM STORAGE IN TISSUE CULTURE - NORMAL AND MINIMAL GROWTH

SYNOPSIS: Tissue cultures can be maintained under normal growth conditions virtually indefinitely, provided that nutrients are supplied and accidents avoided. For reasons of stability, this may only be completely suitable for meristem cultures. Growth can be suppressed by altering the constituents of the culture medium, reducing the culture temperature, reducing the gas pressure over the culture, dessication and mineral oil overlay Only low-temperature growth has been given sufficient attention to make it a realistic storage method, wherein the subculturing interval can be extended to one year.

3.1 Growth at normal (elevated) temperatures

In all tissue culture laboratories where cultures are maintained in growth as stocks for use in experimental work, this form of culture storage is occuring. Of course, it would be facetious to suggest that such stocks form an important plant germplasm repository. However, attempts to conserve specific genomes in culture under normal or near normal conditions have been carried out.

Henshaw, Roca, Westcott and colleagues (Roca et al., 1978; 1979; Westcott et al., 1977; Henshaw et al., 1980) described the initiation and maintenance of cultures from meristem explants of a range of cultivars of Solanum species. Stem segments were sterilized, then their shoot-tip region excised and placed onto a Murashige and Skoog (1962) type semi-solid medium. The explant normally gives rise to organogenetic callus which eventually yields adventitious buds. Alternatively callus may develop at the base of the explant and from this leaves arise. In this case the main apex grows slightly and its leaves expand (rosette culture; Roca et al., 1978). In some cultivars, single shoot cultures develop.

The various forms of culture depend upon the cultivar under consideration and the hormonal complement of the culture medium. In all 38 cultivars of Solanum tuberosum subsp. tuberosum (tbr), S. tuberosum subsp. andigena (adg), tbr x adg, and a cross between tbr and S. phureja tested by Roca et al. (1978), either organogenetic or rosette callus could be induced. Transfer of the growing explants into liquid shake culture induced a proliferative phase yielding up to 18 shoots, each with up to 4 nodes per shoot. Again the culture medium was critical, the greatest proliferation being recorded in the presence of NAA, gibberellic acid (GA_3) and benzyl amino purine (BAP) (at 0.01,0.4 and 0.5 mg l^{-1} respectively). Nodal cuttings could be taken from the proliferating cultures for the regeneration of whole plants in vitro. Further multiplication can be carried out in vivo using stem cuttings and tubers. Meristem plantlets developed in vitro have been characterised morphologically and biochemically (see Section 2.3) and shipped to international locations. Survival upon receipt ranged from 10 - 90%. It was necessary to take precautions to safeguard against damage and temperature shock during transit (Roca et al., 1979). Westcott et al., (1977) described the introduction into culture and storage therein of shoot tips of 13 clones including some of the above and

Solanum goniocalyx, S. x chaucha, S. x juzepcjukii and S. x curtilobum, covering the ploidies 2x to 5x. Single shoot cultures could be established in 30% of the explants of the tuberosum subspecies, but in all the other cases the frequency was lower (5 - 10%). The situation was reversed with the production of multiple shoot cultures wherein the latter all responsed to an appropriate hormonal treatment to give multiple shoots with or without basal callus in 80 - 100% of the explants, but tuberosum only yielded multiple shoots with basal callus in 20% of the explants, Nodal segments of the developing shoots can be rooted, maintained in a propagator for 7 days and then treated as normal seedlings.

Examples could be given of many other crop plants including Dioscorea spp. (Mantell et al., 1978), Manihot spp. (Roca, 1978), Xanthosoma sagittifolium and Colocasia esculentum (Hartman, 1974) being introduced into and regenerated from shoot-tip derived cultures (see also Narayanaswamy 1977; Murashige, 1978).

In the studies described above, the cultures were maintained under conditions free of the risks of field growth and required little attention individually. However, there is the possibility of the reintroduction of pathogens or non-specific microbial overgrowth, equipment failure or other accident, and collectively, a large germplasm stock would be technically demanding. Therefore, despite the simplicity of this storage method and its obvious suitability for meristem cultures, it may more appropriately be considered as a basis for developing a superior storage method involving growth suppression.

3.2 Growth suppression

The normal approach to tissue culture is to find a medium and set of growth conditions which favour the most rapid rate of growth and promote the desired pattern of development. For storage the aproach must be changed. Growth can be limited by one of several methods: temperature reduction, medium modification, desiccation and modification of the gaseous environment.

Temperature reduction has been shown to be very effective and will be described first. Other methods are only reported in the literature in a preliminary form or are clearly fortuitous observation; they will be described subsequently.

Most plant tissue cultures are maintained in growth at a temperature falling between 20 and 30°C. Lowering of the growth temperature will reduce the growth rate, but the scope for temperature reduction depends upon the species in question. The first relevant report by Galzy (1969) described the successful suppression of growth in cultured shoot-tips of Vitis rupestris exposed to 9°C. Normally culture is carried out at 20°C and when cultures were returned to this temperature, after 50, 185 or 290 days of 'cold-storage; growth resumed at the control rate. Storage at 2 or 7°C was far less successful, giving recovery pecentages after 90 days exposure to the lower temperature, of 13 and 46% respectively compared with 100% at 9°C. These figures relate to cultures from a pregrowth passage of 24 days at 20°C; extending the pregrowth passage to 42 days resulted in no survival at 2°C and only 9% at 7°C. Survival at 9°C was not affected. 1/ In adopting low temperature growth as a storage method, Galzy was able to reduce the subculture frequency to once a year.

1/ See also Section 4.3.5: Pregrowth effects in freeze-preservation

In the case of **Solanum** spp. meristem cultures, subcultured at yearly intervals (Henshaw et al., 1980), survival at the end of the subculture period was 14% when grown at 22°C but 61% at 6°C. Providing alternating day and night temperatures (of 12 and 6°C respectively) increased the survival rate to 83%. Storage below 6°C was unsuccessful. For **Ipomea batatas**, storage cannot be carried out at temperatures below 14°C (Alàn, 1979). **Manihot esculentum** is similarly sensitive: storage of nodal cultures at 15°C being unsuccessful, although growth at 20°C reduced the growth rate to below one tenth of that in a 30°C day, 25°C night temperature regime (Roca, 1978).

Other reports of successful growth suppression of organised cultures by exposure to low temperatures include: a significant reduction in the growth of plantlets of **Beta vulgaris** by lowering the culture temperature from 20°C to 12°C (Hussey and Hepher, 1978); the maintenance of meristem tip cultures of species of **Lolium**, **Festuca**, **Dactylis** and **Phleum** at 2 - 4°C under an 8 hour photoperiod (Dale, 1978; 1980); and storage for up to 6 years of stock meristem-derived plantlets of **Fragaria vesca** on filter paper bridges in tubes incubated in the dark at a temperature in the range of -1 to +6°C (Mullin and Schlegel, 1976). The latter cultures were not subcultured during the storage period but the liquid medium was renewed whenever necessary.

Chilling to suppress growth has been of limited success with other types of culture. In addition to the occasional suggestion, unsupported by experimental data (e.g. Potrykus in Alfermann and Reinhard, 1978), there are few relevant reports. Withers (1978c; 1980f) has stored callus of **Capsicum annuum** at a temperature of 4°C for 70 days but only after pretreatment with ethanolamine (see Ilker et al., 1976). Bannier and Steponkus (1972) described storage of callus **Chrysanthemum morifolium** on a medium with an enhanced level of sugar (10%) for up to 28 days at -3.5°C. This report is erroneously described as 'freeze-preservation' (see comments in Section 5.4). Suspension cultures cannot be stored at chilling temperatures according to our present knowledge, but this may be largely a technical problem involving aeration of the culture. Generally, the suggestions that cultures can undergo cold acclimatization (Bannier and Steponkus, 1976; Sugawara and Sakai, 1978) would indicate that there is scope for improving upon the few successful reports cited here. The caution concerning selection, given in Section 2.3, must be borne in mind.

Henshaw et al. (1979, 1980) have used several modifications of the culture medium to reduce the growth rate of meristem tip cultures of **Solanum** spp. and reduce their subculturing requirement accordingly. Raising the level of sucrose from 3 to 8% and increasing the culture volume from 3.5 to 60 ml improved the net survival of cultures maintained at 10°C for one year from an initial 39% up to 56 and 88% respectively. In cultures grown at 22°C, the addition of mannitol at 6% or abscissic acid (ABA) at 5 mg l^{-1} gave mean survival rates after one years growth of 63 and 43% respectively compared with 14% without the supplements (see above).

Desiccation as a means of storage is described by Jones (1974c). Early stages of somatic embryogenesis in a culture of **Daucus carota** were left on semi-solid medium for up to 2 years at 25°C, whereupon supply of a sucrose solution to the cultures stimulated regrowth. The situation is likened to seed germination. (Note: Nemmer and Luyett, 1954, studied the resistance of seedlings of **Pisum sativum** to drying and demonstrated that the younger the seedling the greater its tolerance of drying; see also Sun, 1958). Nitzsche (1972) has shown survival in callus of **Daucus carota** dehydrated after preculture on medium containing an enhanced sugar content and ABA and then enclosed in a gelatin capsule. The callus could resist freezing down to -80°C .

One report has been made of growth suppression brought about by altering the atmospheric conditions in culture. By lowering the atmospheric pressure to 40mm Hg and the partial pressure of oxygen to 7.6mm Hg, Bridgen et al. (1978) have achieved four fold reductions in the growth rate of callus cultures of Chrysanthemum morifolium, Lycopersicon esculentum and Nicotiana tabacum. Mineral oil overlay, (a method used to store cultures of micro-organisms) has been shown to be effective in the case of callus cultures of Daucus carota (Caplin, 1959). The subculture period of the callus was increased from 4 - 6 weeks up to 5 months. The efficacy of the oil as an overlay is attributed to the higher solubility of oxygen in it compared with in water. In effect this report may be placed in the same category as that of Bridgen et al. (1978).

In Section 6, the storage methods described herein are evaluated critically in comparison with freeze-preservation (see Sections 4 and 5).

4. FREEZE-PRESERVATION: BACKGROUND AND BASIC TECHNIQUES

SYNOPSIS: Freezing at a rapid rate exposes the cell to the risk of ice damage during freezing and ice recrystallization damage during thawing - unless the rates of cooling and warming are sufficiently rapid. Slow freezing permits protective dehydration and thereby prevents ice damage. Either slow continuous cooling or stepwise cooling may be used. Rapid or slow thawing may follow slow freezing. Nucleic acids are very resistant to freezing and thawing; the majority of the components of net cryodamage relate to membrane structure and function. Chemical cryoprotection may relieve damage in either rapidly or slowly frozen specimens. Rates of freezing and thawing, and cryoprotectant treatments must, at present, generally be determined empirically. Commercial freezing units can be used for slow freezing and improvised units for either type of programme. For long-term storage a liquid nitrogen refrigerator is essential. It is important to choose a specimen appropriately in terms of its pregrowth history and/or to apply preparative treatments such as cold hardening and pregrowth in supplemented medium. Appropriate post-thaw treatments are required to support the recovering specimen but this area and that of pregrowth have not been fully explored.

4.1 Introduction

It will be obvious that this section and also the succeeding one are considerably more lengthy than the foregoing one. This reflects the broader attack which has been made through freeze-preservation upon the problems of genetic conservation, the high degree of technical involvement and the potential which it is considered to have for the future. Before describing techniques in freeze-preservation, some general points will be made concerning the freezing and thawing process in living organisms. Then practical aspects of freezing, storage and thawing will be examined, followed by physiological considerations. Detailed results for specific botanical specimens are confined to Section 5.

4.2 The freeze-thaw process

In the literature, extensive treatments of this process have been given; hence only a brief description necessary to an understanding of the principle of freeze-preservation in plant tissues will be given here. The majority of specimens to be frozen will be multicellular, but in the first instance, we can consider a single cell which is being exposed to temperature reduction over a period of time:

Initially, the cell, surrounded by liquid (either intercellular fluid or extracellular culture medium) is cooled to the freezing point of the liquid. Super-cooling may occur but eventually it will freeze due to ice nucleation. Physical disturbance or 'seeding' with an ice crystal will precipitate freezing. The freezing point of the intracellular liquid is likely to be much lower, especially in a chemically cryoprotected

system (see Section 4.3.1), thus the protoplast will not yet freeze. Provided that heat removal does not proceed at too rapid a rate, a process of cellular dehydration will be initiated due to the water vapour pressure deficit established by extracellular ice formation. The concentration of intracellular solutes resulting from dehydration further depresses the freezing point of the protoplast, and the cell can proceed to an even lower temperature without freezing. The process whereby the intracellular water is gradually withdrawn is termed '<u>protective dehydration</u>' since it effectively prevents ice formation in the cytoplasm or vacuole. Ice formation will only occur when the freezing rate overtakes the rate of dehydration and in theory may never occur since all of the 'unbound' water in the cell (see Luyett, 1966 and Meryman, 1966b) may be removed, followed simply by solidification of the cell contents at an ultralow temperature.

If we now consider the case where the freezing rate is so rapid that the freezing point of the intracellular milieu is reached in the early stages of freezing, then ice nuclei will form in the cytoplasm and vacuole, and dehydration will be prevented even with progress to lower temperature (Farrant <u>et al</u>., 1976). The majority, or all of the unbound intracellular water will form ice <u>in situ</u>. The potential disruptive effect of such ice in a highly hydrated specimen can readily be appreciated and it is therefore imperative that the configuration of the ice be compatible with the maintenance of cellular organization if the specimen is to have any chance of survival. Ultra-rapid freezing will be most likely to lead to the formation of microscopically small ice crystals. An intermediate rate of freezing which is too rapid to support protective dehydration may result in the formation of fewer, large, disruptive ice crystals. The size and number of the crystals will be partially dependent upon the presence and nature of chemical cryoprotectants in the system (see Section 4.3.1).

The rate of freezing necessary to induce either extracellular or intracellular freezing will depend upon a number of factors including the permeability of the cell to water; the temperature coefficient of permeability; the number of osmoles of solute in the cell; and the cell surface area to volume ratio. These factors permit prediction of the rate of freezing necessary to prevent intracellular ice formation (in effect the probability of intracellular ice formation at any given rate; Mazur, 1963, 1977).

A complicating factor is that often we are dealing with multicellular systems, and/or closely packed cell aggregates (not necessarily symplastically connected). Theoretical treatments of such systems suggest that the kinetics of water loss in slow freezing can greatly be modified (Levin <u>et al</u>., 1977; Meryman, 1966b). For these reasons, empirical estimations of adequately slow or rapid freezing rates to promote the two contrasting frozen states are more reliable and essential for practical application of freeze-preservation. Furthermore, most specimens will include cells with differing characteristics e.g. cell size and water content.

If the process of thawing is examined for the relatively slowly frozen and the relatively rapidly frozen cell, further potentially hazardous events may be identified. The highly dehydrated cell may simply pass through the melting points of the extracellular and intracellular milieu and achieve complete rehydration during or shortly after complete thawing. The rate of thawing <u>may</u> not be critical but if there is any intracellular ice present rapid thawing may be beneficial (see below) or, conversely if dehydration is extreme, too rapid a rate of rehydration may lead to deplasmolysis injury (see: Asahina, 1978; Farant <u>et al</u>., 1977; Mazur, 1969; Meryman <u>et al</u>., 1977; Towill and Mazur, 1976; Withers, 1979). The rapidly frozen cell containing microscopically

small ice crystals must be thawed rapidly to prevent these ice crystals from thawing momentarily and then 'recrystallizing' to a larger size, thus causing mechanical damage similar to that which would have occurred during freezing at a sub-optimal rate. The actual temperature at which recrystallization may occur can be as low as ca. -80°C (see: Asahina et al., 1970, Nei, 1973; Sakai et al., 1967, 1968) and can persist to relatively high temperatures, thus demanding a very rapid rate of heat supply throughout thawing.

The above considerations have concentrated upon the disposition of intracellular free water/ice during freezing and thawing but fail to take into account the consequences of intracellular solute concentration (beyond the problem of deplasmolysis injury; see above). A high degree of hydration is essential to the maintenance of normal metabolic processes at temperatures compatible with growth. In nature, extreme dehydration always corresponds to a specialised dormancy/storage condition. The cell can tolerate a certain degree of dehydration, but beyond that point the cell will suffer from 'solution effect damage'(see Meryman et al., 1977)due to solute concentraton per se and may suffer mechanical stress due to the development of osmotic resistance to dehydration and compression of the cell contents ('The Minimum-Cell-Volume-Theory: see Meryman, 1974). Solution effects are little understood although familiar in their consequences to those working with plant and animal systems.

From observations that animal cells frequently exhibit an optimum freezing rate - apparently limited in one direction by overdehydration and in the other direction by ice formation within the cell - it has been possible to formulate the 'Two Factor Hypothesis of Freezing Damage' (see Mazur et al., 1972). This hypothesis can meaningfully be applied to many plant systems which have been frozen at a relatively slow rate (see below). Nucleic acids would appear to be remarkably stable in freezing and thawing; (Levitt, 1966) the cell membranes are the main sites of freezing damage. Rupture of organelle and cell boundaries by gross ice formation will lead to a breakdown in inter- and intra-cellular compartmentation. Other manifestations of cryodamage include, quantitative loss of membrane material, selective loss of specific membrane components, increased membrane permeability, loss of specific membrane activities, the accumulation of enzyme reaction intermediates and other solutes including electrolytes, changes in intracellular pH, disturbances in the structured water in proteins, and cross linking of proteins due to molecular proximity (see: Chen and Gusta, 1978b; Heber, 1967, 1968; Levitt, 1966; Mazur, 1966, 1969, 1972; Meryman, 1966b, 1971; and references in Morris, 1980; Withers 1980a; Withers 1978b; Withers and Davey, 1978; Yoshida and Sakai, 1974). Damage in cells and tissues may be detected by the electron microscope (see Robards, 1974; Withers, 1978b; Withers and Davey, 1978) or biophysical/biochemical examination. (Palta et al., 1977c; Yoshida and Sakai, 1974).

Taking into account the above observations, it is possible to predict that there will be two basic approaches to preservation through freezing to ultralow temperatures; namely, rapid freezing which increases in success as the rate of freezing is taken towards the maximum technically possible, or slow freezing at a rate towards the lower end of the scale but limited in that direction by the onset of solution effects. Experimental findings bear out this prediction (see Fig. 1) although it is more appropriate to class the second approach as preservation through extracellular freezing since slow freezing is only one of the possible ways of achieving this process. This being relatively clear, complications only arise when it is necessary to choose the most appropriate method for a given specimen. Despite apparently simple directions in the literature (e.g. in Bajaj and Reinert, 1977), largely based on early studies, clear predictions of the most likely successful rate cannot yet be made although in the relatively near future, it may become possible to make such predictions (see Section 7).

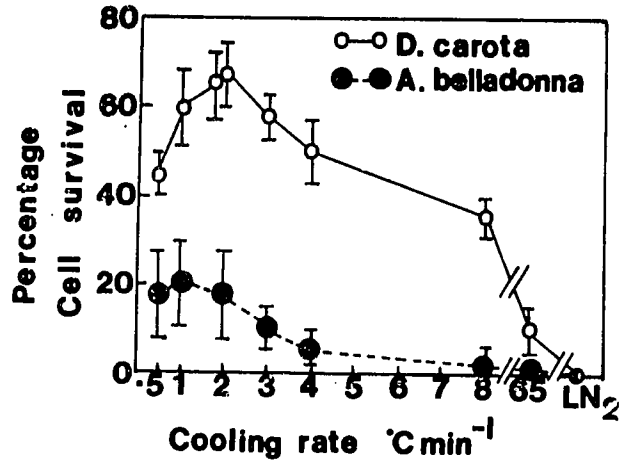


Figure 1a

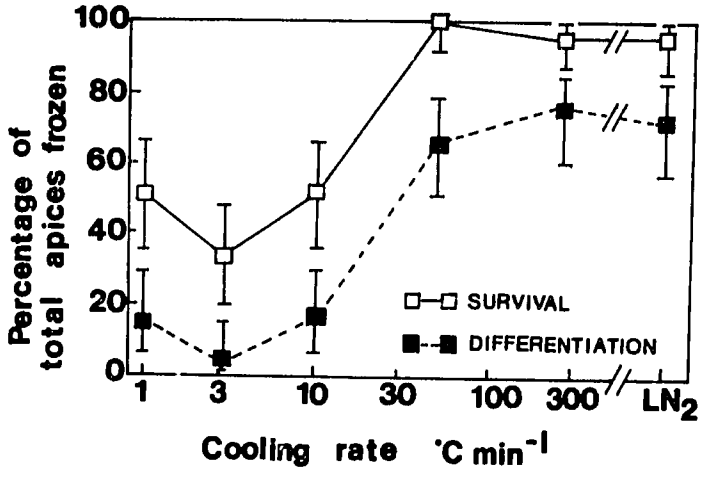


Figure 1b

Figure 1: Freeze-preservation protocols. Plant specimens may be frozen by a method which achieves extracellular freezing and therefore protective dehydration (Figure 1a) or a method which induces the formation of microscopically small intracellular ice crystals (Figure 1b). For convenience these are termed 'slow' and 'rapid' freezing respectively. The relationships between freezing rate and survival can be seen in two contrasting types of specimen: the suspension culture (Figure 1a) and the meristem (Figure 1b). The suspension culture cells survive best when frozen slowly although this is less clear when the basic level of response is poor as in the cells of Atropa belladonna. Rapid freezing (LN = direct immersion in liquid nitrogen) is entirely unsuitable. The survival in the case suspension culture cells is estimated by fluorescein diacetate staining (see Table 2). The meristems of Dianthus caryophyllus shown in Figure 1b survive best at a rapid rate of freezing (more than 50°C min $^{-1}$) although there are indications of a very slow rate of freezing being suitable. Survival was estimated by observation one week after thawing. Differentiation was observed after one month.

(Figure 1a modified from Nag and Street, 1975b; Figure 1b modified from Seibert and Wetherbee, 1977)

4.3 Freeze-preservation protocols

The stages in a freeze-preservation protocol can be broken-down as follows: pre-growth - long term pretreatments, cryoprotective pretreatments, freezing, storage, thawing and recovery. The first and last stages relate to the physiological condition of the specimen over a relatively long time scale as distinct from the other four stages which depend largely upon externally imposed factors. The latter category will be dealt with first.

4.3.1 Cryoprotective pretreatments (short-term)

Specimens can be cryoprotected by reducing the net water content (a process analogous to seed maturation) prior to freezing. The 'drying' occurring during protective dehydration is also a cryoprotective process within the strict definition of the term. However, the term is normally applied to chemical pretreatments, without which very few botanical specimens can survive freezing and thawing (see Withers, 1980a).

Bearing in mind the vast range of lesions against which cryoprotectants must be effective (see Section 4.2), it is not surprising that the range of cryoprotectants is equally wide, often with an increased protection being provided by the use of mixtures. Among the proposed mechanisms by which cryoprotectants are able to reduce cryodamage are: a reduction in the growth rate and size of ice crystals, colligative action in which the effective concentration of solutes in equilibrium with ice inside and outside the cell is lowered, osmotic dehydration before freezing, lowering the freezing point of the intracellular milieu thus facilitating protective dehydration in the early stages of freezing, increased membrane permeability thus aiding water removal from the cell, and stabilization of macromolecules and membranes.

Dimethyl sulphoxide (DMSO) and glycerol are the most commonly employed cryoprotectants. However, despite their frequent use, they do not answer all needs and may in some circumstances be detrimental to the specimen. A number of cryoprotective compounds and mixtures will be effective in slow freezing protocols etc., whereas DMSO alone is likely to be most effective in rapid freezing. (Although the effective action of glycerol can be modified by the temperature of application; see McGann, 1978). The various categories of cryoprotectant are given in Table 1. Further details of the choice of cryoprotectants available and their modes of action can be found in the references cited in connection with freezing injury and: Boutron and Kaufmann, 1978 Darbyshire, 1975; Heber et al., 1971, Heber and Santarius, 1964, Leibo and Mazur, 1970, Sakai and Sugawara, 1978).

Mode of application: once sterilized, preferably by microfiltration (Withers, 1980a) the cryoprotectants are applied to the specimen. This can be done in one of two ways. If the specimen is relatively large it can be transferred through an ascending series of cryoprotectant solutions until the one of desired strength is reached. Small specimens and those already in suspending medium are treated by gradually adding an equal volume of double strength cryoprotectant. Conventionally the application

is carried out after chilling both the specimen and the cryoprotectant but recent findings by Kartha et al., (1979, 1980), and the comment by Morris (1980) to the effect that cryoprotection may be achieved equally satisfactorily by a brief exposure at relatively elevated temperatures as by prolonged exposure at a reduced temperature, with less risk of toxicity (the original reason for the temperature reduction; Nag and Street, 1975a; Withers and Street, 1977a) suggest that this justifies re-examination. Recent observations of the author (Withers, 1980f) in using concentrated cryoprotectant mixtures indicate that the practical nuisance of slow addition and the attendant increased risk of introducing contaminants, may outweigh the advantages of so doing (e.g. reduced plasmolysis damage). Enhanced cryoprotection by a prolonged exposure (more than one or two hours) has been reported in three cases (Kartha et al., 1979; Nag and Street, 1975a; Withers and Street, 1977a) but otherwise there is little justification for this practice.

The cryoprotected specimen is normally dispensed into sterile ampoules, closed and then taken on to the next stage of the procedure. Polypropylene ampoules are superior to glass despite the fact that a flame seal cannot be made. The slightly enhanced exposure to background radiation (see Section 3.3) is balanced by the fact that they are shatterproof thus eliminating the risk to personnel and the risk of cross contamination of specimens in mixed storage. In fact it is very difficult to completely seal glass vials such that liquid nitrogen is excluded and explosions may result during warming (see Simione et al., 1977). In two cases specimens are frozen other than in ampoules: 'dry-frozen' and very rapidly frozen specimens, the latter exposed directly to the coolant (see Sections 4.3.2, 5.5 and 5.6).

4.3.2 Freezing

The freezing process can be carried out using a programmable controlled freezing apparatus to give linear slow freezing rates to a desired sub-zero temperature or step-wise freezing to one or more intermediate temperatures. The Planer (Sunbury on Thames, England) R 201 and 202 (Fig. 2) series have been used in several laboratories and are very suitable for freezing very large numbers of specimens at relatively slow rates. By suspending the specimen at a certain distance above the surface of a volume of liquid nitrogen, it can be cooled at a constantly changing, but highly reproducible rate. The commercially available Biological Freezer Plug modification of the Union Carbide (Co. Durham, England) LR-33 Refrigerator works on this principle, or it is an easy matter to construct such a freezer in the laboratory (Fig. 3a). Placing the specimen within a vacuum flask inside a commercial deep freeze at -70°C has been reported to give steady freezing at ca. 0.1°C min^{-1} (Sala et al., 1979). Other methods of cooling dependent upon the partial insulation of the specimen from a coolant can be found in the literature (see e.g. Finkle et al., 1974). Cooling in an alcohol bath (or one of another low freezing point liquid, e.g. isopentane; Sakai, 1971) with an electrically powered cooling coil can give cooling rates similar to the suspension method if the bath is at ice temperature when the specimen is inserted and the cooling then switched on. Alternatively the bath can be held at a given temperature for step-wise or pre-freezing. An improvised cooling unit as used by Withers and King (1980a) is shown in Fig. 3b.

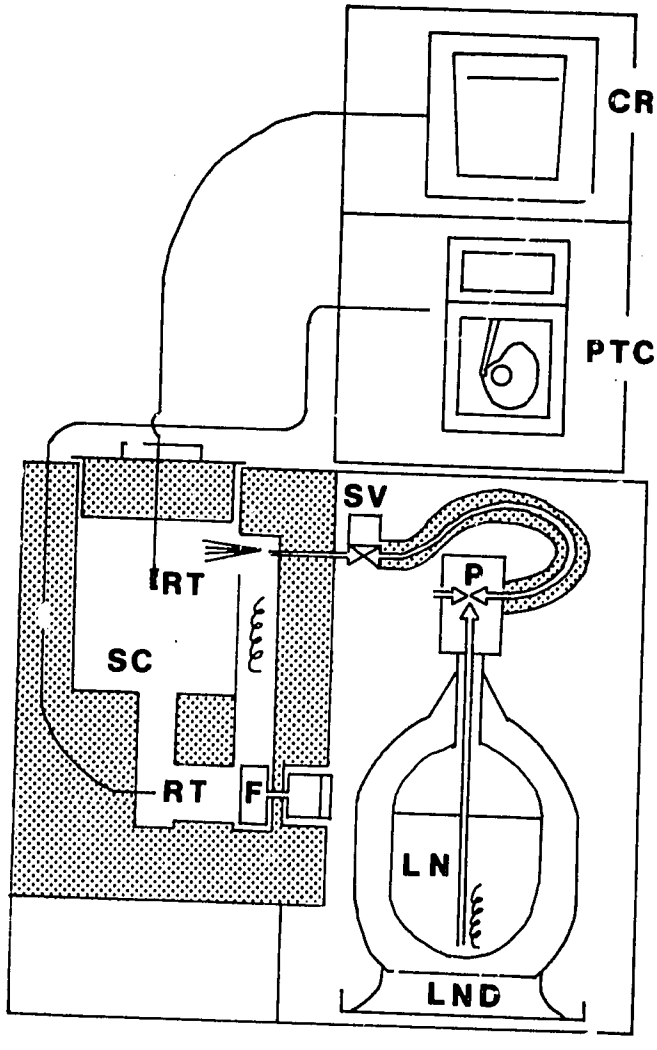


Figure 2: Planer R201 Programmed Freezer
Specimens are placed within the specimen chamber (SC). Liquid nitrogen (LN) from a Dewar vessel (LND) is conveyed by a vapour pressure pump (P) through a solenoid valve (SV) into the chamber. Resistance thermometers (RT) monitor the chamber and specimen temperatures. The rate of cooling is controlled by cam and follower in the programmed temperature control unit (PTC). Nitrogen and warm air are directly alternately into the chamber and the air in the chamber is circulated by a fan (F). A chart recorder (CR) records the specimen temperature. This unit was used in experiments described by Nag and Street, 1975a,b; Withers and Street, 1977a; Withers, 1979; Withers and King 1979a.

Rapid freezing by the direct immersion of the specimen ampoule in liquid nitrogen or a similar coolant can result in rates of freezing of approximately 1000°C min $^{-1}$ but it is extremely difficult to ascertain the true freezing rate of all areas of the specimen. More rapid cooling may be achieved by leaving the ampoule open whilst dipping it into the nitrogen and pouring nitrogen into the open end of the ampoule (Seibert and Wetherbee, 1977). Even more rapid freezing occurs when the specimen is exposed to the coolant without the insulating effects of the ampoule and medium. The specimen is treated with cryoprotectants and then, supported

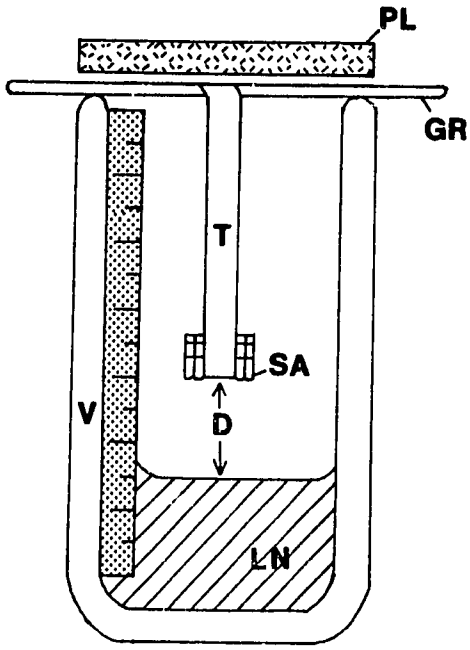


Figure 3a

Figure 3a: Simple freezing unit 1
The specimen ampoules (SA) are suspended by tape (T) from a glass rod (GR) across the top of a Dewar vacuum flask (V). The rate of cooling of the specimens depends upon the distance (D) between the ampoules and the liquid nitrogen (LN). A polystyrene lid (PL) rests over the neck of the flask. (Note: it is dangerous to seal the neck of a flask containing liquid nitrogen).

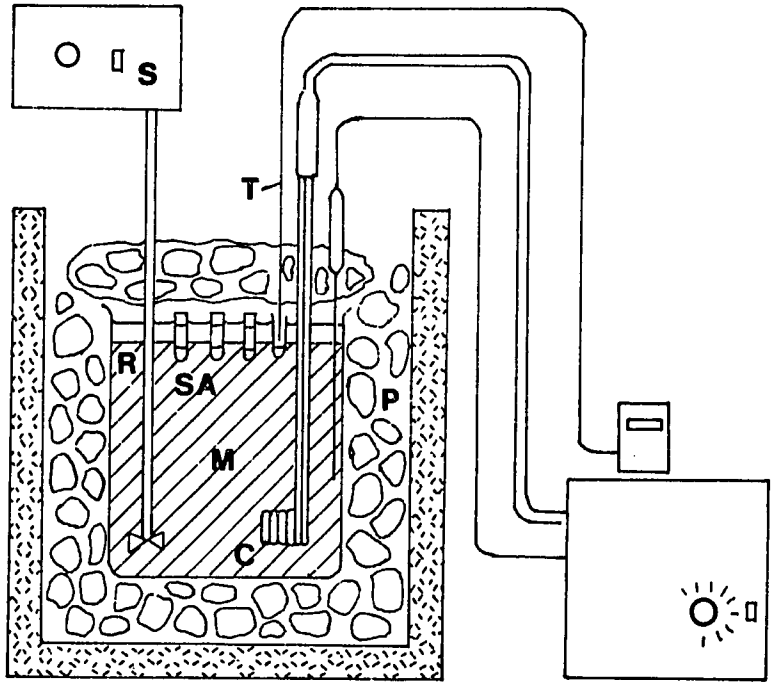


Figure 3b

Figure 3b: Simple freezing unit 2
The specimen ampoules (SA) are inserted in a polystyrene raft (R) floating in a methanol bath (M). The methanol is cooled by a cooling coil (C) and circulated by a stirrer (S). The beaker (B) holding the methanol is separated from an outer bin or box by polystyrene chips (P). A resistance thermometer (T) is inserted into a dummy specimen. The cooling unit is thermostated and can therefore hold a required temperature for a stepwise freezing programme. Alternatively, knowing the cooling capacity of the coil and the volume of methanol, a reproducible non-linear slow cooling may be achieved (from Withers and King 1980a).

on a hypodermic needle, plunged into liquid nitrogen to a predetermined depth (Grout and Henshaw, 1978). The authors do not report any difficulties in maintaining sterility with the latter two methods but it is felt here that caution should apply. Possibly more rapid freezing might be achieved by the use of liquid nitrogen or another coolant 'slush' (which avoids insulation by gaseous nitrogen), by using a high pressure cryostat (Bald and Robards, 1978) or spray-freezing (Plattner et al., 1972).

Fig. 4, illustrates typical freezing and thawing profiles. Details of actual freeze-preservation protocols are given in Section 5 and Table 3.

4.3.3 Storage

Storage conditions can be defined more unequivocally than any other aspect of the process. It is essential to store specimens at a temperature sufficiently low to prevent ice recrystallization (see Section 4.2). Thus laboratory deep-freezers running at -70 to -78°C may be suitable in the short term (Nag and Street, 1973) but an ultralow temperature refrigerator giving a storage temperature of -196°C in the liquid nitrogen or ca. -150°C in the nitrogen vapour is essential for long-term storage. The maintenance of such a refrigerator is technically undemanding and in any case, the most reliable ultralow temperature electrically powered refrigerators running at ca. -100°C normally have a liquid nitrogen dependent emergency back-up system.

4.3.4 Thawing

As can be predicted from comments in Section 4.2, rapid thawing will be beneficial for many specimens and for others there may be no disadvantage in it. Rapid thawing is most easily carried out by exposing the specimen ampoule to water at ca. 40°C. In transferring specimens to and from the storage refrigerator, it is virtually impossible to avoid exposure of the outer surface of the ampoule to microbial contamination. A warm water bath is also a rich source of micro-organisms. Thus it is advisable to carry out the simple precaution of using a small sterile container (e.g. a beaker) of frequently changed sterile water within the water bath, or even on its own for a small number of specimens. Surface sterilization of the ampoules would not appear to be necessary.

Where relatively slow warming can be used, the specimen is simply left in air at room temperature (e.g. Withers, 1979; 1980e) or placed in a current of warm air (Seibert and Wetherbee, 1977).

We can now turn to the remaining two stages listed at the beginning of Section 4.3, pregrowth and recovery. While they do not relate to the freeze-thaw protocol per se it is becoming increasingly clear that if success is to be achieved in all but the most amenable specimens, attention must be paid to the long term preparation for freezing and to nursing cultures after thawing. The attitude that freeze-preservation is a suspension of time only, is fallacious; cells simply cannot be frozen at any stage and 'take up where they left off' after thawing.

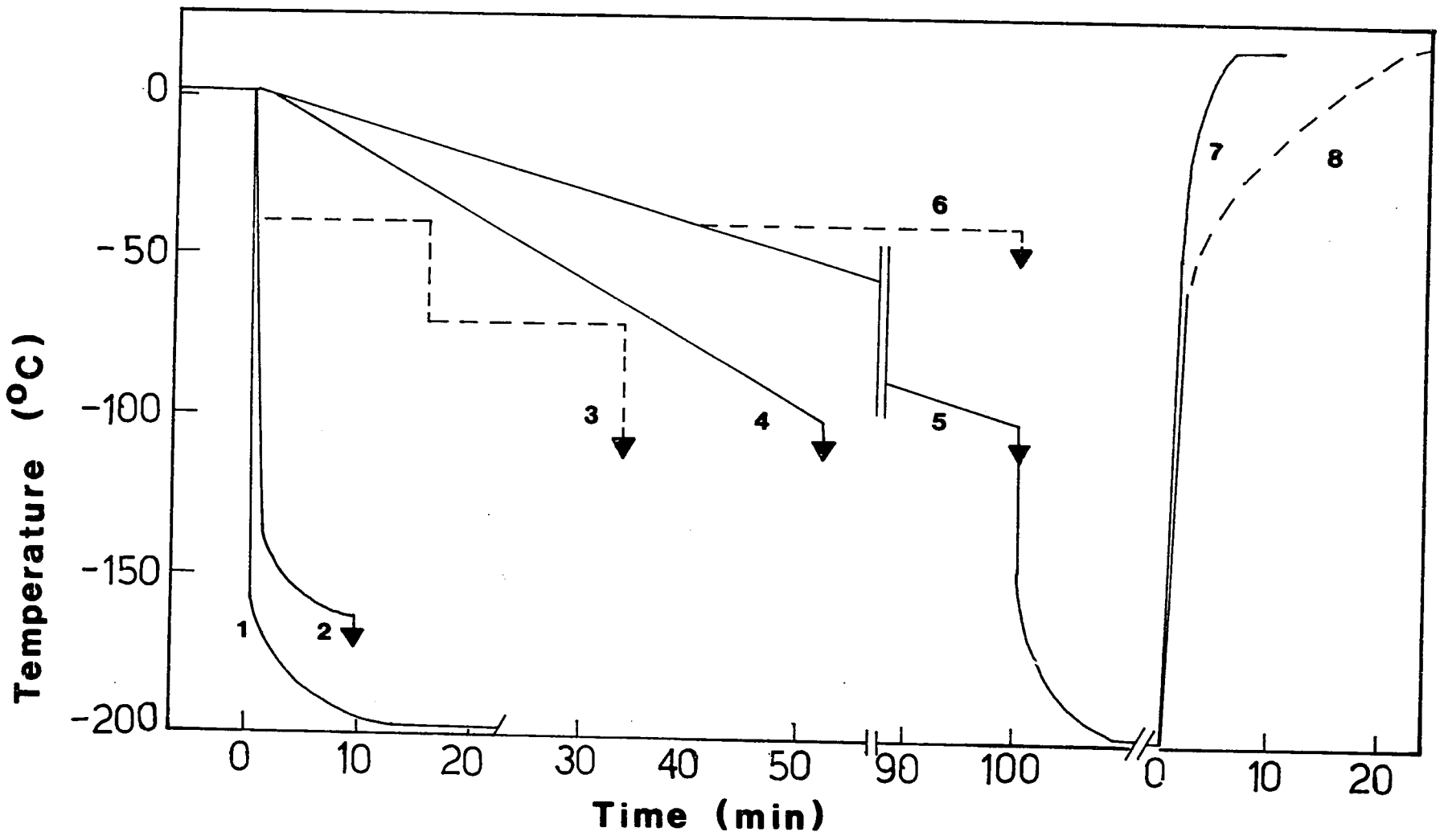


Figure 4 Typical freeze-thaw profiles.

1. Rapid freezing produced by immersing specimen/ampoule in liquid nitrogen
2. Relatively rapid freezing produced by suspending specimen over liquid nitrogen; slope of curve depends upon distance between specimen and liquid nitrogen (see Figure 3)
3. Step-wise or pre-freezing; transition from one step to next may be steep as shown here or more gentle as in the case of a larger specimen
4. Slow freezing at 2°C min $^{-1}$
5. Slow freezing at 1°C min $^{-1}$
6. Slow freezing interrupted by a period at a holding temperature
7. Rapid thawing as in warm water at 35 - 40°C
8. Slow thawing as in air at room temperature

The arrows indicate the termination of the cooling by transfer to liquid nitrogen

4.3.5 Pregrowth

Examples of pregrowth manipulations can be found thoughout the literature, in descriptions of freeze-preservation of a wide range of specimens. Since effective treatments vary considerably according to the type of specimen in use, only general points will be made here, details being given in Section 5.

Cold hardening by exposure to low temperatures prior to storage at ultralow temperatures can be carried out with meristems, callus and twigs, but no evidence of cold hardening in suspension cultures has been presented. Protoplasts isolated from cold hardened tissues may share the low temperature resistance of the parent tissue (e.g. Singh, 1979a). Where it is hoped that the specimen for freezing will share stress tolerance with the tissue or plant from which it is derived, it is important that 'dehardening' does not occur during the period of time prior to actual preservation. There is evidence that this may in fact occur (see Section 5.5). Without entering the controversial area of the physiological basis for cold hardening, two possible contributory phenomena, namely membrane phospholipid modification and solute accumulation are relevant here. The application of compounds such as ethanolamine in order to induce changes in membrane constitution (Ilker et al., 1976) has not been used with any success to increase freeze tolerance. Two reports in the literature lead to the suggestion that there may be scope for manipulating the hormonal content of the culture medium to bring about changes in the composition of the cell membranes. Thus it would appear that pregrowth in the presence of a range of levels of 2,4-D might be worth attempting (Sugawara and Sakai, 1978; Warren and Fowler, 1979). Only one finding in suspension cultures of Daucus carota subjected to 2,4-D withdrawal supports this suggestion (see Section 5.6) but it is worth pursuing. Algal cells grown under autotrophic conditions are more tolerant of freezing than those grown heterotrophically and some relationship to membrane composition can be found (Morris et al., 1977). This might suggest that in cultured higher plant tissues there is scope for increasing freeze tolerance through manipulation of the environmental conditions or the carbon source.

A wide spectrum of higher and lower plants accumulate intracellular solutes in response to stress (e.g. Brown and Hellebust, 1976; Paquin, 1977; Stewart and Lee, 1974; and references in Withers and King, 1979a). Proline is accumulated by plants including Sorghum bicolor and Zea mays exposed to cold or drought (Blum and Ebercon, 1976). This compound is also accumulated by some dicotyledons, many of which also accumulate glycine betaine when under stress (Storey et al., 1977). The precise mechanism of action of these compounds is unknown but in vitro experimentation reveals them to be excellent milieu for carrying out biochemical reactions (Pollard and Wyn-Jones, 1979). It is possible that they in fact 'buffer' the cell against stresses similar to solution effects. (The intracellular effects of apparently different stresses are often very similar; see Levitt, 1978; Trump et al., 1971). From this, two points relevant emerge: that proline or glycine betaine may be effective as applied cryoprotectants and that the application of various stresses prior to freezing may lead to solute

accumulation and increased freeze-tolerance. Both of these suggestions can be borne out in investigations with suspension cultures. Proline is indeed an effective cryoprotectant (Withers and King, 1979a) and both compounds are effective in cryoprotectant mixtures (Withers and King 1979b; 1980b). Osmotic stress applied through the addition of mannitol or proline to the culture medium for a pregrowth period of at least several days is highly effective for suspension cultures (Withers and Street, 1977a; Withers and King, 1979a) but has not been explored for callus, meristem or other cultures. Linking together the possibility of inducing intracellular solutes and hormonal manipulation during pregrowth, is the phenomenon of ABA induced proline accumulation (Rajagopal and Anderson, 1978). Such an effect may underly the improved growth suppression method described by Henshaw et al., 1979; 1980). Preliminary experiments by Withers (1980f) indicate that there may be scope for applying ABA pretreatments to suspension cultures (see Section 5.3).

A final aspect of pregrowth to be considered here is that of choice of culture. A 'passive' means of increasing freeze-tolerance is to time a pregrowth passage in order that cells are at their peak of suitability at the time of freezing. For suspension cultures this normally means cells in exponential growth i.e. when the mean cell size is lowest and the cell has a high cytoplasm to vacuole ratio (Sutton-Jones and Street, 1968; Yeoman and Street, 1977). Examples are given in Section 5.3. Little information is available for other culture forms beyond the observation of an optimum pregrowth culture period for excised meristems (Henshaw et al., 1979; Stamp, 1978).

4.3.6 Post-thaw recovery

It is important to separate factors involved in the initial maintenance of viability in the vulnerable immediate post-thaw period and those involved with the progress and pattern of regrowth. Nursing the culture through an initial recovery period must be the prime aim, after which the manipulation of the culture towards a desired pattern of regrowth is a matter of tissue culture expertise. The latter aspects will be left entirely for consideration in Section 5.

In considering the recovery process per se we should bear in mind the nature of cryoinjury. It can be deduced from observations in Section 4.2 that the cell will be likely to have impaired transport faculties, rendering it susceptible to imbalances in solute and water levels, as uncontrolled influx of medium components or loss of intracellular solutes. Palta and colleagues (1977a,b,c,d) have carried out extensive studies into the condition and responses of tissue pieces exposed to chilling and freezing. An approach similar to that of these workers would greatly help to develop appropriate post-thaw treatments for freeze-preserved specimens, through clearly identifying cellular damage and following post-thaw healing or deterioration.

A wide range of plant and animal systems experience deplasmolysis injury in the immediate post-thaw period (see references cited appropriately in Section 4.2). In an attempt to alleviate this, post-thaw washing procedures involving dilution of the cryoprotectant containing medium over a period of time may be used in preference to a single-step washing (Gresshoff, 1977; Hope, 1977; Jenkins and Weed, 1977; Shillito, 1978; Towill and Mazur, 1976). However, few reports relating to plant tissue cultures include comparisons of differing post-thaw procedures and it is difficult to determine whether indeed the procedures quoted are optimal. Drawing on experiences with suspension cultures, somatic embryos and clonal plantlets, the author has come to the conclusion that any form of post-thaw washing may be harmful and that cultures are most likely to recover if given the minimum of osmotic and manipulative disruption (Withers, 1979; Withers and King, 1979a; 1980a). The assumption that it is necessary to completely remove all traces of cryoprotectant from a specimen is unfounded: in fact there is no concrete evidence for carrying out exhaustive post-thaw washing procedures.

Since deplasmolysis injury is most likely to occur in slowly frozen systems, due to loss of membrane surface area during dehydration and shrinkage (see Meryman et al., 1977; Rochat and Therrien, 1975) it is possible that rapidly frozen specimens, although subject to some membrane lesions, may be less damaged and therefore less sensitive during the post-thaw period. This would appear to be so from an appraisal of the literature but again careful experimentation is lacking in this area.

The possibility of applying supportive post-thaw treatments is virtually unexplored. Suggestions in the literature that certain ions (e.g. Ca^{++}, Sugawara and Sakai, 1975; Ca^{++} and Cl^{-}, Palta et al., 1977d) may alleviate membrane damage or its symptoms when applied either during or after freezing indicate scope for investigation. Shillito (1978) and Withers (1980e) have found that the composition of the medium in which cryoprotectants are prepared can affect viability; this may be due to ionic or organic components of the medium.

Moving to a level more complex than the cellular: it is possible that damaged regions in a specimen may adversely affect neighbouring areas through the release of toxins, and may adversely affect the progress of recovery in the specimen as a whole through destroying symplastic continuity. In the case of recovering somatic embryos and clonal plantlets, the former problem can be tackled by the use of a specific medium supplement but the latter presents a more serious problem (Withers, 1979; see Section 5.6). The physical removal of damaged areas of tissue has been attempted in one case only (Dale and Withers, 1980; see Section 5.6); and the dissection out of the desired region from a frozen specimen in one other case (Bajaj, 1977a; see Section 5.5).

Little attention has been given to an examination of the environmental conditions during the post-thaw period. One report indicates that reduced lighting should be used but there has been no investigation of other physical aspects (Grout and Henshaw, 1978).

Much of the information available relating to the progress of recovery in frozen and thawed specimens involves the use of specific viability tests. These are not definitive indicators of survival potential but are a valuable means of screening multiple specimens if used with caution (Palta et al., 1978; Withers, 1980a). Details of three commonly used ones are given in Table 2. Biomass estimation may also be used to express recovery growth. Technical details of appropriate methods can be found in Street, 1977b).

5. FREEZE - PRESERVATION: RESULTS

SYNOPSIS: Success in freeze-preservation has been reported across the spectrum of tissue culture systems from protoplasts to highly organised plantlets. Pollen and protoplasts are responsive to a wide range of techniques indicating their greater tolerance of freezing and thawing. Pollen alone can be freeze-dried. Anthers are difficult to preserve although pollen embryos can survive slow freezing. In the case of suspension cultures, the response varies widely from species to species. By manipulating pregrowth, cryo-protection, freezing, thawing and post-thaw conditions, survival at a very high percentage may be obtained but not yet in all species. For suspension cultures, slow or step-wise freezing may be used; rapid freezing is entirely unsuitable. Little work has been done with callus. Meristems and shoot-tips of various origins can be preserved by rapid slow or step-wise freezing, but only slow freezing has been successful with *in vitro* propagated meristems. Somatic embryos show a decline in survival potential as their development progresses. This can partially be overcome by the use of the 'dry-freezing' technique, but only meristem regions survive in advanced clonal plantlets. Germinated seedlings are similarly vulnerable and are most responsive to relatively rapid freezing. Drying prior to freezing may improve survival potential in clonal plantlets, zygotic embryos and seed. Buds and twigs excised from mature plants can be preserved by pre-freezing.

5.1 Introduction

In this section the various types of tissue culture will be examined and an assessment made of the present level of expertise in their preservation. The distinctions between the types are not absolutely rigid (especially in the suspension/callus/organ region) but an approach through the ascending order of complexity will be made. Seed, zygotic embryos and mature plant pieces are also considered here for two reasons: *in vitro* approaches or related techniques may be useful for their preservation; and, through attempting to preserve these structures, information may be gained concerning the response of various true tissue culture systems to freezing and thawing.

5.2 Protoplasts

Some effort has been devoted relatively recently to an examination of the response of protoplasts to freezing and thawing. From such studies it may be possible to learn much of relevance to other systems in terms of plasmalemma damage and osmotic responses of cryodamaged cells. However, such studies serve cryopreservation and cryobiology as a whole rather than relating directly to conservation technology (see: e.g. Siminovitch *et al.*, 1978; Weist and Steponkus, 1978). A consequence of the generally biophysical approach is that there has been little interest in long-term maintenance of viability in preserved protoplasts - let alone wall regeneration and development of plants from the protoplasts.

Two examples wherein cultures were recovered from preserved protoplasts indicate that, given a system within which protoplast regeneration is an established procedure, regeneration from freeze-preserved protoplasts should be possible. Withers and Street (1977b, Withers 1980a) have demonstrated survival after slow or rapid freezing, recovery in droplet culture, regeneration and somatic embryogenesis in protoplasts of Daucus carota. Details of the preservation procedure are given in Table 2. Generally, viability percentages are relatively high for protoplasts. This is certainly due in part to the fact that damaged protoplasts will disintegrate thereby biasing the viability data. However, the protoplast isolation procedure involves plasmolysis which in itself is cryoprotective, and the trauma of protoplast isolation is likely to eliminate very sensitive protoplasts before freezing is attempted. In the case of protoplasts of Daucus carota, the initial viability was ca. 90%. In the second example of culture regeneration from freeze-preserved protoplasts of Zea mays (Withers, 1980f) a lower initial viability (ca. 25%) was recorded but control preparations were similarly unhealthy. Mazur and Hartmann (1979) have compared the survival after freezing and thawing of cells and protoplasts of Bromus inermis and Daucus carota cryopreserved with either glycerol or DMSO. With the former cryoprotectant protoplasts survived twice as well as cells (e.g. 68% and 38% respectively). This difference was not found in the case of DMSO protected specimens. The authors indicated that "an equilibrium period prior to freezing was essential for the survival of the protoplasts". Recovery was not reported.

5.3 Pollen

The storage of pollen can play a valuable role in conservation through facilitating fertilization outside seasonal and geographical limitations and ensuring supplies of recalcitrant pollen (Roberts, 1975). Additionally, in the present context, pollen can be thought of as a potential tissue culture system from which haploid and homozygous diploid tissues and plants can be regenerated for use in novel and conventional breeding studies (see: Kasha, 1974; and Keller and Stringam, 1978).

Pollen alone can be preserved by freeze-drying or lyophilization (King, 1965). This is almost certainly due to its low water content and unique morphology. It is highly unlikely that such a technique could be developed for other systems in higher plants. Reports of successful freeze-preservation of pollen span a quarter of a century (e.g. Visser, 1955; Nath and Anderson, 1975; Barnabas and Rajki, 1976, Weatherhead and Grout, 1979 and references therein). Both rapid and slow freezing would appear to be appropriate, without the necessity for chemical cryoprotection, although drying prior to freezing is beneficial in some cases (Barnabas and Rajki, 1976). The pollen is enclosed in a vial, frozen appropriately, transferred to storage and then thawed by immersion in a warm water bath. Viability can be tested by in vitro germination or fertilization. Weatherhead et al. (1979) demonstrated that storage at -196°C was superior to storage at -20°C for pollen of Solanum spp. when preservation for more than a matter of days is required, indicating that recrystallization may be occurring at -20°C. Thus although the basic response of pollen suggests that it may be relatively easy to preserve, it would appear that deterioration phenomena common in other systems may be operating here.

Where pollen storage is required for subsequent pollen plant or haploid tissue induction, it may be more appropriate to preserve the anther as a whole since the culture of isolated pollen may present problems. Withers and Batchelor (1980) have attempted to freeze-preserve anthers of Nicotiana sylvestris and N. tabacum with little

success. The only suggestion of viability being maintained was in the case of anthers which had been perforated prior to preservation to permit cryoprotection penetration and were then frozen very rapidly. Bajaj (1978b,1979) has carried out more extensive studies using anthers of N. tabacum, Petunia hybrida and Atropa belladonna. Slow freezing was unsuccessful and rapid freezing only yielded occasional callus formation after a period of 2 - 3 months. A preculture period of 3 - 4 weeks improved the response and survival was recorded in 1.5 to 5.2% of the anthers. A further improvement resulted from transverse cutting of the anthers (up to 6.6% survival). Further details are given in Table 3. Since the callusing and embryogenesis are occurring during the pregrowth period, this method would appear to have no advantage over freeze preservation of induced tissues or embryos which can be handled more easily (see Sections 5.4 and 5.6).

To the author's knowledge, there are no reports of the successful preservation of ovules, ovaries or derived tissue cultures. This area warrants attention along with further investigations with pollen and anthers.

5.4 Suspension cultures and callus

'Suspension cultures' are most frequently used for physiological studies, being as near as one can approach to the ideal single cell systems of microbiology envied by the tissue culturist. It is important however, to envisage the range of structures which can be embraced by the term. Single cells interspersed with relatively small aggregates, containing tens of cells, most of which will be in direct contact with the culture medium, can be found at one extreme. At the other extreme are large dense aggregates containing thousands of cells. In the latter case there may be a distinct stratification of the aggregate, meristematic cells lying superficially and older, more vacuolated cells lying centrally. Additionally malformed structures such as roots and shoots, and even perfectly formed primordia can be found intermingled with unorganised cell masses (see King et al., 1978). In many cases it must be said that the use of the term suspension culture reflects the technical approach, i.e. growth in liquid medium, rather than the morphology of the culture. For this reason, although the majority of this section is devoted to suspension cultures, so called, the few examples describing 'callus' freeze-preservation are included here also.

The data available on the freeze-preservation of suspension cultures are relatively extensive. Therefore, in this section some of the general points made in Section 4 will be expanded upon using illustrative examples; a full review of techniques and results is given in Table 3.

Selection of the most appropriate growth stage is essential for suspension cultures. Sala et al. (1979) and Withers and Street (1977a) provided quantitative data of viability and regrowth potential substantiating the superiority of cells in late lag and early/mid exponential phase (see Street, 1977b). Cells in early lag phase and stationary phase are large, highly vacuolated and have a high percentage water content. These factors reduce survival potential by increasing the requirement for protective dehydration whilst reducing the cell's capacity to dehydrate through a reduction in the cell's surface area to volume ratio (see Section 4.2), and increasing its susceptibility to plasmolysis and deplasmolysis injury. The elimination of stationary and lag phases by a rapid subculture routine, e.g. four-fold dilution in fresh culture medium every 7 days (Withers and Street, 1977a) can improve the response in some cases, and is technically advantageous since it removes the necessity to monitor the growth stage of the

culture continuously. In the cases of suspension cultures of **Capsicum annuum** and **Acer pseudoplatanus** this rapid subculture routine improved the survival potential of the cells but much more marked improvements were brought about by supplementing the culture medium with mannitol (Withers and Street, 1977a). Growth in the presence of mannitol at a level of 3.3% will reduce the mean cell volume of **A. pseudoplatanus** by ca. 55% within a few passages and the survival potential is increased accordingly. More recent experiments in which cells of this species and **Rosa** spp., **Nicotiana tabacum**, **Hyoscyamus muticus**, Sorghum bicolor and Zea mays are pregrown in the presence of mannitol at up to 6% or in the presence of proline at up to 10%, suggest that this type of pregrowth manipulation may have a wide application (Withers and King, 1979a; 1980a). Latta (1971) used a pregrowth supplement of 6.5% sucrose in the preservation of cultures of **Ipomoea** sp.

Two cautions should be applied to the use of rapid subculturing and the use of pregrowth supplements: firstly, it would appear that a species which has an intrinsically small cell size (e.g. Daucus carota) will not respond by either a cell size reduction or improvement in freeze-tolerance (Withers and Street, 1977a). Secondly, as demonstrated with a suspension culture of A. pseudoplatanus, rapid subculturing may induce a partial synchronization of cell division and a consequent oscillation in post-thaw viabilities (Withers, 1978a; Withers and Street, 1977a). Subsequent examination of an intentionally synchronised culture, indicated that the apparent cell cycle effect on freeze-tolerance was due to an enhanced survival capacity in cells which had newly entered G_1 stage (or G_0) as determined by nuclear DNA estimations. Cells actually in mitosis were particularly sensitive to freezing and thawing (Withers, 1978a). The relatively poor degree of synchrony possible at any one time and the rather demanding manipulations necessary serve as an argument against synchronization as a routine pretreatment.

Pregrowth in the presence of cryoprotectants is reported as a means of testing for cryoprotectant toxicity (e.g. Dougall and Wetherell, 1974) and as a possible freeze-tolerance inducing pretreatment. Nag and Street (1975a) describe an improvement in the viability of cells of Atropa belladonna after pregrowth in the presence of 5% DMSO (40% cf. 30%); a more dramatic increase (60% cf. 15%) being reported in the case of cells of Zea mays pregrown in the presence of 10% proline (Withers and King, 1979a; see above also). The latter treatment is unique in that it is the only example of cells being pregrown and frozen in the same culture medium; thus the additive is compatible with both growth and cryoprotection. In the case of the cells of **A. belladonna** further cryoprotection in addition to the 5% DMSO was required before freezing (Nag and Street, 1975a).

Hormonal manipulations in the pregrowth period have not been reported. Withers (1979) noted that in the early stages of embryogenesis in Daucus carota, 2,4-D withdrawal coincides with a transient increase in freeze tolerance. At this time the cells are highly cytoplasmic and are embarking on a rapid series of divisions involved with embryo formation; this, or a physiological change relating to cold hardening (see Section 4.3.1) may account for the increase. In an attempt to improve the response of cells of Zea mays to freezing and thawing, Withers (1980f) applied ABA during a pregrowth period of several days. Cells frozen immediately after pregrowth in the presence of this supplement did not survive any better than controls, but cells released from pregrowth in the presence of ABA and grown for several days in standard medium did show an increased survival. Rather than through the cold-hardening effect and solute

accumulation expected (see Section 4.3.1), it is possible that this treatment improved freeze tolerance by inducing a growth spurt once the cells had been released from ABA inhibition of growth. This phenomenon justifies further investigation.

Cryoprotection

A wide range of cryoprotectants have been used for the preservation of suspension cultures. DMSO or DMSO plus glycerol have been used widely at levels between 0.5 and 1.5 molar (total molarity). There is little evidence for serious cryoprotectant toxicity. Recently there has been an increased interest in developing complex cryoprotectant mixtures for use with suspension and callus cultures. Finkle and Ulrich have examined closely the response of cells of Saccharum sp. to cryoprotectant mixtures containing two or three of the following: DMSO, glycerol ethylene glycol, glucose, polyethylene glycol (Finkle and Ulrich, 1978, 1979). They conclude that mixing cryoprotectants is beneficial and improves freeze-tolerance more than would be expected on a molar basis. Generally a total molarity of 1.9 is optimum with DMSO plus glucose, or polyethylene glycol plus glucose plus DMSO being the most effective combinations. Withers and King have attempted to preserve cells of Zea mays after cryoprotection with three component mixtures (Withers and King, 1979b; 1980a,b). In addition to a basic mixture of DMSO and glycerol, a third component is chosen from a range of sugars, sugar alcohols or amino acids (see Table 1), giving a total molarity of 2. Many such mixtures are suitable and, in combination with a appropriate freeze preservation programme can yield very high viability levels and rapid recovery (see Table 3). It is interesting that all of the cryoprotectant mixtures tried are most effective when made up in culture medium. If the medium constituents are omitted viability is reduced except when sucrose is the third component of the cryoprotectant mixture (Withers 1980e). Further, all cells are very sensitive to deplasmolysis injury, but those for which proline was the third component of the mixture were least sensitive. Where cryoprotectants were used singly, made up in water, only proline was effective. The membrane stabilizing properties of this compound are relevant here (Heber et al., 1971). It is important to point out, in the light of the above results, that reports in which cryoprotectants are described as being used 'singly', they are in fact almost invariably made up in culture medium, not water.

The choice of a suitable freezing programme

There are no examples of successful preservation when rapid freezing is employed. It is essential to use a freezing method which brings about extracellular freezing. Thus slow (Figure 1a), stepwise or pre-freezing may be suitable. Often the difference between these is virtually a semantic one; e.g. the tight progression through steps of -15, -23, -30, -40, -50, -70°C before immersion in liquid nitrogen as described by Sugawara and Sakai (1974) is not likely to be very different in its effects from a linear or non-linear continuous cooling. Bajaj (1976) described a simpler three-step (-20, -70, -196°C) series and Withers and King (1979a,b; 1980a) combined slow (linear or non-linear) freezing with holding at a sub-zero temperature. These authors found that the programme: 1°C min^{-1} to -30 or -35°C followed by holding at that temperature for 30 - 40 minutes then immersion in liquid nitrogen was suitable for a wide range of suspension cultures cryoprotected with 2 molar concentrations of three-component mixtures. This type of programme is in fact superior to continuous linear slow cooling wherein the specimen is cooled beyond 30 - 35°C before immersion in liquid nitrogen. This is thought to be due to the fact that once protective dehydration has ensued during

the holding period or the initial slow cooling period, further slow cooling is detrimental since it permits slow growth of ice crystals from any remaining intracellular free water, whereas the rapid immersion in liquid nitrogen from the holding temperature prevents dehydration progressing towards a level where solution effects become serious and leads to microscopical ice crystal formation. It is likely that in early reports, where slow cooling was continued to temperatures as low as -100°C potentially viable cells were lost during the latter stages of freezing (e.g. Nag and Street, 1975a,b; Withers and Street, 1977a); (see also Section 5.6).

A further comment which must be made concerning the terminal freezing temperature relates to studies where cells were cooled only to ca. -30 or -40°C before thawing and testing (e.g. Finkle et al., 1975; Finkle and Ulrich, 1979; Withers and Street, 1977a) or where it is unclear whether cells were cooled only to an intermediate temperature (ca. -20°C) or from that temperature to -196°C (e.g. Bajaj and Reinert, 1977; Bajaj, 1976). At such intermediate temperatures the intracellular milieu may well not have frozen and the procedures are erroneously termed 'freeze-preservation'.

Suspension cultures are thawed rapidly in the vast majority of cases; this underlines the comment made above concerning transfer temperatures. The beneficial effect of rapid thawing suggests that the cells do indeed contain ice even when frozen slowly. Exceptions to this include survival in cells of Daucus carota (Dougall and Wetherell, 1974, Nag and Street, 1975b) and Zea mays (Withers, 1980e) after slow thawing. In the former case the generally lower water content of these small, highly cytoplasmic cells may be responsible; in the latter case slow thawing was only possible when the cells had been cryoprotected with DMSO, glycerol and sucrose. Overall, the range of freezing and thawing conditions which are possible with cells cryoprotected with this three component mixture is wider than might be expected - suggesting that its use may reduce the presently strict requirement to determine optimum freezing and thawing conditions. Further investigation is necessary to substantiate this.

In much of the early work on the freeze-preservation of suspension cultures, it was generally assumed that the cells could be returned to culture in liquid medium and achieve recovery growth if the initial inocculum was sufficiently high (see Withers and Street, 1977a). Indeed there are several reports of successful regrowth under these circumstances (e.g. Sala et al., 1979; Nag and Street, 1975a.b; Sugawara and Sakai, 1974; Dougall and Wetherell, 1974; Withers and Street, 1977a). It now appears likely, however, that this treatment may be deleterious and may account for many instances where initially high viabilities suffered a protracted or rapid decline followed by death of the culture. An examination by Withers and King (1979a) of the scope for variation of the post-thaw culture conditions for suspension cultured cells of Zea mays reveals that dilution in liquid medium is harmful but less so than washing with medium or water to remove cryoprotectants completely. Droplet culture (Potrykus et al., 1979) may sustain viability but requires renewal of medium after a few days. Only cells reintroduced into culture by layering over semi-solid medium recovered growth rapidly. When this growth method is combined with an optimised freeze-thaw protocol it is possible to recover cultures which resume mitotic activity within two days of thawing and which can be transferred to liquid medium within one or two weeks. It is assumed that recovery ensues rapidly because the cells are not subjected to deplasmolysis injury and have access to fresh nutrients by diffusion from the semi-solid medium. A further observation that removal of the suspending medium once the cells have been returned to growth over semi-solid medium is detrimental would suggest that uptake of constituents of the suspending medium is an important component of recovery.

The pattern of recovery growth in quantitative terms has been monitored in two studies. Sala et al. (1979) reported that cells of Oryza sativa will resume growth within a very few days of thawing and increase in biomass at the same rate as control cultures. Withers and Street, (1977a) examined the rate of increase in cell number in suspension cultured cells of Daucus carota and found that the rate was reduced during the recovery passage (in liquid medium) but picked up in subsequent passages. More recent observations of a range of cultures recovering over semi-solid medium (Withers and King, 1980a) suggest that the rate of growth (as measured by increase in percentage viability) will normally approximate to that of control cultures although there will almost always be a lag period before growth is resumed.

Little work has been done on the physiology of cells during the recovery period. Cella, Sala and colleagues (Cella et al., 1978; Sala et al., 1979; Sala et al., 1980) have examined cells of Oryza sativa, looking at tetrazolium chloride reduction (TTC test; see Table 2), coupled and uncoupled respiration, and stability of protoplasts isolated from the cells. It appears that the thawed cells have both a respiratory lesion and structural/osmoregulatory lesion leading to protoplast instability, but both of these are 'healed' within the three to four days after which growth is resumed. Withers and Davey (Withers, 1978b; Withers and Davey, 1978) have carried out electron microscopical examination of frozen and thawed cells in which it is clear that disturbance of the osmoregulatory capacity of organelles is occurring (a common symptom of cytotoxicity or injury; Trump et al., 1971). These observations are consistent with the quantitative data on recovery growth which suggest that a brief recovery period is inevitable during which essential healing processes occur. A preliminary observation by Withers and Street (1977a) suggests that the supply of 'conditioning factors' (Stuart and Street, 1971) to the recovering cells may aid regrowth. The scope for applying supportive post thaw treatments is vast and largely unexplored.

Once cultures have become established, it is important that specific characteristics are tested. This has been covered largely in Section 2.3 but the main point will be reiterated here. There is no evidence for the loss of morphogenic potential during freeze-storage, or evidence for the modification of the pattern of growth in non-morphogenic species. Unfortunately some of the suspension cultures to which most attention has been devoted (e.g. Zea mays, Rosa sp. and Acer pseudoplatanus) are in the form of very fine suspensions and very suitable for freezing but they are 'cell lines' (see Section 6.1) and therefore will not regenerate plants. Hyoscyamus muticus is however morphogenic and in terms of its physical characteristics is far from suitable for freeze-preservation, being highly aggregated and containing deformed primordia. Therefore it is very encouraging that plants can be recovered from freeze-preserved cultures of this species (Withers and King, 1980a), presaging well for the use of this type of culture and preservation method for conservation (see Section 6.1).

The latter example of successful recovery is placed in the category of suspension cultures by virtue of the fact that it was pregrown in liquid shake culture. However it could easily have been pregrown on semi-solid medium and yielded a similarly structured culture. Looking now at cultures which have been pregrown in this way and which will be referred to as 'callus': rapid freezing can be ruled out for callus

pieces since it would be virtually impossible to ensure an even rate of cooling. Cryoprotectant penetration is likely to be a problem in the case of callus cultures, and it is interesting that one example of successful preservation omits a chemical cryoprotectant stage. Sakai and Sugawara (1973) induced callus formation in cambial tissue excised from twigs of Populus euramericana and hardened the callus by, for example, 60 days under an 8 hour short day regime with a daytime temperature of 15°C and a night time temperature of 0°C, followed by 20 days at 0°C. Freezing at a rate of 5°C per day to -30°C, followed by immersion in liquid nitrogen and then slow thawing, yielded specimens which could resume growth normally. The method is attractive in its simplicity involving only the cold-hardening and the transfer of the callus to fresh medium after thawing; the possibility of wider use with tissues which will respond to cold-hardening should be explored.

A second example of successful freeze-preservation of callus is described by Ulrich et al. (1979). Callus of Saccharum sp. cryoprotected with a mixture of DMSO, glycerol and polyethylene glycol was frozen slowly (2°C min $^{-1}$ to -40°C, then 5°C min $^{-1}$ to -80°C followed by immersion in liquid nitrogen). A 'rapid' thawing rate is quoted without an actual figure being given and it must be assumed that only rates slower than in the case of fine suspension cultures are likely to be possible with relatively large callus pieces. Viability values are difficult to determine in callus but the authors reported regrowth of pieces of callus within a few weeks of thawing. This result is significant since Saccharum plants cannot tolerate prolonged exposure to temperatures in the range +2 to -3°C, yet with adequate cryoprotection the callus can be preserved at ultra-low temperatures. From this callus, rootlet formation was observed and the authors anticipated (but do not confirm) whole plant regeneration, control callus having this capacity.

Sakai and Nishi (1978) quote an unpublished finding of Yoshida in which callus of Sambucus racemosa could be hardened such that it could tolerate exposure to -196°C. No further details are given.

5.5 Shoot-tips and meristems

In this category are included shoot meristem tissues consisting of an apical dome and up to 4 sets of leaf primordia. Larger structures (meristems enclosed within buds, seedling apical regions and embryo meristems are included in Section 5.6) with the one exception of meristems of Manihot utilissima (Bajaj, 1977a) which were frozen as buds but cultured as meristems after dissection.

Several studies have been carried out to explore the possibility of using this type of culture for genetic conservation through freeze-preservation and the results justify optimism. Some reports suggest that rapid freezing provides the most suitable preservation method and this is a conclusion reached in a recent overview (Henshaw, 1979) however, when the literature are closely examined, the picture is less conclusive.

One report of successful preservation of meristems describes a cold-hardening treatment prior to freezing. Recovery of meristems of Dianthus caryophyllus was increased by more than 100% by exposure of the parent plant to a temperature of 4°C for at least 3 days before meristem excision (Seibert and Wetherbee, 1977). Other attempts to induce cold hardiness have failed or were even detrimental (Kartha et al., 1979; Sakai et al., 1978). In the case of meristems excised from plants of Solanum goniocalyx, a preincubation period is necessary for optimisation of survival (Stamp,

1978; Henshaw et al. 1979). Freshly excised meristems exhibit a survival percentage of ca. 7% whereas incubation for ca. 28 to 73 hours increases freeze tolerance such that recovery occurs in ca. 55 to 60% of the meristems. Longer incubation periods are detrimental. Kartha et al. (1979; 1980) recommend a two day incubation in the presence of 5% DMSO for the preservation of meristems of Pisum sativum and Fragaria x ananassa. However, in these cases, the authors do not appear to have carried out a preincubation in medium alone. The control used is a freshly excised preparation; therefore, the real requirement may be for incubation per se, rather than a long term exposure to cryoprotectant. Such a period may permit healing of the excision wound.

In the most extensive study to date into the relationship between survival and freezing rate, Seibert and Wetherbee (1977) concluded that for the successful preservation of meristems of Dianthus caryophyllus, a freezing rate of at least 50°C min $^{-1}$ is necessary. In fact if their data are examined (Fig. 1b), it can be seen that there is some survival at intermediate freezing rates and an upturn as the lowest rate is reached. Perhaps an even lower rate would be superior (see below)? Rapid thawing at an initial rate of between 1000 and 2000°C min $^{-1}$ is vastly superior to slow thawing ca. 130°C min $^{-1}$) although even at the latter rate there is some survival (20 - 30%).

Support for the use of rapid freezing is offered by the work of Grout and Henshaw (1978) in which meristems of Solanum goniocalyx were preserved successfully by freezing at the most rapid rate available - by plunging into liquid nitrogen, meristems carried on hypodermic needles (see Section 4.3.2). Survival percentages of up to 60% are recorded for this species. Attempts to extend the method to include other species within this genus failed initially (Grout, personal communication) but more recently Stamp (1978) and Henshaw et al. (1979) have succeeded in recovering up to 36% of frozen meristems of S. tuberosum subsp. andigena and between 2 and 32% of meristems of S. tuberosum subsp. tuberosum (see below). In all of the above examples the cryoprotectant used was DMSO at a level of up to 15%. (See also Bajaj, 1978a).

Several workers have attempted to preserve meristems of Manihot spp. but with less success than in earlier examples. Stamp (1978) has achieved 3-8% recovery using the same method as for Solanum spp. and Bajaj (1977a) reported a survival rate of 21% (13% plantlet formation plus 8% callusing) for meristems dissected from buds which had been cryoprotected with 10% glycerol plus 5% sucrose, frozen rapidly and thawed rapidly (other studies with Manihot esculentum are under way: Kartha personal communication; Roca (1978)).

Five further examples of the successful preservation of meristems suggest that freezing methods dependent upon extracellular freezing may be very suitable for this type of specimen. Kartha et al. (1979, 1980) have successfully preserved meristems of Pisum sativum and Fragaria x ananassa by a slow freezing technique. In the former case upto 73% plantlet regeneration was achieved using a freezing rate of 0.6°C min $^{-1}$ to -40°C followed by storage in liquid nitrogen and rapid thawing. In the latter case a freezing rate of 0.84°C was most suitable, yielding between 50 and 95% survival and plant regeneration (see Section 2.3). (A similar approach with meristems of Cicer arietinum has given ca. 40% survival in a preliminary study (Kartha personal communication)). Sakai et al. (1978) have preserved excised runner meristems/apices of Fragaria sp. by a pre-freezing technique. After freezing to -20 or -30°C, in a stepwise manner (ca. 2.5°C min -1) the specimens, supported on cover glasses were plunged into liquid nitrogen. A survival rate between 60 and 80% was recorded. Only ca. 16%

survival resulted if the meristems were frozen in a vial by plunging from a pre-freezing temperature of -40°C, or 40 - 60% survival if rapidly frozen from room temperature using the cover glass method. The open vial method of Seibert and Wetherbee (above and Section 4.3.2) failed completely with this material. Preliminary observations by Towill (1979) and Anderson (1979) indicate that slow freezing may be successful for meristems of Solanum tuberosum and Dianthus caryophyllus respectively.

To summarize the findings described above, it is clear that meristems may be preserved by a range of methods and not just variations on the theme of rapid freezing. This is discussed further in Section 6.2.

After thawing and washing, regeneration of plants has been attempted in all of the cases quoted above, usually successfully. (It is interesting that in both studies by Kartha et al. (1979, 1980) the use of glycerol was unsuccessful - this could be an indication of deplasmolysis injury during post-thaw washing; no immediate post-thaw viability test was carried out). Plant regeneration values are usually less than the total 'recovery' percentages. Thus in the case of Dianthus caryophyllus (Seibert and Wetherbee, 1977) of the 90% survival recorded after one week, only 70% differentiation into plants occurred (Figure 1b); for 60% survival, only 17% differentiation was recorded. In the case of Solanum goniocalyx (Grout and Henshaw, 1978, 1980) the (maximum) appropriate values are 76 and 63%; for Manihot utilissima (Bajaj, 1977a) 21 and 13%, as indicated above. Comparable data are not available in the remaining cases. The incidence of disorganised growth as an alternative to plant regeneration or in specimens which do eventually regenerate plants through direct outgrowth of the original apex or through adventitious meristem formation, is very relevant to a decision regarding the choice of a suitable specimen for genome preservation. This is discussed further in Section 6.1.

The reasons for disorganised growth after thawing are not fully understood. In an electron microscopical examination of frozen and thawed meristems of Solanum spp., Grout and Henshaw (1980) noted large areas of tissue destruction in the leaf primordial and apical dome regions of the explant, with ruture of the epidermis of the shoot-tip. In the regeneration of frozen meristems of Pisum sativum, Haskins and Kartha (1980) note that active growth often occurs from leaf primordia and axillary bud or stipule meristematic areas. Growth occurs only rarely from the apical dome. Sakai et al. (1978) noted that serious damage is occuring in the subapical tissues at the base of the explant. It is thus possible that disturbances in the symplastic continuity within the specimen may disrupt hormonal gradients or even destroy sources of endogenous hormone. Conversely, the production of compounds with hormone like activity as a result of cytolysis (Sheldrake and Northcote, 1968) may induce disorganised growth. In the future, it is important that careful examination of the recovering explant be carried out to establish the origin of regenerating structures and reveal the nature and extent of cryoinjury.

Few investigations have been carried out into the scope for manipulating post-thaw conditions to improve the level or pattern of recovery. Grout and Henshaw (1978) recommended a low light level during the recovery of meristems of Solanum goniocalyx. Within the same genus, Henshaw et al. (1979) demonstrated a striking increase in the survival of meristems of S. tuberosum subsp. tuberosum by addition of NAA at 0.5 mg l^{-1} and GA_3 at 0.2 mg l^{-1} to the recovery medium (meristems were on filter paper bridges in contact with liquid medium). On standard medium containing benzyladenine at 1 mg l^{-1} the survival percentage was 2; with the alternative supplements it was increased to 32%. The meristems of Fragaria x ananassa preserved by Kartha et al. (1980) could be induced to undergo multiple meristem proliferation for rapid clonal multiplication by transfer to semi-solid medium containing a high level of benzyl aminopurine (10 μM). The results quoted in the latter paper are very significant in the present context since they represent the first successful preservation of meristems which had formed in vitro. In all other cases the meristems were excised from individual plants (see Section 6).

Note: In a patent application filed by Seibert the method published in connection with the preservation of meristems of Dianthus caryophyllus (Seibert and Wetherbee, 1977) is applied to the freeze-preservation of meristems of Asparagus officinalis and Lactuca sativa (U.S. Patent No. 4,052,317; Oct.11,1977).

5.6 Somatic and zygotic embryos, clonal plantlets and seedlings

These specimens have been grouped together by virtue of the fact that they all have a high degree of actual or potential organization and contain both root and shoot meristem regions. The foregoing section relates entirely to shoot meristem preservation. To the author's knowledge there are no reports of successful root-tip meristem preservation other than in the structures considered in this section.

Embryos developed in vitro, from two sources, have been committed to freeze-storage: those derived from haploid pollen within cultured anthers of Atropa belladonna (Bajaj, 1977d), and those derived from diploid suspension culture cells of Daucus carota (Withers, 1979). In both cases a slow freezing type of programme was used, involving the application of DMSO at 5 - 10%, freezing at a rate of 1 - 3°C min $^{-1}$ storage in liquid nitrogen and rapid thawing. Survival potential declined with advancing embryo development. In the case of the haploid embryos this is revealed as a reduction in viability test response: 31% of the globular embryos survived but only 9% of the early heart shaped and 2% of the late heart shaped embryos. In the case of the diploid somatic embryos of Daucus carota, the decline is revealed as a reduction in the mass of regrown material produced by recovering embryos in a given period of time. Structural examination of the recovering embryos in the latter case indicates that at the peak of recovery entire embryos are not surviving but secondary embryogenesis is occurring in the epidermal cells of the individual globular and early heart shaped embryos (Withers, 1979). As embryo maturation proceeds, secondary embryogenesis declines with the reduction in the proportion of meristematic cells in the embryo/plantlet and the increase in vacuolate and elongated cells (see McWilliam et al.1974; Street and Withers, 1974).

In order to improve the survival potential of more advanced embryos and promote survival of the entire embryo rather than secondary developments, the 'dry-freezing' technique has been developed (Withers, 1979). This is not to be confused with freeze-drying (e.g., King, 1965). The technique is a simple modification of slow freezing in which the specimen is cryoprotected (DMSO at 2.5 to 20%) and then removed from the suspending medium using forceps or by filtration. After blotting dry of superficial moisture and enclosing in a foil envelope, slow freezing is carried out. There is a pronounced interaction of freezing rate, transfer temperature and thawing rate in dry-frozen specimens. For example: after freezing at 1°C min $^{-1}$ to -40°C and plunging into liquid nitrogen, rapid thawing is most beneficial, but if freezing is continued below -40, e.g. to -100°C, then slow thawing is superior. At a freezing rate of 2°C min $^{-1}$, cooling can be continued down to -100°C and rapid thawing employed. These findings can be interpreted in terms of cell water content and the risk of deplasmolysis injury (see Section 4.2), a conclusion corroborated by an examination of the response of specimens which have been partially desiccated prior to freezing. As the extent of desiccation proceeds from 0 to 2 hours exposure to dry air in a vacuum desiccator, there is a progressive increase in the advantage of slow thawing over rapid thawing, indicating a reduction in ice recrystallization damage but an increase in deplasmolysis injury as the net water content is reduced (Withers, 1979). The slower freezing rate is far more suitable for the type of specimen involved here since freezing renders the tissue very brittle and prone to physical damage if handled in the frozen state.

The use of the dry freezing method has permitted the preservation of entire torpedo stage embryos and the meristem regions of clonal plantlets (up to 20 mm long) derived from the somatic embryos. In the best examples of the latter, recovery from 100% of the meristems (both root and shoot) could be obtained. The pattern of development depends upon the medium to which the thawed specimen is exposed. In all cases recovery would only ensue on semi-solid medium, use of liquid medium or even washing before transfer to semi-solid medium entirely precluding survival. Semi-solid medium containing 2,4-D promoted callus formation; medium without this hormone supported organised shoot growth (from both meristems). At superoptimal levels of DMSO (e.g. more than 10%) callusing was extensive even on 2,4-D-free medium; in many other cases callus development preceeding organised growth could be observed by ultrastructural examination of recovering plantlets. This problem can be resolved by the avoidance of excessive DMSO levels and initiating recovery growth on medium containing activated charcoal (Fridborg and Eriksson, 1975). Again it is possible that cytolysis is producing hormone-like compounds which affect the surviving cells.

The dry-freezing technique has now been used by Withers (1978c) to preserve zygotic embryos and small callus pieces of Zea mays. Immature embryos require a cryoprotectant treatment but mature dry embryos can be frozen without any such application. Recent experiments with mature imbibed seed of Zea mays (Withers, 1980f) indicate that the seed or embryos excised from it can be dry-frozen but cryoprotection and desiccation are essential to accomodate the large size and high water content of the specimen. Grout (1979) has also shown that chemical cryoprotection is a useful way of supporting viability in imbibed seed. Using a putative recalcitrant seed model system - imbibed seed of Lycopersicon esculentum, he demonstrated that up to a degree of hydration of ca. 42% water content, viability may be maintained in ca. 90% of the seed provided that DMSO at a level of 15% is applied prior to freezing. At a water content of more than 70% however, although seed would not germinate after thawing, meristems dissected from the seed could be grown in vitro. It would be valuable to repeat this with a known recalcitrant seed species with individual seed of a larger size. It must be said that the small seed of Lycopersicon esculentum is unlikely to present a very taxing model system. Other attempts to cryopreserve seeds are well documented elsewhere (see: Mumford and Grout, 1978, 1979; Sakai and Noshiro, 1975 and references therein).

Seedlings have been studied in two cases, essentially as model systems for the development of techniques for in vitro systems. Seedlings of Lycopersicon esculentum with up to 20 mm of emerged radicle can be freeze-preserved by a rapid freezing method (immersion of the ampoule in liquid nitrogen) after cryoprotection with DMSO at 10 - 15% (Grout et al., 1978). Higher levels of DMSO are deleterious. Up to 8% of the shoot meristems excised from the seedlings after rapid thawing and washing, resumed growth when cultured on filter paper bridges. Of the 8%, up to 5% produced adventitious buds, at least 3% callusing. An alternative method of cooling involving suspending the ampoules 7 cm above the surface of liquid nitrogen (35 - 40°C min -1 between 0 and -100°C) before plunging into liquid nitrogen and rapid thawing, yielded up to 20% surviving and callusing explants of which 15% bore adventitious shoots. In neither case did outgrowth of the original meristem occur. In a subsequent experiment the authors showed that up to 45% recovery involving outgrowth of the original meristem can be achieved by using a recovery medium containing GA_3. This observation indicates that specific treatments may be required to permit expression of the survival potential of the specimen.

In a recent preliminary study by Dale and Withers (1980) in which the freeze-preservation of seedlings of Dactylis sp. was attempted, the suitability of DMSO as cryoprotectant and a rapid freezing programme are confirmed for this type of specimen. Excision of damaged leaf material from the rapidly thawed and washed seedlings promotes recovery growth. Addition of activated charcoal to the semi-solid medium reduces callusing in the recovering seedling shoot meristem. The root meristem regions did not survive. This work is continuing and it is hoped that a procedure may be developed for use with herbage grass plantlets developed in vitro to provide genetic reserves for future seed production.

It is pertinent to mention here the early work of Sun (1958) in which it was demonstrated that excised seedlings of Pisum sativum could survive rapid freezing and rewarming provided that they had been desiccated to a final water content of 27 - 41% (equivalent to a water loss of 72 - 77% of the fresh weight) in seedlings 7 - 12 mm long, or 45% (77% water loss) in seedlings 13 - 20 mm long. Drying beyond ca. 14% water content damaged the root meristem region severely. Overall, roots were more sensitive to drying and freezing damage. Complete survival of the entire seedling only occurred in the smallest specimens, as in the case of somatic embryos of Daucus carota, cited above (Withers, 1979).

5.7 Mature plant pieces

This section describes the preservation of relatively mature plant pieces which are subjected to a minimum, if any, in vitro manipulation before or after freezing and thawing. A strong foundation was provided for experimental plant cryobiology by the work of Sakai and colleagues in Japan, who have studied the effects of low and ultralow temperatures on tissues. From this work we are able to learn a great deal about the interactions of freezing and thawing rates, the effects of cold-hardening upon low temperature tolerance and the possibility of storing 'in vivo' somatic tissues at ultra-low temperatures. It would be beyond the scope of this report to describe all of the work, but it is accessible through the bibliography at the end of this report and through citations in all major works on cryobiology.

Buds and twigs are known to survive low environmental temperatures especially in species which can cold-harden. Differential thermal analysis of freezing in buds indicates that death is associated with a marked exotherm occurring at a temperature below the exotherm corresponding to freezing of the extracellular milieu (close to 0°C). The position of the former exotherm relates to the season and degree of cold hardiness (Sakai, 1978, 1979).

Very small sections of hardened stem tissue of Morus bombycis, 20 - 30 µm thick can be frozen rapidly and then thawed rapidly, retaining viability as determined by vital staining and plasmolysis tests (Sakai and Yoshida, 1967). Less hardy tissue can be pre-frozen before the rapid freezing stage to achieve protective dehydration (Sakai and Otsuka, 1972), or can still be rapidly frozen if cryoprotected with e.g. 1M glucose (Sakai and Otsuka, 1972). Moving to larger tissue pieces: Sakai (1965) has shown that it is possible to preserve twigs of Salix spp., Populus spp., Betula spp. and Rosa pendulina by a pre-freezing technique. The temperature at which effective pre-freezing is achieved tends to be higher in winter twigs, but in all cases the preservation technique is basically the same: the twigs are transferred at intervals of 1 hour through a series of chambers at 5°C intervals and then into liquid nitrogen once pre-freezing has been achieved. The twigs can then be thawed slowly, and placed in water

or sand to await bud development. More recent studies by Sakai and Nishiyama (1978) demonstrate the successful preservation by pre-freezing and liquid nitrogen immersion of hardy dormant shoots of a wide range of fruit trees. Storage at -5 or -20°C was unsuitable, not surprisingly. In one species studied (*Malus domestica*), buds are shown to recover growth after storage and slow warming, and can be grafted onto 2 year old seedlings. The authors suggest that a cryoprotectant treatment might extend the method to include less hardy twigs, but point out that ultra-rapid freezing and thawing techniques are unlikely to be successful for such large specimens.

6. IN VITRO STORAGE - AN EVALUATION

Synopsis: Meristem cultures are valuable for utilization in normal and minimal growth storage but may have limitations when adopted for freeze-preservation programmes allied to in vitro virus elimination and mass propagation. Freeze-preservation technology is not well developed for cultured meristems; the indications are that methods quite different from those employed for newly excised meristems may be required, calling into question the choice of model systems for technique development. The high incidence of callusing and adventitious shoot formation both after freeze- preservation and in normal propagation procedures suggest that it may be timely to look at less formally organised but nonetheless stable, morphogenic systems such as embryogenic suspension cultures or rapidly proliferating liquid cultured meristems. Growth suppression through low temperature growth and medium modification provide the only realistic alternatives to freeze- preservation at the present. Freeze-preservation is technically simple, but requires development in physiological aspects. Within the broad category of freeze-preservation methods, rapid freezing is unlikely to have many applications in a true in vitro storage programme unless great advances are made in cold-hardening and otherwise pretreating cultures appropriately for such a freezing method. Slow, stepwise or pre-freezing are likely to meet most requirements in freeze-preservation.

6.1 Which is the most suitable specimen for storage?

This question and the following one related to choice of preservation method are not inseparable, but an attempt will be made here to look at each in turn. The choice of tissue culture or other specimen for storage must depend upon two major criteria: efficacy and cost-effectiveness. The latter is not just a cynical comment; it will be essential that the system adopted for use be proved to be of sufficient economic value in germplasm work to justify the necessary expenditure as well as for the introduction of novel propagation and growth procedures.

Firstly the question of efficacy: this has two components; which system will best do the job in terms of plant germplasm storage and which will best do the job in terms of plant regeneration after storage? The answer must be that there is no one system. Pollen and pollen embryos have a special role to play in providing for future fertilizations and the generation of haploid and homozygous diploid plants for breeding purposes (Kasha, 1974). In the latter case embryos must be favoured rather than anthers or pollen itself since their recovery values are higher (Bajaj, 1977d, 1978b, 1979; Table 3) and the induction of embryogenesis provides an opportunity for the embryogenic capacity of the pollen to be demonstrated.

In a few isolated cases, protoplasts might be the only system from which a particularly unresponsive plant may be regenerated. Additionally, cell, tissue or organ cultures may be unresponsive to preservation techniques through, for example their overall bulk or an excessive individual cell size. A case in point is **Nicotiana tabacum**

which is very difficult to freeze-preserve as a morphogenic suspension culture but for which protoplast isolation, wall regeneration and plant growth methodology are highly developed. With progress in the development of plants from protoplasts of economically important species, and the interest in looking at in vitro methods for crop plant improvement (see King et al., 1978; Thomas et al., 1979; Vasil and Vasil 1979, 1980), preservation of protoplasts may become of increasing importance.

At the other end of the organizational scale, the storage of twigs may offer a very simple convenient means for storing the germplasm of trees and other perennials, particularly those which can cold-harden. In developing interest in the use of strict in vitro methods, this direct type of approach should not be overlooked. Seedlings are not of any great potential value except as rather questionable model systems (see below) or perhaps as providers of explant material for recalcitrant seed storage (see Section 7). Highly developed clonal plantlets must be dismissed similarly, although early stage somatic embryos are a different matter (see below). Seed and zygotic embryo storage represent a special situation and will certainly meet specific needs in conservation, but along with all of the systems considered above are not serious alternative in the choice of a major tissue culture system for future conservation programmes. The real 'contest' is between unorganised callus or suspension cultures and organised meristems.

Before considering the main issue, the suitability of callus for storage can be examined. Very few examples of its successful use can be quoted (see Sections 3 and 5.4). Drawbacks in its use relate to the frequent bulk of the tissue making it difficult to freeze and thaw at uniform rates and the mode of culture which restricts the application of compounds through a liquid medium. However cold-hardening has been demonstrated in this type of culture and in some cases, although callus may be generated from an explant it may be difficult to culture either meristems or suspensions derived from the callus (see King et al., 1978). Therefore callus alone may be available for preservation. Thus we are left with the problem of making a choice between suspension cultures and meristems for the majority of species.

In examining the efficacy of the two systems in relation to storage, the statement will be made here, to be expanded upon in the subsequent sub-section, that only freeze-preservation and minimal growth storage answer conservation needs. For minimal growth storage, meristems are ideal and suspensions are entirely unsuitable. This needs no further expansion. In the case of freeze-preservation, the picture is less clear. It is difficult to directly compare the efficiency of viability maintenance in the two culture systems. On the one hand we are dealing with a dispersed cell/tissue and on the other hand, a symplastically connected structure with a high degree of organization and polarity. All that is needed for 100% survival in the latter system, is the maintenance of viability in a sufficient proportion of the cells in all specimen units. In the case of suspension cultures, percentage cell death is a more obvious event, being revealed as loss of viability in a certain percentage of the specimen units. Thus the very high survival data for meristems (e.g. Seibert and Wetherbee, 1977) should not lead to the assumption that 90 or 100% of the cells in the specimen survive and that the viability data for suspension cultures (particularly with recent advances in methodology: see Withers and King, 1979a; 1980a) are inferior. The suspension culture has the advantage here in that it has only to survive as a certain number of totipotent units; the meristem must survive both in terms of the number of cells in the unit being adequate to resume growth and in terms of the retention of organization such that recovery growth follows the established pattern of organization. The data presented in Section 5.5 indicate that in many cases, the results fall short of this requirement, particularly when the basic level of survival is low.

Now turning to the second component of 'efficacy', namely plant regeneration after storage. In terms of genetic stability, no-one would question the superiority of the organised, cultured meristem yielding progeny from terminal or axillary buds and which can be maintained in culture with little risk of deviation (see Morel, 1975). However, here we are not really talking about maintenance of cultures, but their storage. Beyond the initiation stage and the initial period within which the culture suitable for preservation is established, there need be no prolonged period of exposure to conditions which threaten stability and regenerative capacity. In the following discussion, the poor reputation of suspension cultures and the apparent superiority of meristems will be questioned.

As suggested in Section 3, we are learning more all of the time about factors which generate instability at the culture initiation stage and in maintenance, and the feasibility of taking measures to stabilize cultures genetically. The answer to the problem may lie in a careful choice of inoculum material (e.g. a meristem) to avoid mixoploidy and a careful control over culture induction and maintenance conditions prior to preservation, avoiding over-administration of growth substances and nutrient limitation. Much of the poor reputation of suspension cultures relates to the wide use of non-totipotent cell lines in physiological/biochemical areas of tissue culture, and the apparent lack of interest by the biochemically inclined worker in the karyotype of these cell lines. These cultures are often highly dispersed and consist of large, highly vacuolated cells. They are morphologically distant from the morphogenic type of culture from which plants may be regenerated. The latter consists of groups of highly cytoplasmic cells interspersed with single highly vacuolated cells (see Yeoman and Street, 1977; Street and Withers, 1974).

The cell groups in the latter type of culture may be looked upon as permanently proliferating meristematic zones, and their relationship to in vivo and in vitro meristems may be elucidated by proposing the existence of 'meristemoids' which are morphologically distinct, tightly grouped, relatively isodiametric and thin walled cells, which have a capacity to respond to hormonal stimulation and give rise to shoots, roots and embryos (from Torrey in Murashige, 1974). Meristems proper may consist of the original meristemoids, and (apparently disordered) suspension cultures may in fact consist in part of progeny meristemoids which maintain the totipotency of the original ones, and even a highly ordered (stabilizing?) pattern of cell divisions. (Street and Withers, 1974, describe a familiar cell group configuration in embryogenic cell suspensions before the development of embryos is observed). This is the type of culture which may permit the maintenance of genetic stability while embodying the characteristics of a specimen ideally suited to freeze-preservation. Of course, good, fine, morphogenic cell suspensions are not available for many species at the present, but progress in recent years and the current direction of effort by tissue culturists towards their production should generate optimism (see Winton, 1978).

To look now at meristems: if the data on clonal propagation from in vitro systems are examined, the superiority of meristems as the central culture system must be doubted. In a review published by Murashige in 1974, ca. 150 species were listed as having potential for in vitro propagation. Only 3 of these species produced clonal plants by outgrowth of pre-existing meristems; the remainder produced callus and adventitious buds or asexual embryos, although many cultures in this latter category had developed from lateral buds or shoot, root and rhizome apices. Since the above publication, more examples could be quoted of plants being propagated through non-adventitious bud development and then rooting of cuttings (e.g. Mantell et al., 1978). However, the fact remains that the majority of plants are not propagated in such a manner.

As suggested in Section 3, cultures involved in mass propagation may be open to factors which generate variation once the strict organization is lost. Cryptic comments such as "sporting ....is especially prevalent after the fourth division of the culture material. Thus in commercial practice few labs go beyond the third division" (Anon, 1976), underline the reality of the situation and reveal that it may be pragmatic not to ask too much of cultured meristems, and their progeny. However, the findings of Roca et al. (1979) indicate that adventitiously derived cultured plantlets may be acceptable according to the tests that they apply, providing evidence for the adequacy of adventitiously regenerated structures. Clearly each case must be considered on its own merits, but the general lesson to be learned from the above is that meristem culture may neither meet the need for propagation through pre-existing meristem development, nor automatically yield identical progeny.

The cost effectiveness of plant production from various culture systems has been examined (de Fossard, 1977). For a particular species of gymnosperm, the cost (in 1977) of producing a seedling was 1 cent; production of a plantlet through adventitious bud development had a unit cost of 25 cents, but predictions for a suspension culture system producing e.g. embryos, reduced the cost to 2 cents per plantlet. This argument is supported by comments of Winton (1978). The issue of conservation must be separate from the wider one of vegetative propagation in vitro, but it would be short sighted to encumber conservation technology with a system which becomes out-dated through developments in propagation technology, when the latter must be used to utilize many in vitro stored accessions. The cost effectiveness of the various culture systems in relation to storage has not properly been examined but many factors will be in common with propagation.

All in all, a picture must be emerging of trends in tissue culture technology leading towards the production of plants through embryogenesis or a similar process involving liquid culture in particular, this production relying on the maintenance of genetic stability during multiplication phases of the culture process, and a receding importance of meristem culture per se. When these factors are considered alongside the evident suitability of highly cytoplasmic 'meristemoid' type suspension cultures for freeze-preservation, and the high incidence of adventitious regeneration after freeze-preservation of meristems, there can be little doubt that the former type of system can answer most conservation needs. The role of meristem culture may then lie in its suitability for minimal growth storage and as an ideal inoculum for culture initiation, but a strong case can no longer be made for its adoption in favour of all other systems for freeze-preservation.

6.2 Which is the most satisfactory storage method?

In answering this question, several 'fringe' methods can be ruled out for the forseeable future. A number of growth suppression techniques may be of interest to the physiologist but they require a great deal of developmental work to yield a widely applicable method with a clear advantage over presently exploited methods. Minimal growth storage through the imposition of a low temperature or manipulation of the culture medium composition, are the only methods which can seriously be entertained alongside freeze-preservation, for effective germplasm storage.

Minimal growth storage has demonstrated its value with a number of species (see Section 3.2) and it is relatively clear where effort needs to be directed in making its use more widespread. The individual requirements of particular species demand attention (compare the minimum temperatures tolerated by different species listed in Section 3.2),

and more needs to be known about the role of the pregrowth period in rendering the specimen capable of surviving storage. The observation of Galzy (1969) that the length of the pre-culture period may be critical in terms of cold tolerance is a case in point. The drawbacks of any 'growing' method need not be reiterated here beyond saying that no relaxation in the strict observance of aseptic laboratory procedures is offered, nor are the risks inherent in culture maintenance reduced; they are merely spread over a longer period of time. Minimal growth storage has advantages in that accessions can be seen to be alive and any loss of condition may be spotted relatively rapidly. Regeneration from stored material involves a speeding up of a continuing process rather than a re-initiation of a suspended process as in freeze-preservation. In terms of disseminating germplasm, although Roca et al. (1979) conclude that material in a growing, non-frozen state can be flown long distances without complete loss of viability, it is evident that there are risks. A temporary period of neglect may destroy growing material but it would require an accident or a specific act of sabotage to endanger material deep frozen in a purpose built transport vessel. It is now possible to obtain small, secure vessels with long static holding times, and which are acceptable to commercial passenger airlines, since they are no risk to personnel and require no attention or back-up facilities in flight. On balance, minimal growth storage is of value as a relatively simple conservation method, which may even be the only one available for certain unresponsive tissues, but it can not answer all needs. It is likely to be of most value as a component of a two-system conservation programme (see Section 7) running in parallel with and possibly interchanging material with ultralow temperature storage.

For very-long term storage, particularly of accessions which have no immediate place in field growth or breeding, a method which has no periodical renewal procedures must be superior. Ultralow temperature storage has so many advantages that there is little future in trying to overcome the problems of intermediate temperature storage. The assumption of stability of specimens in storage may still be something of an act of faith and technical aspects may, at first, seem a little daunting, but these are not serious grounds for pessimism. The facilities required for freeze-preservation are relatively unsophisticated. Tissue culture facilities are a requirement for any in vitro storage method and for freeze-preservation the facilities required may be less expensive than for minimal growth. A liquid nitrogen refrigerator is easily maintained requiring only (say) weekly coolant renewal and a routine (say) monthly check that the vacuum seal is intact. The feasibility of maintaining small scale germplasm stocks over a wide geographical area has been demonstrated in the livestock 'industry'. Liquid nitrogen is cheap and readily available in many locations. Failing an existing supply, compression equipment is relatively cheap. The preservation apparatus need not be costly. Indeed, the most successful reports of preservation published in recent years, involve the use of 'home-made' units (Grout et al., 1978; Grout and Henshaw, 1978; Withers and King, 1980a; Seibert and Wetherbee, 1977). Drawbacks at present to freeze-preservation lie not in the hardware, but in physiological aspects of pregrowth and recovery, and in the choice of preservation protocol.

The vast majority of examples of tissue culture preservation have involved the use of a freezing method based upon extracellular freezing and the achievement of protective dehydration. Superiority of rapid freezing is apparent in some cases but in others it must be questioned. The interpretation of the data of Seibert and Wetherbee (1977) offered in Section 5.5, indicates that meristems may indeed respond to rapid freezing or slow freezing. Other workers have tried to use the rapid freezing method

as described by Seibert and Wetherbee (1977) for similar specimens but with no success (Kartha et al., 1979, 1980; Grout and Henshaw, 1978; Sakai et al., 1978). In one case problems were overcome by the development of a more rapid freezing procedure (Grout and Henshaw, 1978) and in the others, slow or stepwise/pre-freezing was used. Risks involved in both the open vial method of Seibert and Wetherbee (1977) and the hypodermic needle method of Grout and Henshaw (1978) (see Section 4.3.2) cannot be overlooked. Sterility must be ensured in valuable accessions. Remaining examples of the use of rapid freezing (Bajaj, 1977a; and Stamp, 1978), both involving the storage of Manihot spp. are relatively unsuccessful. Claims that slow freezing is unsuitable for certain specimens may conceal the fact that it is necessary to 'finely-tune' the preservation protocol. Only Kartha et al. (1979, 1980) and Seibert and Wetherbee (1977) have made any effort to use a range of freezing rates sufficiently exacting to reveal the requirements of the tissue culture system. The data of Kartha et al. (1980) relating to the preservation of cultured meristems of Fragaria x ananassa suggest that the necessary freezing rate for optimal survival may indeed be very precise. Deviation from the most successful rate of 0.84°C min $^{-1}$ with a recovery percentage of 95, to either 0.56°C min $^{-1}$ or 0.95°C min $^{-1}$, lowers the recovery to 62 and 33% respectively. This may at first appear surprising, the difference in rate being small in view of the range used for freeze-preservation (from 0.5 to more than 1000°C min $^{-1}$). However, bearing in mind the effect which period of exposure to sub-zero temperatures can have on the progress of protective dehydration (Rall and Mazur, 1977), we can redefine the freezing conditions used by Kartha et al. (1980) in terms of number of minutes in the temperature range of (say) -30 to -40°C and derive the values of ca. 18, 12 and 10.5 for the increasing rates of freezing. These differences are quite large enough to under or over dehydrate a specimen (see Rall and Mazur, 1977). In view of the fact that dehydration may be achieved by step-wise freezing with fewer risks of overdehydration than slow freezing, and the fact that sample specimens can quickly be frozen and thawed to test the progress of dehydration actually during the programme, this type of freezing programme might be of more general use than slow freezing, and permit a more exact definition of optimum freezing requirements. The recent observation by Withers and King (1980a, Withers 1980e) that three component cryoprotectant mixtures including sucrose may alleviate some of the technical demands of optimising the freezing and thawing conditions during step-wise freezing, give further support for this type of method.

The most critical fact pertaining to the findings of Kartha et al. (1980), is that this is the only example of freeze-preservation of meristems which have developed in vitro. If freeze-preservation is to be incorporated into a germplasm conservation and utilization programme including virus elimination and clonal propagation in vitro, it is of paramount importance that methods for preservation be suitable for material other than recently explanted meristems. Galzy (1969) and Stamp (1978) both noted the deterioration in terms of cold and freeze-tolerance respectively, occurring during the early preculture period. It is possible that although a minimum period may be necessary for healing of an excision wound and stabilization after the trauma of isolation, too long a period leads to 'de-hardening'. Unfortunately, by present indications, the method of Henshaw et al. (1979, 1980; Grout and Henshaw, 1978, 1980; Stamp, 1978) involving ultra-rapid freezing, would not even accomodate a single-step clonal multiplication of a particularly valuable meristem, or a virus elimination stage in excess of ca. 100 hours; not very realistic (Walkey, 1978). In view of the fact that this method of preservation is entirely unsuccessful in preserving in vitro propagated meristems of cultivars of Solanum spp. which can be preserved in the form of seedling or auxillary bud meristems (Grout, pers. com.) and the reduced response of meristems

from seedlings of Solanum species with ploidies higher than diploid, it is possible that the 'de-hardening' phenomenon may involve an increase in mean cell size and/or an increase in the water content of the meristem, both contributing to an increased succulence. The need for the freezing rate be taken to the maximum possible to achieve preservation of the meristems examined by Grout and Henshaw (1978) and Henshaw et al. (1979, 1980) means there is no scope for increasing the rate for material which may have an even greater need for control over ice crystal formation within the cell (see Section 4.2). In contrast, slow and step-wise/pre-freezing involve an optimum freezing rate or holding time giving scope for variation on either side of the optimum according to the special requirements of the tissue. Perhaps this type of method should be examined closely with a view to its adoption for the Solanaceous and other meristem systems. One further point detracts from the suitability of rapid freezing in general. Since it involves a mechanism based on the minimising of ice crystal size and the prevention of recrystallization, it is essential that the freezing and thawing rates be adequately rapid. If freezing has been carried out too slowly, no improvement in thawing conditions can recover the specimen. In contrast, a slowly frozen specimen which has been underdehydrated may be recovered by increasing the thawing rate, and one which has been over dehydrated may be recovered by reducing the thawing rate (e.g. somatic embryos of Daucus carota; Withers, 1979).

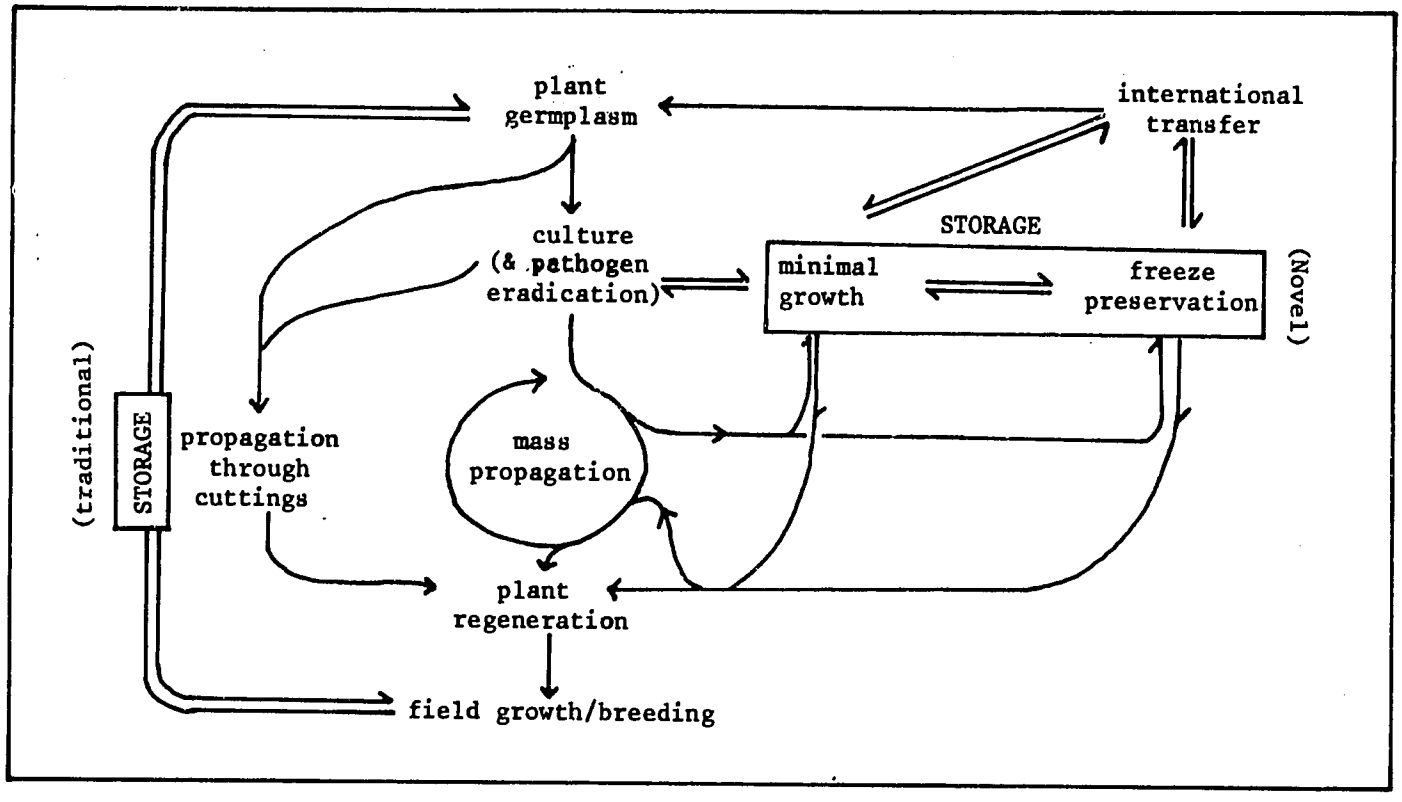
Criticism must be made here of the use of seedling terminal or axillary bud meristems as model systems to develop protocols for in vitro cultivated counterparts (e.g. Grout and Henshaw, 1978; Grout et al., 1978; Dale and Withers, 1980). The physiological condition of such material must be very different from cultured meristems and the likelihood of variation within the seedling material must present the possibility of recovering only freeze-tolerant genotypes (i.e. selection). Is this a valid process in view of the critical importance of pregrowth conditions (Section 4.3.5) and the "strong heterozygosity of many potato cultivars" (Grout and Henshaw, 1978), which motivated the development of an in vitro preservation method for this genus?

In conclusion, an examination of the options within freeze-preservation suggests that slow freezing or a related 'extracellular freezing method' is more likely to offer a preservation protocol for cultured plant material, and that in many instances, there is a strong case for going back to the beginning and examining critically the relationship between the condition of the specimen, its pregrowth history and its response to freezing and thawing. Within the basic approach of freeze-preservation dependent upon extracellular freezing and protective dehydration, there is scope for variation. Dry-freezing is particularly suitable for certain specimens (see Section 5.6) whereas freezing in suspending medium will be more suitable for others. The choice between slow, stepwise and pre-freezing will depend, in part, upon available apparatus. The unit described in Figure 3b offers all of these possibilities, especially if more than one bath is available to provide for multiple step pre-freezing.

7. THE FUTURE

SYNOPSIS: A scheme is proposed for the incorporation of the two favoured storage methods (freeze-preservation and minimal growth) into a genetic conservation programme. Ideally, the two systems should be able to interchange material and provide ready supplies for field growth, breeding and dissemination. Clonal mass-propagation is likely to become of increasing importance in the future and therefore forms a component of the scheme, permitting multiplication of accessions for utilization and exchange, and also for the storage of replicates (an important factor in the conservation and distribution of valuable germplasm). In examining the proposal in the light of current techniques, several areas demanding particular attention can be identified. These relate to physiological aspects of culture induction, propagation, pregrowth and recovery. Further, it is essential that a clear indication be given of the most urgent cases where survival of germplasm is is threatened, in order that research effort may be directed most usefully. An analysis of the economics of the scheme in comparison with traditional methods would be useful, but this is not a central biological issue. In the future, it is most important that research into the *in vitro* conservation of specific plants be initiated or its continuation encouraged, with an emphasis on bringing together expertise from the fields of plant tissue culture and genetic resources. In the context of the plant groups identified in the Introduction, no generalisations can be made. Some staple food crops (notably *Solanum* spp.) are benefiting from attention in the areas of tissue culture and preservation, but in others neither the basic culture nor storage methods have gone beyond exploratory stages. For recalcitrant seed species, freeze-preservation might be valuable but this awaits examination. Either zygotic or somatic tissue may be appropriate for culture initiation and storage, as in the case of woody perennials. The situation with Palms is under-explored, but encouraging.

From the discussion in Section 6, it may be deduced that minimal growth and freeze-preservation of morphogenic cell cultures or proliferating meristems, are the most appropriate approaches to genetic conservation through tissue culture. In view of the fact that advantages lie in the two different approaches, that some specimens are unresponsive to one or the other treatment at present, and that both short and long-term stocks may be required, both approaches are incorporated into the scheme below. The interrelationships between accession, storage and cultivation may seem over involved. However, if this type of scheme is to be acceptable, it is important that it be as flexible as possible to meet the requirements of the specimen in question.



At all stages there is the requirement for technical development. Introduction into culture and in vitro propagation are possible for many plant species, but many others remain difficult or impossible to handle, necessitating the use of inferior seed or propagule storage, or maintenance of stocks in the field. It is important that plants which come into this category be clearly identified. In terms of international transfer, the feasibility of exchange of germplasm in the form of stored, tissue culture material, has only been examined in the case of potato (Roca et al., 1979) where growing stock cultures were exchanged. The feasibility, cost and administrative (phytosanitary) requirements for exchanging stored (growing or frozen) specimens should be explored.

Transfer of material from a growing state under normal culture conditions, to a storage mode is being attempted for an ever increasing number of plants. In 1976, when the 'prototype' for Table 3 was constructed, fewer than half as many successful reports of preservation had been made and survival values were relatively poor, especially in terms of plant regeneration. However, there are still many cases where in vitro storage has either not been attempted or has proved unsuccessful. There is considerable interest among tissue culturists in carrying out research into germplasm storage (if only for their own laboratory stock storage), as evidenced by the publication record and debate. (Two of the featured articles in the Newsletter of the International Association for Plant Tissue Culture, over the past 5 years have related to genetic conservation: Henshaw, 1979, and Street, 1975). Guidance from those working in the field of plant genetic resources would aid the most effective direction of effort on the part of the tissue culturists towards tackling the problems of conservation.

Failures, to date, in achieving storage relate as much to chilling sensitivity, morphological impediments, and inappropriate handling during pregrowth and post-thaw recovery phases, as to inferior storage techniques per se. If an understanding could be gained of the role that is played by these morphological and physiological factors, it might become possible to develop a 'key' indicating the most suitable storage procedure for a given specimen. On the basis of, say, cell size, maximum tissue unit dimensions, percentage water content, response to chilling, and possibly certain taxonomic criteria, it might become possible to deduce the most suitable storage protocol, in terms of whether it be minimal growth or freeze-preservation, and if the latter, the most suitable pregrowth, cryoprotectant, freeze/thaw, and recovery treatments. As yet this is in no way possible.

A further impediment to the scheme outlined above, is the current lack of clear information relating to the transfer of material from clonal propagation and/or minimal growth storage to freeze-preservation. In some cases, this is not possible (e.g. Solanum spp.), in others it is (e.g. Fragaria x ananassa; Daucus carota), in yet others it has not even been attempted. The answer to the problem must lie in the physiological changes occurring during culture establishment and propagation. It is felt, by the author, that until essential, detailed physiological/biophysical research is carried out into the phenomena accompanying culture establishment, and operating during pregrowth and recovery growth, in relation to cryodamage, it will not be possible to achieve routine storage which dispenses with laborious developmental work, nor to realise the convenience of exchanging material between different culture and storage modes. The empirical approach may take too long for some threatened plants.

The above recommendations, in terms of a suggested storage scheme and areas in which work still needs to be done for the full potential of the scheme to be realised, have only been given in general terms. The final consideration in this report will be given to evaluating the current situation relating to the development of in vitro storage methods for the groups of plants identified in the Introduction.

The prerequisite of an adequately developed tissue culture technology cannot really be claimed in the case of any of the plants under consideration. In the case of Solanum spp., most work has been done and it is now possible to propagate plants from meristems, callus, and protoplasts, although there are persistent interspecific and intervarietal differences in response (Westcott et al., 1977; Roca, et al., 1978, 1979; Melchers, 1978; Bragadø-Aas, 1977). Not surprisingly, storage technology is quite highly developed here, with the possibility of minimal growth and freeze-preservation being available. However, it is essential that in the future, attention be turned away from the use of seedling material alone, and an attempt be made to refine freezing methods for the more demanding in vitro propagules. Latterly attention has been directed towards other staple food species. In the case of Manihot spp. clonal propagation through nodal cuttings and culture of terminal buds is possible (Bajaj, 1977a; Kartha and Gamborg, 1979; Kartha et al., 1974; Liu, 1975; Berbee et al., 1973). Preservation has been carried out only with limited success. (Bajaj, 1977a; Roca, 1978; Stamp, 1978). Both minimal growth storage and freeze-preservation appear to be feasible.

Interest in many crop plants from the point of view of tissue culture originates through the demand for virus free plants. It is fortunate that this is the case, as it provides a foundation, if fragile, for future tissue culture work on relevant species. Manihot spp. (Kartha and Gamborg, 1975; Bertee et al., 1973), Dioscorea spp. (Mantell pers. com.), Colocasia esculenta (Hartman, 1974), Xanthosoma sagittifolium (Hartman, 1974) and Ipomoea batatas (see Murashige, 1978) have all been freed of virus infections through tissue culture techniques. However, the development of clonal propagation, other than through the relatively laborious process of nodal cutting, and the control of morphogenesis in callus or suspension cultures are distant targets in most cases. Nonetheless encouraging signs of the development of tissue culture systems appropriate for germplasm conservation and utilization are: the report of somatic embryogenesis in Dioscorea floribunda (Ammirato, 1978), organogenesis in tuber-derived cultures of Ipomoea batatas (Yamaguchi and Nakajima, 1973), and the production of protocorm like structures on callus initiated from shoot tips of Colocasia esculenta (Abo el-Nil and Zettler, 1976). Other publications of relevance here relate to somatic embryogenesis in Allium sativum (Abo el-Nil, 1977) and Carica papaya (de Bruijne et al., 1974), proliferative growth in meristem cultures of Musa sp. (Guzmann and Decena, 1978), and micropropagation in Ananas sativus (Mathews et al., 1976). Perhaps the overall picture should lead to some optimism since systems appropriate for minimal growth storage would appear to be available now, with technical developments leading towards systems suitable for the preservation of, and development of clonal plants from friable embryogenic callus, now being attempted.

The second group of plants to be considered now, are those which have recalcitrant seed, and/or which would benefit from a vegetative propagation method permitting multiplication of valuable hybrids etc. Two basic approaches to their conservation can be considered. In those where seed recalcitrance is the main problem, the entire seed might be preserved using cryogenic methods. Presently available techniques might be refined by drawing upon the more sophisticated techniques used for tissue culture (see: Sakai and Noshiro, 1975; Stanwood and Bass, 1978): the use of cryoprotectant mixtures incorporating DMSO to aid penetration and compounds such as proline, which are known to be effective against drying and freezing stress; the optimising of freeze-/thaw protocols, then possibly a post-thaw in vitro passage to germinate the seed under controlled conditions or even culture the excised embryo dissected from the thawed seed. As an alternative, the embryo, the embryonic meristem or a seedling meristem might be preserved, with an in vitro proliferative phase either preceeding or following freezing and thawing. In the cases of particularly large embryos, as are not uncommon in recalcitrant seed, culture prior to freezing would be most suitable and the derived tissue could be divided into smaller units.

The second basic approach relevant particularly to species wherein the parental genotype is valuable, but also appropriate to some recalcitrant seed species, involves culture initiation from somatic tissues. The juvenility/maturity phenomenon may present some problems but progress in regenerating plants from mature explants is encouraging. (Note: Winton (1978) suggests that embryogenesis may in fact be a 'mature' response.)

It would not be possible here to review fully the prospects for adopting in vitro methods for the genetic conservation of recalcitrant seed species and woody perennials etc. However, the review of Murashige (1978) in which propagation in vitro of species categorised as flower crops, ornamental foliage crops, succulents and landscape ornamentals, ferns, vegetable and condiment crops, agronomic crops, fruits and nuts, and finally medicinal plants (in all more than 200 species) should give an indication

of the potential source material for in vitro conservation. Successes in the freeze-preservation of a number of woody species including timber and fruit crops indicate that the second part of the process may indeed be feasible; all that remains may be for the effort to be made to bring together the tissue culture and the preservation expertise.

Special mention should be made of the palms, before leaving this area. Much effort has been devoted recently towards developing tissue culture propagation systems for Eleais guineensis, Phoenix dactylifera and Cocos nucifera. All three species show some response in culture and the former two would appear to yield organogenetic and embryogenic cultures capable of producing transplantable plantlets (Anon, 1978; Apavatjrut and Blake, 1977; Corley et al., 1977, Eeuwens and Blake, 1977; Jones, 1974a,b; Rabéchault et al., 1970; Staritsky, 1970b). Both somatic and zygotic tissues are used for culture induction. No report has been made of successful preservation through either minimal growth storage or freeze-preservation of this group of species, and it might be anticipated that difficulties would be experienced in freezing the relatively large adventitious structures produced. Preservation by freezing of suspensions or minimal growth storage of meristems or proliferating embryos, might be more successfull approaches.

Finally, mention must be made of the possibilities for preserving germplasm which has arisen in vitro. Tissue cultures of importance in somatic cell genetics, aimed at crop improvement or industrial processes (Zenk, 1978) are likely to be difficult or even impossible to store as regenerant plants or seed. The obvious economic importance of these applications of tissue culture will almost certainly ensure that effort continues to be devoted to the development of germplasm preservation technology. Among the species which can be preserved to date, are several which have a place in either plant breeding or secondary product synthesis. The Solanaceae are particularly important in both contexts, and this is a very fortunate coincidence - up to a point. However, likely trends, especially in industrial areas may take interest away from morphogenic systems and concentrate more upon e.g. cell-lines. In consequence, although in vitro preservation owes a lot to the past interest on the part of those with rather different motives, if research is to go in the required directions in the future, interest and support must come from the conservation side of the subject.

Table 1 Cryoprotectants

Compound or group of compounds	Approximate effective concentration	Appripriate freeze-preservation protocol	Notes	Examples of use and relevant references
Dimethyl sulphoxide (DMSO)	0.5 - 2 molar	Rapid or slow 1/	Can be applied in pregrowth; some evidence of toxicity at high levels; effective alone or in mixtures, especially with glycerol	Kartha et al, 1979, 1980; Nag and Street, 1975a,b; Seibert and Wetherbee, 1977; Bajaj et al, 1970; Withers, 1979
Glycerol	0.5 - 2 molar	Rapid or slow	Uptake reduced at low temperatures; may predispose cell to deplasmolysis injury; naturally occurring 'antifreeze'	Araki, 1977; Meryman, 1966b; Nag and Street, 1975a,b; Sakai and Otsuka, 1972, Withers, 1978c; 1980a; McGann, 1978
Sugars, sugar alcohols: sucrose, glucose, honey, sorbitol, mannitol	various 2/ up to 2 molar	Rapid or slow	Naturally occurring in some cases (in relation to cold hardiness); may protect against salt stress in nature; rarely effective alone; effective in pregrowth	Sakai, 1971; Brown and Hellebust, 1978; Sakai and Yoshida, 1968; Ohomoto et al, 1978
Amino acids: proline, hydroxyproline, GABA, aspartate, also glycine betaine	0.5 - 2 molar	Slow	Effective with whole cells and organelles; naturally occurring in some cases; effective pre-growth additives; very low toxicity; some only effective in mixtures	Withers and King, 1979a, b, 1980a; Paquin, 1977, Pollard and Wyn-Jones, 1979; Stewart and Lee, 1974; Heber et al, 1971

1/ 'Slow freezing' is used to cover all methods effective through extracellular freezing (including slow, linear or non-linear) freezing, stepwise freezing and pre-freezing.

2/ Sugars may occur at levels as low as 2% in the culture medium and as such are present in most cryoprotectant preparations; used singly, higher levels are necessary.

Table 1 Cryoprotectants (Continued)

Compound or group of compound	Approximate effective concentration	Appropriate freeze-preservation protocol	Notes	Examples of use and relevant references
Methanol	up to 2.5 molar	Slow	Effective with animal, and algal cells; virtually unexplored with higher plants cells but promising	Ashwood-Smith and Lough, 1975; Morris, 1980; Grout. pers. comm.
High molecular weight polymers: PVP, hydroxyethyl starch	-	Rapid	Controversy exists over efficacy and mode of action; may be effective during post-thaw period; not recommended for preservation of viability; effective cryofixatives	Franks et al, 1977; Skaer et al, 1978; Allen et al, 1978; Morris, 1976a,b; Connor and Ashwood-Smith, 1973
Mixtures: 2 to 3 component combinations of the low molecular weight compounds above; may also include ethylene glycol or polyethylene glycol	1 - 2 molar	Slow	Widely effective; vast range of mixtures possible; usually contain at least one conventional cryoprotectant (DMSO or glycerol) may have greater physical stability at ultralow temperatures than single cryoprotectants	Finkle and Ulrich,1978, 1979; 1979; Withers and King, 1980a,b; Nag and Street, 1975a,b; Withers 1980a,e; Boutron and Kauffmann,1978; Pozner et al, 1977; Bajaj, 1979b

Table 2 Protocols for carrying out viability tests (from Withers, 1980a)

Test	Reagent	Application	Examination	References
Fluorescein diacetate staining	0.5% w/v solution of solid in acetone; store stock solution at -20°C; for use dilute to 0.1 ml in 5 ml of culture medium	Add one drop of dilute solution to one drop of cell suspension on a microscope slide	Compare light microscope images under white and UV light illumination; score fluorescing units	Widholm, 1972; Nag and Street, 1975a
2,3,5-triphenyl tetrazolium chloride reduction	1% w/v solution of solid in 0.05M phosphate buffer (pH 7.5)	Add one volume of reagent to one volume of cell suspension in a narrow necked test tube, mix well and incubate at 20°C in the dark for ca. 15 h.; wash cells twice with distilled water, then extract red formazan with 95% ethanol at 70 - 80°C	Read adsorption at 485 nm and compare with a standard curve	Steponkus and Lanphear, 1967; Towill and Mazur, 1974b; Withers, 1978a; Cela et al, 1979
Evan's blue staining	0.025% w/v solution of solid in culture medium	Add one drop of solution to one drop of cell suspension	Observe in light microscope; score unstained units	Gaff and Okongo-Ogola, 1971; Withers, 1978a

Table 3. A review of freeze-preservation protocols applied to plant tissues (pollen, other than as cultured anthers/pollen, seed, zygotic embryos, and mature plant pieces are excluded)

Species	Culture type	Cryoprotectant	Freezing regime	Storage	Thawing	Recovery Viability &/or Growth		Reference
Acer pseudoplatanus	suspension	12% DMSO[d] 5% glycerol	prefreezing to -30/-50°C, transfer to LN[a]	-196°C	+40°C	25% TTC[b]	+	Sugawara and Sakai, 1974
	"	10% glycerol	2°C min $^{-1}$ to -100°C, transfer to LN	"	+37°C	25% FDA[c]		Nag and Street. 1975a
	"	10% glycerol 5% DMSO	"	"	"	22% FDA		"
	"	"	1°C min $^{-1}$ to -100°C, transfer to LN	"	+40°C	30% FDA		Withers and Street, 1977a
	synchronised suspension	"	"	"	"	25% FDA[f] 45% TTC		Withers, 1978a
	suspension[e] pregrown in medium plus 3.3% mannitol	"	"	"	"	40% FDA	+	Withers and Street, 1977a
	as above but with 6% mannitol	0.5M DMSO, 0.5M glycerol plus 1M sucrose or proline	1°C min $^{-1}$ to -35°C, held for 40 min, transfer to LN	"	"	75 - 90% FDA	+	Withers and King, 1980a,b
Acer saccharum	suspension	DMSO[g]	less than 3°C min $^{-1}$	-40°C[h]	Rapid[g]	20% TTC		Towill and Mazur, 1974a
Arachis hypogaea	meistems precultured for 3-5 days	5% DMSO, 5% glycerol plus 5% sucrose	slow or rapid[g]	-196°C	+37°C	23-31% gld[i]	+	Bajaj, 1979b
Asparagus officinalis	shoot apices from cold treated plants	5% DMSO	at least 400°C min $^{-1}$	-196°C	ca. 180° min $^{-1}$	38% g/d	+	Seibert, (Personal communication)

Table 3. A review of freeze-preservation protocols applied to plant tissues (pollen, other than as cultured anthers/pollen, seed, zygotic embryos, and mature plant pieces are excluded) (Continued)

Species	Culture type	Cryoprotectant	Freezing regime	Storage	Thawing	Recovery Viability &/or Growth	Reference
Atropa belladonna	suspension	10% glycerol, 5% DMSO	2°C min $^{-1}$ to -100°C, transfer to LN	-196°C	+37°C	15% FDA	Nag and Street, 1975a
	"	"	"	-85°C	"	g +	"
	"	5% glycerol	1°C min $^{-1}$ to -100°C, transfer to LN	-196°C	"	25% FDA	Nag and Street, 1975b
	"	5% DMSO	"	"	"	30% FDA	"
	suspension pregrown in medium plus 5% DMSO	"	2°C min $^{-1}$ to -100°C, transfer to LN	"	"	40% FDA	Nag and Street, 1975a
	pollen embryos	5-7% DMSO	1-3°C min $^{-1}$	"	"	31% FDA (globular) to 2% FDA (late heart shaped)	Bajaj, 1977d
	anthers (fresh)	DMSO[g]	rapid[g]	"	g	'occasional callus'	Bajaj, 1979a
Bromus inermis	protoplasts	0.7 M glycerol	g	"	g	68%	Mazur and Hartmann, 1979
	'cells'	"	g	"	g	38%	"
Capsicum annuum	suspension	10% glycerol, 5% DMSO	1°C min $^{-1}$ to -100°C, transfer to LN	"	+40°C	1% FDA	Withers and Street, 1977a

Table 3. A review of freeze-preservation protocols applied to plant tissues (pollen, other than as cultured anthers/pollen, seed, zygotic embryos, and mature plant pieces are excluded) (Continued)

Species	Culture type	Cryoprotectant	Freezing regime	Storage	Thawing	Recovery Viability &/or Growth		Reference
Capsicum annuum	suspension[e] pregrown in medium plus 5.2% mannitol	10% glycerol, 5% DMSO	chilled overnight then frozen as above	-196°C	+40°C	25% FDA		Withers and Street, 1977a
	"	"	chilled overnight then frozen at 1°C mid $^{-1}$ to 30°C	-30°C[h]	"	50% FDA	+	"
Chrysanthemum morifolium	callus pregrown on medium plus 10% sucrose	-	2 weeks at 4.5°C	-3.5°C[h]	g	8% TTC	+	Bannier and Steponkus. 1972
Cicer arietinum	meristems precultured in 4% DMSO	5% DMSO	0.5 to 1°C min $^{-1}$ to -40°C, transfer to LN	-196°C	+37°C	40% g/d	+	Kartha, pers. comm.
	meristems precultured for 3-5 days	5% DMSO, 5% glycerol, plus 5% sucrose	slow or rapid[g]	-196°C	+37°C	27-36% g/d	+	Bajaj, 1979b
Dactylis sp.	seedlings	5 - 15% DMSO	direct immersion in LN	"	+40°C	*ca.* 30% g/d		Dale and Withers, 1980
Datma Stramarium	suspension	7% DMSO	1°C min $^{-1}$ to -100°C, transfer to LN	-196°C	+37°C	g		Bajaj, 1976
Daucus carota	suspension	5% glycerol or 10% DMSO	2 - 4°C min^{-1} to -40°C	-40°C[h]	"	g	+	Latta, 1971
	"	5% DMSO	2 - 4°C min^{-1} to -196°C	-196°C	"	g	+	"
	"	"	1.8°C min^{-1} to -196°C	"	"	65% FDA	embryos	Nag and Street, 1973
	"	5 or 10% DMSO	1 - °2°C min^{-1} to -70°C, transfer to LN	"	+30°C or air at RT[j]	g	"	Dougall and Wetherell, 1974
	"	5% DMSO	2°C min^{-1} to -100°C, transfer to LN	"	+40°C	65% FDA	"	Withers and Street, 1977a

Table 3. A review of freeze-preservation protocols applied to plant tissues (pollen, other than as cultured anthers/pollen, seed, zygotic embryos, and mature plant pieces are excluded) (Continued)

Species	Culture type	Cryoprotectant	Freezing regime	Storage	Thawing	Recovery Viability &/or Growth		Reference
Daucus carota	callus grown on medium with extra sugar and with ABA[k]	'dried'	g	-80°C	g	g	+	Nitzsche, 1978
	somatic embryos and clonal plantlets	2.5 to 20% DMSO	1°C min^{-1} to -40°C, transfer to LN (dry freezing)	-196°C	rapid	100%g/d	meristem growth	Withers, 1979
	"	"	"	"	slow	0%		"
	"	"	1°C min^{-1} to -100°C, transfer to LN (dry freezing)	"	"	80%g/d	"	"
	"	"	desiccated for 2h, frozen at 2°C min^{-1} to -100°C, transferred to LN (dry freezing)	"	slow	95%g/d	"	"
	protoplasts	5% DMSO in protoplast medium /no cryoprotectant	2°C min^{-1} to -100°C, transfer to LN/rapid freezing	"	+40°C	up to 90% FDA	regeneration and embryos	Withers and Street, 1977b, Withers, 1980a
	"	0.7M glycerol	g	"	g	68%		Mazur and Hartmann, 1979
	suspension	4% glucose, 3% DMSO, 2.5% ethylene glycol	stepwise[g] to -15°C	-15°C[h]	rapid	40%TTC		Finkle *et al*, 1975
	"	"	stepwise to 23°C	-23°C[h]	rapid[g]	20% TTC		"

Table 3. A review of freeze-preservation protocols applied to plant tissues (pollen, other than as cultured anthers/pollen, seed, zygotic embryos, and mature plant pieces are excluded) (Continued)

Species	Culture type	Cryoprotectant	Freezing regime	Storage	Thawing	Recover. Viability &/or Growth	Reference
Dianthus caryophyllus	shoot-tip	5% DMSO	at least 1000°C min^{-1}	-196°C	+37°C	15 - 33% callus, shoots and then plantlets	Seibert, 1976
	shoot-tips from cold hardened plants	"	at least 50°C min^{-1}	"	*ca.* 1450° C min^{-1}	up to 100% survival and plant development	Seibert and Wetherbee, 1977 (but see: Anderson, 1979)
Fragaria x *ananassa*	*in vitro* propagated meristems pregrown with 5% DMSO	5% DMSO	0.84°C min^{-1} to -40°C, transfer to LN	"	+37°C	50 - 95% g/d	Kartha *et al*, 1980
	runner apical meristems	3% sucrose, 10-16% DMSO	prefreezing to -20 to -30°C, immersion in LN, (*ca.* 10^5 °C min^{-1}) (cover glass method)	"	+40°C	60-80% shoot development	Sakai *et al*, 1978
	"	"	immersion of ampoule in LN (*ca.* 420°C min^{-1})	"	"	15% shoot development	"
Glycine max	suspension	5-7% DMSO	2°C min^{-1} to -100°C, transfer to LN	"	+37°C	52% FDA	Bajaj, 1976; Bajaj and Reinert, 1977
Haplopappus ravenii	"	10% DMSO	1.7°C min^{-1} to -20°C	-20°C[h]	g	3 x control TTC value[g] +	Hollen and Blakely, 1975

Table 3. A review of freeze-preservation protocols applied to plant tissues (pollen, other than as cultured anthers/pollen, seed, zygotic embryos, and mature plant pieces are excluded) (Continued)

Species	Culture type	Cryoprotectant	Freezing regime	Storage	Thawing	Recovery Viability	Recovery &/or Growth	Reference
Hyoscyamus muticus	"	0.5 M DMSO, 0.5M glycerol, plus 1M proline or sucrose	$1^{o}C\ min^{-1}$ to $-35^{o}C$, held for 40 min, transfer to LN	$-196^{o}C$	$+40^{o}C$	30% FDA	plantlet development	Withers and King, 1980a,b
Ipomoea sp.	suspension pregrown in medium containing 6% sucrose	2.5% glycerol 2.5% DMSO	$2 - 4^{o}C\ min^{-1}$ to $-40^{o}C$	$-40^{o}C^{h}$	$+37^{o}C$	g	+	Latta, 1971
Lactuca sativa	shoot apices from cold treated plants	5% DMSO	at least $400^{o}C\ min^{-1}$	$-196^{o}C$	" ca. $180^{o}C\ min^{-1}$	up to 67% g/d		Seibert, Personal communication
Linum usitatissimum	suspension	3.3% DMSO	$5 - 10^{o}C\ min^{-1}$ to $-50^{o}C$	$-50^{o}C^{h}$	$+40^{o}C$	14% TTC		Quatrano, 1968
Lycopersicon esculentum	seedlings	10% DMSO	direct immersion of vial in LN	$-196^{o}C$	"	8% callus, 5% adv. shoot formation from excised meristem		Grout et al, 1978
	"	15% DMSO	$35 - 40^{o}C\ min^{-1}$ initial rate to $-100^{o}C$ (vapour cooling)	"	"	20% callus, 8% adv. shoot formation from excised meristem		"
	"	"	"	"	"	22% callus, 45% original meristem growth (recovery medium containing gibberellin)		"

Table 3. A review of freeze-preservation protocols applied to plant tissues (pollen, other than as cultured anthers/pollen, seed, zygotic embryos, and mature plant pieces are excluded) (Continued)

Species	Culture type	Cryoprotectant	Freezing regime	Storage	Thawing	Recovery Viability &/or Growth	Reference
Manihot esculentum	shoot-apices	DMSO[g]	direct immersion of meristem on hypodermic needle	"	"	3-8% g/d	Henshaw *et al*, 1979, Stamp, 1978
Manihot usitatissimum	excised buds	10% glycerol, 5% sucrose	direct immersion of vial in LN	"	+35-37°C	13% plantlets 8% callus	Bajaj, 1977a
Nicotiana sylvestris	suspension	15% glycerol in culture medium with reduced salts	1°C min^{-1} to -100°C, transfer to LN	"	+40°C	15% FDA	Shillito, 1978
Nicotiana tabacum	"	4% glucose, 3% DMSO, 2.5% ethylene glycol	stepwise[g] to -23°C	-23°C[h]	rapid[g]	20% TTC	Finkle *et al*, 1975
	"	0.5M DMSO,0.5M glycerol plus 1M proline or sucrose	6°C min^{-1} to -35°C,held for 40 min.transfer to LN	-196°C	+40°C	up to 60% FDA +	Withers and King 1980b
	haploid cell suspension	5% DMSO	prefreezing at -20/-70°C, or 1-2°C min^{-1} to -196°C	-196°C	+37°C	5-10% g/d, plant regeneration	Bajaj, 1976; Bajaj and Reinert, 1977
	anthers pre-cultured for 3 - 4 weeks	7% DMSO	2°C min^{-1}	"	rapid[g]	1.5% giving 3.5 anthers per plant	Bajaj, 1977d
	as above but cut transversely before freezing	"	"	"	"	6.6%,same yield per anther as above	"
	pollen embryos	5 - 7% DMSO	1 - 3°C min^{-1}	"	+37°C	31% FDA(globular) to 2% FDA (late heart shaped)	Bajaj, 1977d

Table 3. A review of freeze-preservation protocols applied to plant tissues (pollen, other than as cultured anthers/pollen, seed, zygotic embryos, and mature plant pieces are excluded) (Continued)

pecies	Culture type	Cryoprotectant	Freezing regime	Storage	Thawing	Recovery Viability &/or Growth		Reference
Oryza sativa	suspension	5% DMSO	*ca.* 0.1°C min^{-1} to -70°C, transfer to LN	"	+30°C	60-65% TTC	+	Sala *et al*, 1979; Cella *et al*, 1978
Petunia hybrida	anthers pre-cultured for 3 - 4 weeks	7% DMSO	2°C min^{-1}	"	rapid[g]	5.2%		Bajaj, 1977d
Pisum sativum	excised seed-lings	'dried'	direct immersion in LN	"	water at RT	survival according to size and degree of dehydration		Sun, 1958
	shoot-tip meristems pre-cultured in medium with 5% DMSO	4% DMSO	0.6°C min^{-1} to -40°C, transfer to LN	"	+37°C	at least 60% plant regeneration		Kartha *et al*, 1979
Populus euramericana	callus (cold hardened)	-	prefreezing to -30, -70, -120°C, transfer to LN	"	air at RT	g	callus regrowth	Sakai and Sugawara, 1973
Rosa Paul's Scarlet	suspension pregrown in medium plus 6% mannitol	0.5M DMSO, 0.5M glycerol, plus 1M proline or sucrose	1°C min^{-1} to -35°C, held for 40 min, transfer to LN	"	+40°C	*ca.* 70% FDA	+	Withers and King, 1980a,b
Saccharum sp.	suspension	8% glucose, 10% DMSO	stepwise, 4 min. each at -10, -15, -23, -40°C	-40°C[h]	"	TTC +ve[g]	+	Finkle and Ulrich, 1978a, b; 1979

Table 3. A review of freeze-preservation protocols applied to plant tissues (pollen, other than as cultured anthers/pollen, seed, zygotic embryos, and mature plant pieces are excluded) (Continued)

Species	Culture type	Cryoprotectant	Freezing regime	Storage	Thawing	Recovery Viability &/or Growth		Reference
Saccharum sp.	callus	10% polyethylene glycol, 8% glucose, 10% DMSO	1 - 3°C min^{-1} to -40°C, transfer to LN	-196°C	rapid[g]	TTC +ve[g]	+	Finkle and Ulrich, 1978, 1979; Ulrich *et al*, 1979
	suspension	10% glycerol, 5% DMSO	1°C min^{-1} to -100°C, transfer to LN	"	+37°C	30% FDA		Chen *et al*, 1979
Sambucus racemosa	hardened callus	g	g	"	g	g	+	Yoshida (unpublished)
Solanum goniocalyx	shoot-tips precultured for up to 72 h before freezing	10% DMSO	direct immersion of meristem on hypodermic needle	-196°C	+40°C	up to 60% survival and plantlet regeneration		Henshaw *et al*, 1979; Stamp, 1978; Grout and Henshaw, 1978, 1980
S. tuberosum	"	"	"	"	"	up to 36%	"	Henshaw *et al*, 1979; Stamp, 1978 (but see: Towill, 1979
	meristems from tuber sprouts or auxillary buds	5% DMSO, 5% glycerol, plus 5% sucrose	direct immersion of ampoule into LN	"	"	up to 26%	"	Bajaj, 1978a
Sorghum bicolor	suspension	0.5M DMSO, 0.5M glycerol, plus 1M proline or sucrose	1°C min^{-1} to -35°C, held for 40 min, transfer to LN	"	"	up to 25%	+	Withers and King, 1980a,b
Zea mays	"	0.5M DMSO, 0.5M glycerol, 1M proline	"	"	"	75% FDA	+	Withers and King, 1980a,b

Table 3. A review of freeze-preservation protocols applied to plant tissues (pollen, other than as cultured anthers/pollen, seed, zygotic embryos, and mature plant pieces are excluded) (Continued)

Species	Culture type	Cryoprotectant	Freezing regime	Storage	Thawing	Recovery Viability &/or Growth		Reference
Zea mays	suspension	0.5M DMSO, 0.5M glycerol, 1M sucrose	$1^{o}C\ min^{-1}$ to $-35^{o}C$, held for 40 min, transfer to LN	$-196^{o}C$	$+40^{o}C$ or air ar RT	80% FDA	+	– Withers and King, 1980a,b; Withers, 1980e
	"	10% proline	$1^{o}C\ min^{-1}$ to $-30^{o}C$, held for 40 min, transfer to LN	"	$+40^{o}C$	15-20% FDA	+	Withers and King, 1979a
	"	10% DMSO, 10% glycerol	"	"	"	30-75% FDA (ephemeral)	+	"
	suspension pregrown in medium plus 10% proline	10% proline	"	"	"	75% FDA	+	Withers and King, 1979a
	"	–	"	"	"	55% FDA	+	"

a. Liquid nitrogen
b. 2,3-5 triphenyl tetrazolium chloride reduction test, see Table 2
c. Fluorescein diacetate test, see Table 2
d. Dimethyl sulphoxide
e. Rapid subculturing regime
f. Cell cycle maxima
g. No value recorded
h. Lowest temperature to which tissue is exposed
i. Growth and development
j. Room temperature
k. Abscisic acid

Note: In a number of publications, e.g. Bajaj, 1976; Sugawara and Sakai, 1974; Withers and King, 1979a; Nag and Street, 1975a,b; Withers, 1979, many more protocol variations are given. In the above table, the most significant are picked out. Original publications should be consulted for further details of protocols, especially for culture media and recovery conditions. Some reports duplicating data or reports giving preliminary observations without methodological details have been omitted.

BIBLIOGRAPHY

Abo el-Nil, M.M. 1977 Organogenesis and embryogenesis in callus cultures of garlic (Allium sativum, L.). Plant Sci. Lett., 9, 259-264.

Abo el-Nil, M. and Zettler, F.W. 1976 Callus initiation and organ differentiation from shoot-tip cultures of Colocasia esculenta. Plant Sci. Lett., 6, 401-408.

Alan, J.J. 1979 PhD. Thesis, University of Birmingham.

Alferman, A.W. and Reinhard, E. 1978 Production of Natural Compounds by Cell Culture Methods. Gessellschaft fur Strahlen- und Umweltforschung mbH, Munich.

Allen, E.D., Weatherbee, L. and Permoad, P.A. 1978 Post-thaw suspension of red cells cryopreserved with hydroxyethyl starch. Cryobiology, 15, 375-381.

Ammirato, P.V. 1978 Somatic cell embryogenesis in suspension cultures of the medicinal yam, Dioscorea floribunda. Fourth International Congress of Plant Tissue and Cell Culture. Abstract no. 1708, p. 152.

Anderson, J.O. 1979 Cryopreservation of apical meristems and cells of carnation (Dianthus caryophyllus) (Abstract). Cryobiology, 16, 583.

Anon, 1976 Plant propagation by tissue culture moves from academic theory into commercial production. Florist (June, 1976), 72-81.

Anon, 1978 Amélioration des plantes et conservation des ressources génétiques: Le palmier - dattier. Compte-rendu d'un Séminaire Organisé par l'ACCT, Abidajan, Février, 1978.

Apavatjrut, P. and Blake. J. 1977 Tissue culture of stem explants of coconut (Cocos nucifera L.). Oléagineux, 32, 267-271.

Araki, T. 1977 Freezing injury in mitochondrial membranes. II Degradation of phospholipid in rabbit liver mitochondria during freezing and storage at low temperatures. Cryobiology, 14, 151-159.

Asahina, E. 1978 Freezing processes and injury in plant cells. In, Plant Cold Hardiness and Freezing Stress.eds.P.H.Li and A. Sakai, pp. 17-36. Academic Press, New York.

Asahina, E. Shimada, K. and Hisada, Y., 1970 A stable state of frozen protoplasm with invisible intracellular ice crystals obtained by rapid cooling. Exp. Cell Res., 59, 349-358.

Ashwood-Smith, M.J. and Farrant, J. 1980 (eds.) Low Temperature Preservation in Medicine and Biology. Pitman Medicine, London.

Ashwood-Smith, M.J. and Lough, P. 1975 Cryoprotection of mammalian cells in tissue culture with methanol. Cryobiology, 12, 517-518.

Asuwa, N. 1972 Progressive change in organ forming capacity of tobacco callus during single sub-culture period. Jap. J. Genet., 47, 53-60.

Bajaj, Y.P.S. 1976a Gene preservation through freeze-storage of plant cells, tissue and organ culture. Acta. Hort., 63, 75-84.

Bajaj, Y.P.S. 1976b Regeneration of plants from cell suspensions frozen at -20, -70 and -196oC. Physiol. Plantarum, 37, 263-268.

Bajaj, Y.P.S. 1977a Clonal multiplication and cryopreservation of cassava through tissue culture. Crop Improv., 4, 198-204.

Bajaj, Y.P.S. 1977b Initiation of shoots and callus from potato tuber sprouts and axillary buds frozen at -196oC. Crop Improv., 4, 48-53.

Bajaj, Y.P.S. 1977c Regeneration of plants from pollen embryos frozen at ultralow temperature: A method for the preservation of haploids. In, Proc. Intern. Palnyological Conf., Lucknow (in press).

Bajaj, Y.P.S. 1977d Survival of Atropa and Nicotiana pollen embryos frozen at -196°C. Current Science (India), 46, 305-307.

Bajaj, Y.P.S. 1978a Tuberization in potato plants regenerated from freeze-preserved meristems. Crop Improv., 5, 137-141.

Bajaj, Y.P.S. 1978b Effect of superlow temperature on excised anthers and pollen embryos of Atropa, Nicotiana and Petunia. Phytomorphology, (in press).

Bajaj, Y.P.S. 1979a Establishment of germplasm banks through freeze-storage of plant tissue culture and their implications in agriculture. In, Plant Cell and Tissue Culture: Principles and Applications ed. W.M. Sharp, pp. 745-774. Ohio University Press, Colombus.

Bajaj, Y.P.S. 1979b Freeze-preservation of meristems of Arachis hypogaea and Cicer arietinum. Indian J. Exp. Biol., 17, 1405-1407.

Bajaj, Y.P.S., Rathore, V.S., Wittwerl, S.H., and Adams, M.W. 1970 Effect of dimethylsulfoxide on Zn^{65} uptake, respiration and RNA and protein in bean (Phaseolus vulgaris) tissues. Am. J. Bot., 57, 794-799.

Bajaj, Y.P.S. and Reinert, J. 1977 Cryobiology of plant cell cultures and establishment of gene banks. In, Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. eds. J. Reinert and Y.P.S. Bajaj, pp. 757-777. Springer, Berlin.

Bald, W.B. and Robards, A.W. 1978. A device for the rapid freezing of biological specimens under precisely controlled and reproducible conditions. J. Microscopy, 112, 3-15.

Bannier, L.J. and Steponkus, P.L. 1972 Freeze-preservation of callus cultures of Chrysanthemum morifolium Ramat. Hort Science, 7, 194.

Bannier, L.J. and Steponkus, P.L. 1976 Cold acclimation of Chrysanthemum callus cultures. J.Am.Soc.Hort.Sci., 101, 409-430.

Barnabas, B., and Rajki, E. 1976. Storage of maize (Zea mays L.) pollen at -196°C in liquid nitrogen. Euphytica, 25, 747-752.

Batchelor, S. and Withers, L.A. 1980 Unpublished observations.

Bayliss, M.W. 1977a Factors affecting the frequency of tetraploid cells in a predominantly diploid suspension culture of Daucus carota. Protoplasma, 92, 109-115.

Bayliss, M.W. 1977b The Causes of competition between two cell lines of Daucus carota in mixed culture. Protoplasma, 92, 117-127.

Bayliss, M.W. 1977c The effects of 2,4-D on growth and mitosis in suspension cultures of Daucus carota. Plant Sci. Lett., 8, 99-103.

Bayliss, M.W. and Gould, A.R. 1974 Studies on the growth in culture of plant cells. XVIII Nuclear cytology of Acer pseudoplatanus L. J.Exp.Bot., 25, 772-783.

Behnke, M. 1975 Regeneration in Gewebekulturen einiger dihaploider Solanum tuberosum - Klone. Z. Pflanzenzuchtg., 75, 262-265.

Bennici, A., Buiatti, M., D'Amato, F. and Pagliai, M. 1971 Nuclear behaviour in Haplopappus gracilis callus grown in vitro on different media. Collq. Intern. CNRS, 193, 245-250.

Bennici, C.R., Cionini, P.G., and D'Amato, F. 1976 Protoplasma, 89, 251-261.

Berbee, F.M., Berbee, J.G. and Hildebrandt, A.C. 1973. Induction of callus and virus symptomless plants from stem-tip cultures of cassava. In vitro, 8, 421.

Blum, A., and Ebercon, A. 1976 Genotypic responses in sorghum to drought stress. III Free proline accumulation and drought resistance. Crop Science, 16, 428-430.

Boutron, P. and Kaufmann, A. 1978 Stability of the amorphous state in the system water-glycerol-dimethylsulfoxide. Cryobiology, 15, 93-108.

Bragad-Aas, M. 1977 Regeneration of plants from callus of potato tubers. Acta. Hort., 78, 133-137.

Bridgen, M.P. Sharp, W., Staby, G.L. 1978 Effects of low pressure on the preservation of tissue cultures of Chrysanthemum morifolium. International Association for Plant Tissue Culture. Calgary Meeting. Abstract No. 1207, p. 106.

Briedenbach, R.W. and Waring, A.J. 1977 Response to chilling of tomato seedlings and cells in suspension culture. Plant Physiol., 60, 190-192.

Brossard, D. 1976 Influence of kinetin on formation and ploidy levels of buds arising from Nicotiana tabacum pith tissues grown in vitro. Z.Pfla.Physiol., 78, 323-333.

Brown, L.M. and Hellebust, J.A. 1978 Sorbitol and proline as intracellular osmotic solutes in the green alga Stichococcus bacillaris. Can.J.Bot., 56, 676-679.

de Bruijne, E., de Langhe, E. and van Rijck, R. 1974 Actions of hormones and embryoid formation in callus cultures of Carica papaya. Wetenschappen Gent., 39, 637-646.

Burke, M.J., Gusta, L.V., Quamme, H.A., Weiser, C.J. and Li, P.H. 1976 Freezing and injury in plants. Ann.Rev.Plant Physiol., 27, 507-528.

Caplin, S.M. 1959 Mineral-oil overlay for conservation of plant tissue cultures. Am.J. Bot., 46, 324-329.

Cella, R., Sala, F., Nielsen, E., Rollo, F. and Parisi, B. 1978 Cellular events during the regrowth phase after thawing of freeze-preserved rice cells. Abstracts: Federation of European Societies of Plant Physiology. Edinburgh, July 1978, p. 127-128.

Chaturvedi, H.C. and Mitra, G.C. 1975 A shift in Morphogenic pattern in Citrus callus tissue during prolonged culture. Ann.Bot., 39, 683.

Chen, P. and Gusta, L.V. 1978a The role of water in cold hardiness of winter cereals. In, Plant Cold Hardiness and Freezing Stress. eds. P.H. Li and A. Sakai, pp. 165-174. New York, Academic Press.

Chen, P.M. and Gusta, L.V. 1978b Changes in membrane permeability of winter wheat cells following freeze-thaw injury as determined by N.M.R. Plant Physiol., 61, 878-882.

Chen, W.H., Cockburn, W. and Street, H.E. 1979 Preliminary experiments on freeze-preservation of sugar cane cells. Taiwania, 24, 70-74.

Chu, T.M., Jusaitis, M. Aspinall, D., and Paleg, L.G. 1978 Accumulation of free proline at low temperatures. Physiol. Plant., 43, 254-260.

Connor, W. and Ashwood-Smith, M.J. 1973 Cryoprotection of mammalian cells in tissue culture with polymers: Possible mechanisms. Cryobiology, 10, 488-496.

Corduan, G. and Spix, C. 1974. Anther derived plants from **Digitalis purpurea**. In, Genetic Manipulations with Plant Material. ed. L. Ledoux, 576-577. New York, Plenum Press.

Corley, R.H.V., Barrett, J.N. and Jones, L.H. 1977 Vegetative propagation of oil palm by tissue culture. Oil Palm News, 22, 2-7.

Dale, P.J. 1978 Meristem tip culture in herbage grasses. International Association for Plant Tissue Culture. Calgary Meeting. Abstract No. 1206, p.106.

Dale, P.J. 1980 A method for in vitro storage of **Lolium multiflorum** Lam. Ann. Bot. 45, 497-502.

Dale, P.J. and Withers, L.A. 1980 Unpublished observations.

D'Amato, F. 1975 The problems of genetic stability in plant tissue and cell culture. In, Crop Genetic Resources for Today and Tomorrow. eds. O.H. Frankel and J.G. Hawkes, pp. 333-348. Cambridge University Press.

D'Amato, F. 1977 Cytogenetics of differentiation in tissue and cell cultures. In, Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. eds. J. Reinert and Y.P.S. Bajaj, pp. 343-357. Springer, Berlin.

D'Amato, F. 1978 Chromosome number variation in cultured cells and regenerated plants. In, Frontiers of Plant Tissue Culture, 1978 ed. T.A. Thorpe, pp. 287-296. International Association for Plant Tissue Culture.

Darbyshire, B. 1975 The results of freezing and dehydration of Horseradish peroxidase. Cryobiology, 12, 276-281.

Day, P.R. 1980 Tissue culture methods in plant breeding. In, Tissue Culture for Plant Pathologists, eds. D. Ingram and J. Helgeson, Oxford, Blackwell (in press).

Deansley, R. 1957 Egg survival in immature rat ovaries grafted after freezing and thawing. Proc.Roy.Soc.London, Ser B., 147, 412-421.

Denton, I.R., Westcott, R.J. and Ford-Lloyd, B.V. 1977. Phenotypic variation of **Solanum tuberosum** L. cv. Dr. McIntosh regenerated directly from shoot-tip culture. Potato Res., 20, 131-136.

Diller, K.R., Cravalho, E.G., and Huggins, C.E. 1972 Intracellular freezing in biomaterials. Cryobiology, 9, 429-440.

Dix, P.J., and Street, H.E. 1976 Selection of plant cell lines with enhanced chilling resistance. Ann.Bot., 40, 903-910.

Dougall, D.K. and Wetherell, D.F. 1974. Storage of wild carrot cultures in the frozen state. Cryobiology, 410-415.

Drew, R.L.K. 1979. Effect of activated charcoal on Embryogenesis and regulation of plantlets from suspension cultures of Carrot (**Daucus carota** L.) Ann.Bot., 44, 387-389.

Dunwell, J.M. 1978 Division and differentiation in cultured pollen. In, Frontiers of Plant Tissue Culture, 1978, ed. T.A. Thorpe, pp. 103-112. International Association for Plant Tissue Culture.

Earley, E.M. 1973 The influence of different tissue culture media on the stability of embryonic diploid rabbit cell lines. In Vitro, 8, 442.

Eeuwens, C.J. and Blake, J. 1977 Culture of coconut and date palm tissue with a view to vegetative propagation. Acta.Hort., 78, 277-286.

Everett, N.P. 1980 2,4-D Independent cell cultures of Sycamore (**Acer pseudoplatanus** L.): Responses to 2,4-D and kinetin and sensitivity to antimetabolites. J. Exp. Bot. (submitted for publication).

Farrant, J. and Morris, G.J. 1973 Thermal shock and dilution shock as the causes of freezing injury. Cryobiology, 10, 134-140.

Farrant, J., Walter, C.A. and Knight, S.C. 1976 Cryopreservation and selection of cells. Les Colloques de l'Institut National de la Santé et de la Recherche Médicale. Cryoimmunologie, 62, 61-78.

Farrant, J., Walter, C.A., Lee, H. and McGann, L.E. 1977a Use of 2-step cooling procedures to examine factors influencing cell survival following freezing and thawing. Cryobiology, 14, 273-286.

Farrant, J., Walter, C.A., Lee, B., Morris, J.G. and Clarke, K.J. 1977b Structural and functional aspects of biological freezing techniques. J. Microsc., 111, 17-34.

Finkle, B., Sa, E., Pereira, B. and Brown, M.S. 1974 Freezing of non-woody plant tissues. I. Effect of rate of cooling on damage to frozen beet root sections Plant Physiol., 53, 705-708.

Finkle, B.J., Sugawara, Y. and Sakai, A. 1975 Freezing of carrot and tobacco suspension cultures. Plant Physiol., 56 (suppl.) 80.

Finkle, B.J. and Ulrich, J.M. 1978 Effects of combination of cryoprotectants on the freezing survival of sugarcane cultured cells. In, Plant Cold Hardiness and Freezing Strees.eds.P.H. Li, and A. Sakai pp. 373-388. Academic Press, New York.

Finkle, B.J. and Ulrich, J.M. 1979 Effects of cryoprotectants in combination on the survival of frozen sugar cane cells. Plant Physiol. 63, 598-604.

Fishbein, W.N. and Winkert, J.W. 1977 Parameters of freezing damage in simple solutions: Catalase. The characteristics pattern of intracellular freezing damage exhibited in a membraneless system. Cryobiology, 14, 389-398.

de Fossard, R.A. 1977 Tissue culture in horticulture - A perspective. Acta. Hort., 78, 455-459.

de Fossard, R.A. and Bourne, R.A. 1977 Reducing tissue culture costs for commercial propagation. Acta Hort., 78, 37-44.

Frankel, O.H. 1970 Genetic conservation of plants useful to man. Biol. Conservation, 2, 162-169.

Frankel, O.H. and Bennett, E. ed. 1970 Genetic Resources in Plants - Their Exploration and Conservation. Blackwell, Oxford.

Frankel, O.H. and Hawkes, J.G. ed. 1975 Crop Genetic Resources for Today and Tomorrow. Cambridge University Press, Cambridge.

Franks, F., Asquith, M.H. Hammond, C.C., Skaer, H. le. B., and Echlin P. 1977 Polymeric cryoprotectants in the preservation of ultrastructure, 2. J. Microscopy, 110, 223-238.

Fridborg, G.T. and Eriksson, T. 1975 Effects of activated charcoal on growth and morphogenesis in cell cultures. Physiol Plant., 32, 306-308.

Furner, I.J., King, J. and Cambore, O.L. 1978 Plant regeneration from protoplasts isolated from a predominantly haploid suspension culture of **Datura innoxia** (Mill.) Plant Science Letters, 11, 169-176.

Furuya, T. 1978 Biotransformation by plant cell cultures. In, Frontiers of Plant Tissue Culture, 1978 ed. T.A. Thorpe, pp. 191-200. International Association for Plant Tissue Culture.

Gaff, D.F. and Okongo-Ogola, O. 1971 The use of non-permeating pigments for testing survival of cells. J.Exp.Bot., 22, 756-758.

Galzy, R. 1969 Recherches sur la croissance de **Vitis rupestris** Scheele Sain et court nové cultivé in vitro a differentes temperatures. Ann de Phytopathol., 1, 149-166.

Geier, T. 1977 Morphogenesis and plant regeneration from cultured organ fragments of Cyclamen persicum. Acta Hort., 78, 167-174.

Gleba, D. and Gleba, Yu Yu. 1978 Ploidy level evolutions during the culture of tobacco mesophyll protoplasts, and effect of pretreatments with mutagen and fusagen. Fourth International Congress on Plant Tissue and Cell Culture. Abstract No. 504, p. 65.

Green, C.E. In vitro plant regeneration in cereals and grasses. In, Frontiers of Plant Tissue Culture, 1978, ed. T.A. Thorpe, pp. 411-418. International Association for Plant Tissue Culture.

Gresshof, P.M. 1977 **Chlamydamonas reinherdii** - A model plant system. II Cryopreservation. Plant Sci Lett., 9, 23-25.

Gressel, J., Zilkah, S. and Ezra G. 1978 Herbicide action, resistance and screening in cultures vs. plants. In, Frontiers of Plant Tissue Culture, 1978, ed. T.A. Thorpe p. 427-436. International Association for Plant Tissue Culture, Calgary University Press.

Grout, B.W.W. 1979 Low temperature storage of imbibed tomato seeds: A model for recalcitrant seed storage. Cryo-Letters, 1, 71-76.

Grout, B.W.W. and Crisp, P. 1977 Practical aspects of the propagation of cauliflower by meristem culture. Acta Hort., 78, 289-296.

Grout, B.W.W. and Henshaw, G.G. 1978 Freeze-preservation of potato shoot tip cultures. Ann.Bot., 42, 1227-1229.

Grout, B.W.W. and Henshaw, G.G. 1980 Frowth of potato shoot-tip cultures after storage in liquid nitrogen: a microscopical study. Ann.Bot. (in press).

Grout, B.W.W., Westcott, R.J. and Henshaw, G.G. 1978 Survival of shoot meristems of tomato seedlings frozen in liquid nitrogen. Cryobiology, 15, 478-483.

Guzmann, E.V. and Decena, A.C. 1978. Effect of media and irradiation of the culture of banana shoot-tips. 4th Intern. Congr. Plant Tissue and Cell Culture, Calgary, Abstract No. 1745, p. 172.

Harrington, J.F. 1970 Seed and pollen storage for conservation of plant gene resources. In, Genetic Resources in Plants - Their Exploration and Conservation. eds. O.H. Frankel and E. Bennett pp. 501 -521 Blackwell, Oxford.

Hartman, R.D. 1974 Dasheen mosaic virus and other phytopathogens eliminated from caladium, taro and cocoyam by culture of shoot-tips. Phytopathology, 64, 237-240.

Haskins, R.H. and Kartha, K.K. 1980 Freeze-preservation of pea meristems. Can. J. Bot., 58, 833-840.

Heber, U. 1967 Freezing injury and uncoupling of phosphorylation from electron transport in chloroplasts. Plant Physiol., 42, 1343-1350.

Heber, U. 1968 Freezing injury in relation to loss of enzyme activities and protection against frezing. Cryobiology, 5, 188-201.

Heber, U. and Santarius, K. 1964 Loss of adenosine triphosphate synthesis caused by freezing and its relationship to frost hardiness problems. Plant Physiol., 39, 712-719.

Heber, U., Tyankova, L. and Santarius, K.A. 1971 Stabilization and inactivation of biological membranes during freezing and the presence of amino acids. Biochim. Biophys.Acta., 241, 575-592.

Hedtrich, C.M. 1977 Differentiation of cultivated leaf discs of **Prunus mahaleb**. Acta Hort., 78, 177-183.

Heinz, D.J., Krishnamurthi, M., Nickell, L.G. and Maretzki, A. 1977 Cell tissue and organ culture in sugarcane improvement. In, Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. eds. J. Reinert and Y.P.S. Bajaj, pp. 3-17. Springer, Berlin.

Heinz, D.J. and Mee, D.W.P. 1971 Morphological cytogenetic and enzymatic variation in **Saccharum** species hybrid clones derived from callus tissue. Amer.J.Bot., 58, 257-262.

Henshaw, G.G. 1975 Technical aspects of tissue culture storage for genetic conservation. In, Crop Genetic Resources for Today and Tomorrow. eds. O.H. Frankel and J.G. Hawkes, pp. 349-358. Cambridge University Press.

Henshaw, G.G. 1978 Conservation of germplasm as meristem cultures. Proc. II Latin-American Botanical Congress. (in press).

Henshaw, G.G. 1979 Tissue cultures and germplasm storage. IAPTC Newsletter, No. 28, pp. 2-7.

Henshaw, G.G. O'Hara, J.F. and Westcott, R.J. 1980 Tissue culture methods for the storage and utilization of potato germplasm. In, Tissue culture for Plant Pathologists. eds. D. Ingram and J. Helgeson, Oxford, Blackwell (in press).

Henshaw, G.G. Stamp, J.A. and Westcott, R.J. 1979 Tissue cultures and germplasm storage. In, Proceedings: Plant Tissue Culture Conference. Pavia, Italy 1979, ed. Sala, F. and Cella, R. (in press).

Henshaw, G.G., Westcott, R.J. and Roca, W.M. 1978 Tissue culture methods for storage and utilization of potato germplasm. Fourth International Congress on Plant Tissue and Cell Culture. Abstract No. 1204, p. 105.

Hermsen, J.G. Th. 1974 Summation: Haploids in plant breeding. In, Haploids in Higher Plants. ed. K.J. Kasha, pp. 281-285. University of Guelph.

Hollen, L.B. and Blakely, L.M. 1975 Effects of freezing on cell suspensions of **Haplopappus ravenii**. Plant Physiol. (Suppl.), 56, 39.

Hope, H.J. 1977 Mitochondrial damage following sub-lethal and lethal frost damage to young winter wheat plants Cryobiology, 14, Abs. 30. p. 694.

Huskins, C.L. 1948 Segregation and reduction in somatic tissues. I. Initial observations on **Allium cepa**. J. Heredity, 39, 311-325.

Hussey, G. and Hepher, A. 1978 Clonal propagation of Sugar Beet plants and the formation of polyploids by tissue culture. Ann. Bot., 42, 477-479.

Ibrahim, R.K. 1969 Normal and abnormal plants from carrot root tissue cultures. Can.J.Bot., 47, 825-826.

Ilker, R., Waring, A.J., Lyons, J.M. and Briedenbach, R.W. 1976. The cytological response of tomato seedling cotyledons to chilling and the influence of membrane modifications upon these responses. Protoplasma, 90, 229-252.

Jenkins, E.C. and Weed, R.G. 1977 Slow and fast post-thaw dilutions in the cryoprotection of lymphocytes in whole blood. Cryobiology, 14, 696-697.

Jones, L.H. 1974 Plant cell culture and biochemistry: Studies for improved vegetable oil production. In, Industrial Aspects of Biochemistry. ed. B. Spencer Fed.Eur. Biochem. Soc., pp. 813-833.

Jones, L.H. 1974b Propagation of clonal oil palms by tissue culture. Oil Palm News, 17, 1-9.

Jones, L.H. 1974c Long term survival of embryos of carrot (Daucus carota L.) Plant Sci. Lett., 2, 221-224.

Kartha, K.K. and Gamborg, O.L. 1975 Elimination of cassava mosaic disease by meristem culture. Phytopath., 65, 826-828.

Kartha, K.K. and Gamborg, O.L. 1979 Cassava tissue culture - Principles and applications. In, Plant Cell and Tissue Culture: Principles and Applications. ed. W.M. Sharp, pp. 711-723. Ohio University Press, Columbus.

Kartha, K.K., Gamborg, O.L., Constabel, F. and Shyluk, J.P. 1974 Regeneration of cassava plants from apical meristems. Plant Sci.Lett., 2, 107-113.

Kartha, K.K., Leung, N.L. and Gamborg, O.L. 1979 Freeze preservation of pea meristems in liquid nitrogen and subsequent plant regeneration. Plant Sci.Lett., 15, 7-15.

Kartha, K.K., Leung, N.L. and Pahl, K. 1980 Cryopreservation of strawberry meristems and mass propagation of plantlets. J.Amer.Soc.Hort.Sci. (in press).

Kasha, K.J. 1974 Haploids in Higher Plants: Advances and Potential. The University of Guelph.

Keller, W.A. and Stringam, G.R. 1978 Production and utilization of microspore-derived haploid plants. In, Frontiers of Plant Tissue Culture, 1978. ed. T.A. Thorpe, pp. 113-122. International Association for Plant Tissue Culture.

King, J.R. 1965 The storage of pollen - particularly by the freeze-drying method. Bull Torrey. Bot. Club., 92, 270-287.

King, M.W. and Roberts, E.H. 1979 Storage of recalcitrant seeds - Achievements and Possible Approaches. IBPGR, Rome.

King, P.J., Potrykus, I. and Thomas, E. 1978 In vitro genetics of cereals: Problems and perspectives. Physiol.Végétale, 16, 381-399.

Koblitz, H. and Schumann, U. 1976 Tissue cultures of alkaloid plants. 5. Some morphogenic abilities of long-term callus cultures of **Macleaya microcarpa**. Biochem. Physiol.Pflanzen., 169, 289-297.

Kohlenbach, H.W. 1978 Comparative somatic embryogenesis. In, Frontiers of Plant Tissue Culture, 1978. ed. T.A. Thorpe, pp. 59-68. International Association for Plant Tissue Culture.

Lakshmi Sita, G., Raghava Ram, N.V. and Vaidyanathan, C.S. 1979 Differentiation of embryoids and plantlets from shoot callus of sandalwood. Plant Sci.Lett., 15, 265-270.

Latta, R. 1971 Preservation of suspension cultures of plant cells by freezing. Can.J. Bot., 49, 1253-1254.

Lavée, S. 1977 The growth potential of olive fruit mesocarp in vitro. Acta Hort., 78, 115-122.

Leiberman, M., Craft, C.C., Audia, W.V. and Wilcox, M.S. 1958 Biochemical studies of chilling injury in sweet potatos. Plant Physiol., 33, 307-311.

Leibo, S.P. and Mazur, P. 1970 In, The Frozen Cell. ed. G.E.W. Wolstenholme and M. O'Connor p. 235. London, Churchill.

Leibo, S.P., McGrath, J.J. and Cravalho, E.G. 1978 Microscopic observation of intracellular ice formation in unfertilized mouse ova as a function of cooling rate. Cryobiology, 15, 257-271.

Levin, R.L., Cravalho, E.G. and Huggins, C.E. 1977. Cryobiology, 14, 549-558.

Levitt, J. 1964 Cryobiology as viewed by the Botanist, Cryobiology, 1, 11-17.

Levitt, J. 1966 Cryochemistry of plant tissue-protein interactions. Cryobiology, 3, 243-251.

Levitt, J. 1966 Winter hardiness in plants In, Cryobiology ed. H.T. Meryman pp. 495-564, London, Academic Press.

Levitt, J. 1978 An overview of freezing injury and survival and its interrelationships to other stresses. In, Plant Cold Hardiness and Freezing Stress. ed. P.H. Li and A. Sakai. pp.3-15. Academic Press, New York.

Li, P.H. and Sakai, A. 1978 Plant Cold hardiness and Freezing Stress. London, Academic Press.

Liu, K.C. and Sherman, J.K. 1977 Ultrastructural comparison of freeze-drying and freeze-substitution in preservation of the frozen state. Cryobiology, 14, 382-386.

Liu, M-C. 1975 The in vitro induction of callus and regeneration of cassava plants from shoot apical meristems. Taiwan Sugar, 22, 171-177.

Lockett, M.C. and Luyett, B.J. 1951 Survival of frozen seeds of various water contents. Biodymanica, 134, 67-76.

Lovelock, J.E. and Bishop, M.W.H. 1959 Prevention of freezing damage to living cells by dimethylsulfoxide. Nature, 183, 1394-1395.

Luyett, B.J. 1966 Anatomy of the freezing process in physical systems. In, Cryobiology ed. H.T. Meryman pp. 115-138 London, Academic Press.

Luyett, B.J. and Keane, J. 1955 A critical temperature range apparently characterised by sensitivity of bull semen to high freezing velocity. Biodynamica, 7, 281-292.

Lyons, J.M. 1972 Phase transitions and control of cellular metabolism at low temperatures. Cryobiology, 9, 341-350.

Lyons, J.M. 1973 Chilling injury in plants Ann.Rev.Plant Physiol., 24, 445-466.

Maliga, P. 1978 Resistance mutants and their use in genetic manipulation. In, Frontiers of Plant Tissue Culture, 1978 ed. T.A. Thorpe, pp. 381-392. International Association for Plant Tissue Culture.

Mantell, S.H., Haque, S.Q. and Whitehall, A.P. 1978 Clonal multiplication of Dioscorea alata L. and Dioscorea rotundata Poir. yams by tissue culture. J.Hort.Sci., 53, 95-98.

Martin, F.W. 1975 The storage of germplasm of tropical roots and tubers in the vegetative form. In, Crop Genetic Resources for Today and Tomorrow. eds. O.H. Frankel and J.G. Hawkes, pp. 369-378, Cambridge University Press.

Mathews, T., Rangan, S. and Narayanswamy, S. 1976 Micropropagation of Ananas sativus in vitro. Z. Pflanzenphysiol., 79, 450-454.

Mazur, P. 1963 Kinetics of water loss from cells at sub-zero temperatures and the likelihood of intracellular freezing. J.Gen.Physiol., 47, 347-369.

Mazur, P. 1966 Physical and chemical basis of injury in single-celled micro-organisms subjected to freezing and thawing. In, Cryobiology ed. H.T. Meryman pp. 214-316, London, Academic Press.

Mazur, P. 1969 Freezing injury in plants. Ann.Rev.Plant Physiol., 20, 419-448.

Mazur, P. 1977 The role of intracellular freezing in the death of cells cooled at supra-optimal rates. Cryobiology, 14, 251-272.

Mazur, P., Leibo, S.P., and Chu, E.H.Y. 1972 A two-factor hypothesis of freezing injury. Evidence from chinese hamster tissue culture cells. Exp.Cell.Res., 71, 354-355.

Mazur, R.A. and Hartmann, J.X. 1979 Freezing of plant protoplasts. In, Plant Cell and Tissue Culture: Principles and Applications, ed. W.R. Sharp, p. 876. Ohio State University Press, Columbus.

McGann, L. 1978 Differing actions of penetrating and non-penetrating cryoprotective agents. Cryobiology, 15, 382-390.

McGann, L.E. and Kruuv, J. 1977 Freeze-thaw damage in protected and unprotected synchronised mammalian cells. Cryobiology, 14, 503-505.

McWilliam, A.A., Smith, S.M. and Street, H.E. 1974 the origin and development of embryoids in suspension cultures of carrot (Daucus carota L.). Ann.Bot., 38, 243-250.

Meins, F 1974 Mechanisms underlying the persistence of tumour autonomy in grown-gall disease. In, Tissue Culture and Plant Science, 1974. ed. H.E. Street, pp. 233-264. London, Academic Press.

Melchers, G. 1978 Potatoes for combined and sexual breeding methods: Plants from Protoplasts and fusion of Protoplasts of potato and tomato. In, Production of Natural Compounds by Cell Culture Methods, pp. 306-312. Ges. Strahlen- und Umweltforschg.

Meryman, H.T. ed. 1966a Cryobiology. Academic Press. London, New York.

Meryman, H.T. 1966b Review of biological freezing. In, Cryobiology ed. H.T. Meryman, pp.2-106. London, New York, Academic Press.

Meryman, H.T. 1971 Osmotic stress as a mechanism of freezing injury. Cryobiology, 8, 489-500.

Meryman, H.T. 1974 Freezing injury and its prevention in living cells. Ann.Rev.Biophys. Bioeng., 3, 341-363.

Meryman, H.T. and Douglas, M. 1977 Colligative cryoprotection, the only artificial protective mechanism at slow cooling rates. Cryobiology, 14, Abs. 8, p. 684.

Meryman, H.T., Williams, R.J. and Douglas, M. St. J. 1977 Freezing injury from solution effects and its prevention by natural or artificial cryoprotection. Cryobiology, 14, 287-302.

Meyer-Teuter, H. and Reinert, J. 1973 Correlation between rate of cell division and loss embryogenesis in long-term tissue cultures. Protoplasma, 78, 273-283.

Monnier, M. 1978 Culture of zygotic embryos. In, Frontiers of Plant Tissue Culture, 1978, ed. T.A. Thorpe, pp. 277-286. International Association for Plant Tissue Culture.

Morel, G. 1975 Meristem culture techniques for the long term storage of cultivated plants. In, Crop Genetic Resources for Today and Tomorrow, eds. O.H. Frankel and J.G. Hawkes. pp. 327-332. Cambridge University Press.

Morris, G.J. 1976 The cryopreservation of Chlorella I. Interactions of rate of cooling, protective additive and Warming rate. Arch.Microbiol., 107, 57-62.

Morris, G.J. 1976b The Cryopreservation of Chlorella II. Effect of growth temperature on freezing tolerance. Arch.Microbiol., 107, 309-312.

Morris, G.J. 1980 Cryopreservation of Plant Tissues. In, Low Temperature Preservation in Medicine and Biology. ed. M.J. Ashwood-Smith and J. Farrant, Pitman Medicine, London, p. 253-284.

Morris, G.J., Clarke, K.J. and Clarke, A. 1977 The cryopreservation of Chlorella III. Arch.Microbiol., 114, 249-254.

Mott, G.O. ed. 1977 Handbook for the Collection, Preservation and Characterisation of Tropical Forage Germplasm Resources. CIAT, Cali, Colombia.

Mullin, R.H. and Schlegel, D.E. 1976 Cold storage maintenance of strawberry meristem plantlets. Hort.Science., 11, 100-101.

Mumford, P.M. and Grout, B.W.W. 1978 Germination and liquid nitrogen storage of cassava seed. Ann.Bot., 42, 255-260.

Mumford, P.M. and Grout, B.W.W. 1979 Desiccation and low temperature (-196°C) tolerance of Citrus limon seed. Seed Science and Technol. (in press)

Murashige, T. 1974 Plant propagation through tissue cultures. Ann.Rev.Plant Physiol., 25, 135-166.

Murashige, T. 1977 Plant cell and organ cultures as horticultural practises. Acta Hort., 78, 17-30.

Murashige, T. 1978 The impact of plant tissue culture on agriculture. In, Frontiers of Plant Tissue Culture, 1978, ed. T.A. Thorpe pp. 15-26. International Association for Plant Tissue Culture.

Murashige, T. and Nakano, R. 1967 Chromosome complement as a determinant of morphogenic potential of tobacco cells. Am.J.Bot., 54, 963-970.

Murashige, T. and Skoog, F. 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol.Plantarum, 15, 473-497.

Nag, K.K. and Street, H.E. 1973 Carrot embryogenesis from frozen cultured cells. Nature, 245, 270-272.

Nag, K.K. and Street, H.E. 1975a Freeze-preservation of cultured plant cells. I. The pretreatment phase. Physiol.Plantarum, 34, 254-260.

Nag, K.K. and Street, H.E. 1975b Freeze-preservation of cultured plant cells. II. The freezing and thawing phases. Physiol.Plantarum, 34, 261-265.

Narayanswamy, S. 1977 Regeneration of plants from tissue cultures. In, Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. eds. J. Reinert and Y.P.S. Bajaj pp. 197-206. Berlin, Springer.

Nash, T. 1966 Chemical constitution and physical properties of compounds able to protect living cells against damage due to freezing and thawing. In, Cryobiology. ed. H.T. Meryman. pp. 197-211. Academic Press, New York.

Nath, T., and Anderson, J.O. 1975 Effect of freezing and freeze drying on the viability in storage of Lilium longiflorum L. and Zea mays L. pollen. Cryobiology, 12, 81-88.

National Academy of Sciences 1975 Underexploited Tropical Plants with Promising Economic Value. NAS, Washington D.C.

National Plant Genetic Resources Board. 1979 Plant Genetic Resources: Conservation and Use. USDA.

Nei, T. 1973 Growth of ice crystals in frozen specimens. J. Microscopy, 99, 227-233.

Nemmer, M.W. and Luyett, B.J. 1954 Survival of dehydrated pea seedlings. Biodynamica, 7, 193-211.

Nitzsche, W. 1978 Erhaltung der Lebensfahigkeit in getrocknetern Kallus. Z. fur Pflanz. Phys., 87, 469-472.

Novak, F.J. 1974 The changes of karyotype in callus cultures of Allium sativum L. Caryologia, 27, 45-54.

Novak, F.J. 1980 Phenotype and cytological status of plants regenerated from callus cultures of Allium sativum L. Z. Pflanzenzüchtg., 84, 250-260.

Ochoa, C. 1975 Potato collecting expeditions in Chile, Bolivia and Peru, and the genetic erosion of indigenous cultivars. In, Crop Genetic Resources for Today and Tomorrow. eds. O.H. Frankel and J.G. Hawkes. pp. 167-173. Cambridge University Press.

Ohomoto, T., Yoshida, K., Iizuka, H. and San Clemente, C.L. 1978 Relative stability of biological properties on encapsulated strains of Staphylococcus aureus after freezing and freeze-drying. Cryobiology, 15, 461-469.

Ojima, K. and Ohira, K. 1978 Nutritional requirements of callus and cell suspension cultures. In, Frontiers of Plant Tissue Culture, 1978. ed. T.A. Thorpe, pp. 265-276. International Association for Plant Tissue Culture.

Palta, J.P., Levitt, J., Stadelmann, E.J. and Burke, M.J. 1977a Dehydration of onion cells: A comparison of freezing vs. desiccation and living vs. dead cells. Physiol. Plantarum, 41, 273-279.

Palta, J.P., Levitt, J. and Stadelmann, E.J. 1977b Freezing tolerance of onion bulbs and significance of freeze-induced tissue infiltration. Cryobiology, 14, 614-619.

Palta, J.P., Levitt, J. and Stadelmann, E.J. 1977c Freezing injury in onion bulb cells. I. Evaluation of the conductivity method and analysis of ion and sugar efflux from injured cells. Plant.Physiol., 60, 393-397.

Palta, J.P., Levitt, J., Stadelmann, E.J. 1977d Freezing injury in onion bulb cells II. Post-thaw injury or recovery. Plant.Physiol., 60, 398-401.

Palta, J.P., Levitt, J. and Stadelmann, E.J. 1978 Plant viability assays. Cryobiology. 15, 249-255.

Palta, J.P. and Li, P.H. 1978 Cell membrane properties in relation to freezing injury. In, Plant Cold Hardiness and Freezing Stress. eds. P.H. Li and A. Sakai pp. 93-115. Academic Press, New York.

Paquin, R. 1977 Effect des basses temperatures sur la résistance au gel de la luzerne (Medicago media Pers.) et son contenu en proline libre. Physiol.Veg., 16, 657.

Pearce, R.S. and McDonald, I. 1977 Ultrastructural damage due to freezing followed by thawing in shoot meristem and leaf mesophyll cells of tall fescue (Festuca arundinacea Schreb.) Planta, 134, 159-168.

Pearce, R.S., Withers, L.A. and Willison, J.H.M. 1974. Bodies of wall-like material ("Wall Bodies") produced intracellularly by cultured isolated protoplasts and plasmolysed cells of higher plants. Protoplasma, 82, 223-236.

Peterson, W.D. and Stulberg, C.S. 1964 Freeze-preservation of cultured animal cells. Cryobiology, 1, 80-86.

Plattner, H., Fischer, W.M., Schmitt, W.W. and Bachman, L. 1972 Freeze etching of cells without cryoprotectants. J.Cell.Biol., 53, 116-126.

Pogosyan, K. 1971 Effect of freezing rate on survival of grapevine tissues. Fiziol. Rast., 18, 171-183.

Polge, C., Smith, A.U. and Parkes, A.S. 1949 Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature, 164, 666.

Pollard, A. and Wyn Jones, W.G. 1979 Enzyme activities in concentrated solutions of glycine betaine and other solutes. Planta, 144, 291-298.

Potrykus, I., Harms, C.T. and Lorz, H. 1979 Multiple-Drop-Array (MDA) Technique for the large scale testing of culture media variations in hanging micro-drop cultures of single cell systems. I. The technique, Plant Sci.Lett., 14, 231-235.

Pozner, R.I., Shepard, M.L., Cocks, F.H. and Stone, K.R. 1977 Post freeze viability of human renal epithelial carcinoma cells related to phase transformation in ternary solutions. Cryobiology, 14, 653-661.

Quatrano, R.S. 1968 Freeze-preservation of cultured flax cells utilizing DMSO. Plant Physiol., 43, 2057-2061.

Rabéchault, H., Ahee, J. and Guenin, G. 1970 Collonies cellulaires et formes embryöides obtenues in vitro a partir de cultures d'embryons de Palmier a huile (Elaeis guineensis) Jacq. var. dura Becc) C.R.Acad.Sci.Paris., 270, Ser.D., 3067-3070.

Rajagopal, V. and Anderson, A.S. 1978 Does abscisic acid influence proline accumulation in stressed leaves? Planta, 143, 85-88.

Rall, W.F. and Mazur, P. 1977 Calculation of water loss and intracellular freezing in cells frozen by two-step procedure. Cryobiology, 14, Abs. 7. p. 683.

Registry of Animal Cell Lines Certified by the Cell Culture Collection Committee (1964) U.S. Government Printing Office, Washington D.C..

Reid, D. 1978 A programmed controlled temperature microscope stage. J. Microscopy, 114, 241-248.

Reinert, J. 1959 Uber die Kontrolle der Morphogenese und die Induktion von Adventivembryonen an Gewebkulturen aus Karotten. Planta, 53, 318-333.

Reinert, J. and Bajaj, Y.P.S. 1977 Applied and Fundamental Aspects of Plant Cell Tissue and Organ Culture, Springer, Berlin.

Rey, L.R. 1959 Conservation de la vie par la froid. Actualités Sci Indust. Paris, Herman, p. 12[illegible].

Robards, A.W. 1974 Ultrastructural methods for looking at frozen cells. Sci.Prog., 61, 1-40.

Roberts, E.H. 1975 Problems of long term storage of seed and pollen for genetic resources conservation. In, Crop Genetic Resources for Today and Tomorrow. ed. O.H. Frankel and J.G. Hawkes. pp. 269-296. Cambridge University Press, Cambridge.

Roca, W.M. 1978 Report: Genetic Resources Unit, CIAT, Colombia.

Roca, W.M., Espinoza, N.O., Roca, M.R. and Bryan, E.J. 1978 A tissue culture method for the rapid propagation of potatos. Amer.Potato Journal, 55, 691-701.

Roca, W.M., Bryan, J.E. and Roca, M.R. 1979 Tissue culture for the international transfer of potato genetic resources. Amer.Potato Journal, 56, 1-10.

Rochat, E. and Therrien, H.P. 1975 Etude ultramicroscopique des modificacions cytologiques chez le blé d'hiver lors de l'endurcissement au froid. Can.J.Bot., 53, 536-543.

Rueveni, I., Adato, Y. and Lilien-Kipnis, L. 1972 A study of new and rapid methods for the vegetative propagation of date palm. Date Growers' Inst. Report., 19, 17-24.

Sacristán, M.D. and Melchers, G. 1969 The caryological analysis of plants regenerated from tumorous and other callus cultures of tobacco. Molec.Gen.Genet., 105, 317-333.

Sakai, A. 1960 Survival of the twigs of woody plants at -196°C. Nature, 185, 392-394.

Sakai, A. 1965 Survival of plant tissue at super-low temperatures. III Relation between effective prefreezing temperatures and degree of frost hardiness. Plant Physiol., 40, 882-887.

Sakai, A. 1971 Some factors contributing to the survival of rapidly cooled plant cells. Cryobiology, 8, 225-234.

Sakai, A. 1978 Low temperature exotherms of winter buds of hardy conifers. Plant and Cell Physiol., 19, 1439-1446.

Sakai, A. 1979 Deep supercooling of winter flower buds of **Cornus florida** L. Hort.-Science., 14, 69-70.

Sakai, A. and Nishiyama, Y. 1978 Cryopreservation of winter vegetative buds of hardy fruit trees in liquid nitrogen. Hort.Science., 13, 225-227.

Sakai, A. and Noshiro, M. 1975 Some factors contributing to the survival of crop seeds cooled to the temperature of liquid nitrogen. In, Crop Genetic Resources for Today and Tomorrow. eds. O.H. Frankel and J.C. Hawkes. pp. 317-326. Cambridge University Press, Cambridge.

Sakai, A., and Otsuka, K. 1967 Survival of plant tissue at super-low temperatures V An electron microscopical study of ice in cortical cells cooled rapidly. Plant Physiol., 42, 1680-1694.

Sakai, A. and Otsuka, K. 1972 A method for maintaining the viability of less hardy plant cells after immersion in liquid nitrogen. Plant and Cell Physiol., 13, 1129-1133.

Sakai, A., Otsuka, K., and Yoshida, S. 1968 Mechanism of survival in plant cells at super-low temperatures by rapid cooling and rewarming. Cryobiology, 4, 165-173.

Sakai, A. and Sugawara, Y. 1973 Survival of poplar callus at super-low temperatures after cold acclimation. Plant and Cell Physiology, 14, 1201-1204.

Sakai, A. and Sugawara, Y. 1978 Survival of plant germplasm in liquid nitrogen. In, Plant Cold Hardiness and Freezing Stress. eds. P.H. Li and A. Sakai, pp. 345-359. Academic Press, New York.

Sakai, A., Yamakawa, M., Sakato, D., Harada, T. and Yakuwa, T. 1978. Development of a whole plant from an excised strawberry runner apex frozen to -196OC. Low Temp. Sci.Ser.B., 36, 31-38.

Sakai, A. and Yoshida, S. 1967 Survival of plant tissue at super-low temperatures VI. Effects of cooling and rewarming rates on survival. Plant Physiol., 42, 1695-1701.

Sakai, A. and Yoshida, S. 1968 The role of sugar and related compounds in variations of freezing resistance. Cryobiology, 5, 160-174.

Saks, N.M. 1978 The preservation of salt marsh algae by controlled liquid nitrogen freezing. Cryobiology, 15, 563-568.

Sala, F., Cella, R., Rollo, F. 1979 Freeze-preservation of rice cells. Physiol. Plantarum, 45, 170-176.

Sala, F. Cella, R. Nielson, E., Rollo, F. and Parisi, B. 1980 Unpublished observations.

Scheider, O. 1978 Production and uses of chlorophyll deficient mutants. In, Frontiers of Plant Tissue Culture, 1978. ed. T.A. Thorpe, pp. 393-402. International Association for Plant Tissue Culture.

Scherer, W.F. and Hoogasian, A.C. 1954 Preservation of sub-zero temperatures of mouse fibroblasts (strain L) and human epithelial cells (strain Hela) Proc.Soc.Exp.Biol. Med., 87, 480-487.

Seibert, M. 1976 Shoot initiation from carnation shoot apices frozen to -196°C. Science, 191, 1178-1179.

Seibert, M. and Wetherbee, P.J. 1977 Increased survival and differentiation of frozen herbaceous plant organ cultures through cold treatment. Plant Physiol., 59, 1043-1046.

Shamina, Z.B. 1966 Cytogenetic study of tissue culture of **Haplopappus gracilis**. In, The Mutation Process: Mechanisms of Mutation and Inducing Factors. ed. Z. Landa, pp. 337-380. Prague, Academia.

Sharp, W.R. 1979 Plant Cell and Tissue Culture: Principles and Applications. Ohio State University Press, Columbus.

Sheldrake, A.R. and Northcote, D.H. 1968 The production of auxin by tobacco internode tissues. New Phytologist, 67, 1-13.

Shepard, J.F. and Totten, R.E. 1977 Mesophyll cell protoplasts of potato. Isolation, proliferation and plant regeneration. Plant Physiol., 60, 313-316.

Shillito, R.D. 1978 Problems Associated with the Regulation of Auxotrophic Mutants from Plant Cell Tissue Cultures. Ph.D. Thesis, University of Leicester.

Siminovitch, D. 1978 Protoplasts surviving freezing to -196°C and osmotic dehydration in 5 molar salt solutions prepared from the bark of winter black locust trees. Plant Physiol., 63, 722-725.

Siminovitch, D., Singh, J. and de la Roche, I.A. 1978 Freezing behaviour of free protoplasts of winter rye. Cryobiology, 15, 205-213.

Siminovitch, D., Therrien, H., Gfeller, F. and Rheaume, B. 1964 The quantitative estimation of frost injury and resistance in black locust, alfalfa and wheat tissues by determination of amino acids and other ninhydrin reacting substances released after thawing. Can.J.Bot., 42, 637-649.

Simione, F.P. and Daggett, T. 1977 Recovery of a marine dinoflagelate following controlled and uncontrolled freezing. Cryobiology, 14, 362-366.

Simione, F.P., Dagett, P.M., McGrath, M. and Alexander, M.T. 1977 The use of plastic ampoules for freeze-preservation of micro-organisms. Cryobiology, 14, 500-507.

Singh, B.D., Harvey, B.L., Kao, K.N. and Miller, R.A. 1972 Selection pressure in cell populations of Vicia hajastana cultured in vitro. Can.J.Genet.Cytol., 14, 65-70.

Singh, B.D., Harvey, B.L., Kao, K.N. and Miller, R.A. 1975. Karyotypic changes and selection pressure in Haplopappus gracilis suspension cultures. Can.J.Genet. Cytol., 17, 107-116.

Singh, J. 1979a Freezing of protoplasts isolated from cold-hardened and non-hardened winter rye. Plant Sci.Lett., 16, 195-201.

Singh, J. 1979b Ultrastructural alterations of cells of hardened and non-hardened winter rye during hyperosmotic and extracellular freezing stress. Protoplasma, 98, 329-341.

Singh, J., Siminovitch, D. and de la Roche, I.A. 1975 Membrane argumentation in freezing tolerance of plant cells. Nature, 247, 669-670.

Skaer, H. le B., Franks, F. and Echlin, P. 1978 Non-penetrating polymeric cryofixatives for ultrastructural and analytical studies of biological tissues. Cryobiology, 15, 589-602.

Skirvin, R.M. 1978 Natural and induced variation in tissue culture. Euphytica, 27, 241-266.

Skirvin, R.M. and Janick, J. 1976a Tissue culture variation in scented Pelargonium spp. J.Amer.Soc.Hort.Sci., 101, 281-290.

Skirvin, R.M. and Janick, J. 1976b 'Velvet Rose' Pelargonium, a scented geranium. Hort.Sci., 11, 61-62.

Smith, S.M. and Street, H.E. 1974 The decline of embryogenic potential as callus and suspension cultures of carrot (Daucus carota L.) are serially subcultured. Ann. Bot., 38, 223-241.

Sokolov, N.N., Sidorov, B.M. and Durimanova, S.A. 1974. Theor.App.Genetics., 44, 65-70.

Sopory, S.K. and Rogan, P.G. 1976. Induction of pollen divisions and embryoid formation in anther cultures of some dihaploid clones of Solanum tuberosum. Z. Pflanzenphysiol., 80, 77-80.

Stamp, J.A. 1978 Freeze-preservation of Shoot-tips of Potato Varieties. M.Sc. Thesis, University of Birmingham.

Stanwood, P.C. and Bass, L.N. 1978 Ultracold preservation of seed germplasm. In, Plant Cold Hardiness and Freezing Stresses. ed. P.H. Li and A. Sakai. pp. 361-371 Academic Press, New York.

Staritsky, G. 1970 Embryoid formation in callus tissues of coffee. Acta.Bot.Neerl., 19, 509-514.

Staritsky, G. 1970 Tissue culture of the oil palm (Elaeis guineensis Jacq.) as a tool for its vegetative propagation. Euphytica, 19, 288-292.

Staritsky, G. 1974 Tropical Root Crops Newsletter, 7, 38-39.

Steponkus, P.L. and Bannier, L.J. 1971 Cold acclimation of Chrysanthemum callus cultures J. Ann. Soc. Hort. Sci., 101, 409-412.

Steponkus, P.L. and Lanphear, F.O. 1967 Refinement of the triphenyl tetrazolium chloride method of determining cold injury. Plant Physiol., 42, 1432-1436.

Steponkus, P.L. and Weist, S.C. 1978 Plasma membrane alterations following cold acclimation and freezing. In, Plant Cold Hardiness and Freezing Stress. ed. P.H. Li and A. Sakai. pp. 75-91 Academic Press, New York.

Steward, F.C., Kent, A.E. and Mapes, M.O. 1967 Growth and organization in cultured cells: Sequential and synergistic effects of growth regulating substances. Ann.N. Y. Acad.Sci., 144, 326-334.

Stewart, G.R., and Lee, J.A. 1974 The role of proline accumulation in halophytes. Planta, 120, 279-289.

Storey, R., Ahmad, N. and Wyn-Jones, R.G. 1977 Taxonomic and related aspects of the distribution of glycine betaine and related compounds in plants. Oecologia, 27, 319-332.

Strauss, A., Gebhardt, C. and King, P.J. 1980 Selecting variation using plant somatic cells in vitro. In, tissue culture for plant pathologists. eds. D. Ingram and J. Helgeson, Blackwell, Oxford. (in press).

Street, H.E. 1974 Plant cell cultures: Present and projected applications for studies in genetics. In, Genetic Manipulations with Plant Material. ed. L. Ledoux, pp. 231-244. Plenum Press, New York.

Street, H.E. 1975 Present status of the freeze-preservation of cultured plant cells, IAPTC Newsletter, No. 15, pp. 2-4.

Street, H.E. 1977a Plant Tissue and Cell Culture. Oxford, Blackwell, Oxford.

Street, H.E. 1977b Cell (suspension) cultures - techniques. In, Plant Tissue and Cell Culture. ed. H.E. Street pp. 61-102. Blackwell Oxford.

Street, H.E. 1977c Laboratory Organization. In, Plant Tissue and Cell Culture, ed. H.E. Street pp. 11-30. Blackwell, Oxford.

Street, H.E. and Shillito, R.D. 1977 Nutrient media for plant organ, tissue and cell culture. In, Handbook Series in Nutrition and Food, VI. ed. M. Rechcigl Jnr., pp. 305-359. Palm Beach, USA, CRC Press.

Street, H.E. and Withers, L.A. The anatomy of embryogenesis in culture. In, Tissue Culture and Plant Science, 1974. ed. H.E. Street, pp. 71-100. Academic Press, London.

Stuart, R. and Street, H.E. 1971 Studies on the growth in culture of plant cells. X. Further studies on the conditioning of media by suspensions of Acer pseudoplatanus L. cells. J. Exp. Bot., 22, 96-106.

Sugawara, Y. and Sakai, A. 1974 Survival of suspension cultured sycamore cells cooled to the temperature of liquid nitrogen. Plant Physiol., 54, 722-724.

Sugawara, Y. and Sakai, A. 1975 Effect of Ca^{++} on the survival of cultured plant cells after freezing and thawing. Low Temp. Science Ser. B., 33, 21-28.

Sugawara, Y. and Sakai, A. 1978 Cold acclimation of callus cultures of Jerusalem artichoke. In, Plant Cold Hardiness and Freezing Stress, eds. P.H. Li and A. Sakai pp. 197-210. Academic Press, New York.

Sukumaran, N.P. and Weiser, C.J. 1972 Freezing injury in potato leaves. Plant Physiol., 51, 564-567.

Sun, C.N. 1958 The survival of excised pea seedlings after drying and freezing in liquid nitrogen. Bot.Gaz., 119, 234-236.

Sunderland, N. 1977 Nuclear cytology. In, Plant Cell and Tissue Culture. ed. H.E. Street, pp. 177-205. Blackwell, Oxford.

Sunderland, N. and Dunwell, J.M. 1977 Anther and pollen culture. In, Plant Tissue and Cell Culture. ed. H.E. Street, pp. 223-266. Blackwell, Oxford.

Sutton-Jones, B. and Street, H.E. 1968 Studies on the growth in culture of plant cells III Changes in fine-structure during the growth of Acer pseudoplatanus cells in suspension culture. J. Exp. Bot., 19, 114-118.

Syono, K. 1965 Changes in organ forming capacity of carrot root calluses during sub-cultures. Plant.Cell Physiol., 6, 403-419.

Tabata, M., Ogino, T., Yoshioka, K., Yoshikawa, N. and Hiraoka, N. 1978 Selection of cell lines with higher yield of secondary products. In, Frontiers of Plant Tissue Culture 1978. ed. T.A. Thorpe, pp. 213-222. International Association for Plant Tissue Culture.

Takeuchi, M. 1978 An attempt to freeze-preserve some cultured cells (Marchantia sp.) International Association for Plant Tissue Culture: Congress Abstracts, Calgary August 1978, Abstract 1200, p. 103.

Taylor, M.J., Walter, C.A. and Elford, B.C. 1978 The pH dependent recovery of smooth muscle from storage at -13°C in unfrozen media. Cryobiology, 15, 452-460.

Terasima, T. and Yasukawa, M. 1977. Dependence of freeze-thaw damage on growth phase and cell cycle of cultured mammalian cells. Cryobiology, 14, 379-381.

Thomas, E., King, P.J. and Potrykus, I. 1979 Improvement of crop plants via single cells in vitro - an assessment Z.Pflanzenzucht., 82, 1 - 30.

Thomas, E. and Wernike, W. 1978 Morphogenesis in herbaceous crop plants. In, Frontiers of Plant Tissue Culture, 1978. ed. T.A. Thorpe, pp. 403-410. International Association for Plant Tissue Culture.

Thompson, P.H. 1974 The use of seed banks for the conservation of populations of species and ecotypes. Biological Conservation, 6, 15-19.

Thorpe, T.A. 1978 Frontiers of plant tissue culture, 1978 International Association for Plant Tissue Culture, Calgary University Press.

Torrey, J.G. 1961 Kinetin as a trigger for mitosis in mature endomitotic plant cells. Exp.Cell Res., 23, 281-299.

Torrey, J.G. 1967 Morphogenesis in relation to chromosomal constitution in long-term plant tissue cultures. Physiol.Plant., 20, 265-275.

Towill, L.E. 1979 Cryopreservation of potato germplasm using shoot-tip culture methodology. (Abstract) Cryobiology, 16, 583-584.

Towill, L.E. and Mazur, P. 1974a Effect of cooling and warming rates on survival of frozen Acer saccharum tissue cultures. Plant Physiol., 54, (suppl.), 10.

Towill, L.E. and Mazur, P. 1977b Studies on the reduction of 2,3,5-triphenyl tetrazolium chloride as a viability assay for plant tissue cultures. Can.J.Bot., 53, 1097-1102.

Towill, L.E. and Mazur, P. 1976 Osmotic shrinkage as a factor in freezing injury in plant tissue cultures. Plant Physiol., 57, 290-296.

Tran Than Van, K. and Trinh, H. 1978 Morphogenesis in thin cell layers: concept, methodology and results. In, Frontiers of Plant Tissue Culture, 1978. ed. T.A. Thorpe, pp. 37-48. International Association for Plant Tissue Culture.

Trump, B.F., Croker, B.P. and Mergner, W.J. 1971 The role of water, Energy metabolism ion and water shifts in the pathogenesis of cell injury. In, Cell Membranes. Biological and Pathological Aspects. eds. Richter, G.W. and D.G. Scarpelli, pp. 84-129, Williams and Wilkins Co., Baltimore.

Tumanov, I.I., Butenko, R.G. and Ogolevets, I.V. 1968 Application of isolated tissue culture techniques for studying hardening processes in plant cells. Fiziol. Rast., 15, 749-756.

Ulrich, J.M., Finkle, B.J., Moore, P.H. and Ginoza, H. 1979 Effect of a mixture of cryoprotectants in attaining liquid nitrogen survival of callus cultures of a tropical plant. Cryobiology, 16, 550-556.

Vasil, V. and Vasil, I.K. 1979 Isolation and culture of cereal protoplasts. I. Callus formation from pearl millet (Pennisetum americanum) protoplasts. Z. Pflanzenphysiol., 92, 379-383.

Vasil, V. and Vasil, I.K. 1980 Isolation and culture of cereal protoplasts. II. Embryogenesis and plantlet formation from protoplasts of Pennisetum americanum. Z. Pflanzenphysiol. (in press)

Verkleij, A.J., Ververgaert, P.H.J., Vandeenen, L.L.M. and Elbers, P.F. 1972 Phase transitions of phospholipid bilayers and membranes of Acholoplasma laidlawii visualised by freeze-fracture electron microscopy. Biochim.Biophys.Acta., 228, 326-332.

Villiers, T.A. 1975 Genetic maintenance of seeds in imbibed storage. In, Crop Genetic Resources for Today and Tomorrow, eds. O.H. Frankel and J.G. Hawkes, pp. 297-315, Cambridge University Press.

Visser, T. 1955 Germination and storage of pollen. Meded. Landbouwhogesch. Wageningen, 55, 1-68.

Walkey, D.G.A. 1978 In vitro methods for virus elimination. In, Frontiers of Plant Tissue Culture, 1978, ed. T.A. Thorpe, pp. 245-254. International Association for Plant Tissue Culture.

Wang, C.C., Sun, C.S. and Chu, C.C. 1977 Acta Bot. Sinica, 19, 190-199.

Wang, P.J. 1977 Regeneration of virus-free potato from tissue culture. In, Plant Tissue Culture and its Bio-Technological Applcation. ed. W. Barz, E. Reinhard and M.H. Zenk, pp. 386-391. Berlin, Springer.

Wang, P.J. 1978 Gene preservation and foundation stock seed production of potato in vitro. Fourth International Congress of Plant Tissue and Cell Culture. Abstract no. 1205, p. 105.

Warren, G.S. and Fowler, M.W. 1979 Changing fatty acid composition during somatic embryogenesis in cultures of Daucus carota. Planta, 144, 451-454.

Weatherhead, M.A., Grout, B.W.W. and Henshaw, G.G. 1979 Advantages of storage of potato pollen in liquid nitrogen. Potato Research, 21, 97-100.

Webb, K.J. and Street, H.E. 1977 Morphogenesis in vitro of Pinus and Picea. Acta Hort., 78, 259-269.

Weist, S.C. and Steponkus, P.L. 1978 Freeze-thaw injury to isolated spinach protoplasts and its simulation at above freezing temperature. Plant.Physiol., 62, 699-705.

Westcott, R.J., Henshaw, G.G., Grout, B.W.W. and Roca, W.M. 1977 Tissue culture methods and germplasm storage in potato. Acta.Hort., 78, 45-49.

Westcott, R.J., Henshaw, G.G. and Roca, W.M. 1977 Tissue culture storage of potato germplasm: Culture initiation and plant regeneration. Plant Sci.Lett., 9, 309-315.

White, P.R. 1954 The Cultivation of Animal and Plant Cells. London, Thames and Hudson.

Whittingham, D.G. 1978 Freezing embryos of laboratory species. Cryobiology, 15, 367-369.

Whittingham, D.G., Leibo, S.P. and Mazur, P. 1972 Survival of mouse embryos frozen to -196°C and -269°C. Science, 178, 411-414.

Whittingham, D.G., Lyon, M.E. and Glenister, P.H. 1977 Long-term storage of mouse embryos at -196°C: The effect of background radiation. Genet. Res., 29, 171-178.

Widholm, J.M. 1977 The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol., 47, 189-194.

Wilkes, G. 1977 The world's crop plant germplasm - An endangered resources. Bull. Atomic. Sci., 33, 9-18.

Willemot, C., Hope, H.J., Williams, R.J. and Michaud, R. 1973 Changes in patty acid composition of winter wheat during frost hardening. Cryobiology, 14, 87-93.

Willis, J.S. 1972 The possible roles of cellular K for survival of cells at low temperatures. Cryobiology, 9, 351-366.

Wilson, J.M. and Crawford, R.M.M. 1974 Leaf patty acid content in relation to hardening and chilling injury. J. Exp. Bot., 25, 121-131.

Wilson, R.F. and Rinne, R.W. 1976 Effect of freezing and cold storage on phospholipids in developing soybean cotyledons. Plant Physiol., 57, 270-273.

Winton, L.L. 1978 Morphogenesis and clonal propagation of woody plants. In, Frontiers of Plant Tissue Culture, 1978. ed. T.A. Thorpe, pp. 419-426. International association fcr Plant Tissue Culture.

Withers, L.A. 1973 A Study of the Fusion of Higher Plant Protoplasts. PhD Thesis, University of Nottingham.

Withers, L.A. 1978a The freeze-preservation of synchronously dividing cultured cells of Acer pseudoplatanus L. Cryobiology, 15, 87-92.

Withers, L.A. 1978b A fine-structural study of the freeze-preservation of plant tissue cultures. II The Thawed state. Protoplasma, 94, 235-247.

Withers, L.A. 1978c Freeze-preservation of cultured cells and tissues. In, Frontiers of Plant Tissue Culture, 1978. ed. T.A. Thorpe, pp. 297-306. International Association for Plant Tissue Culture.

Withers, L.A. 1979 Freeze-preservation of somatic embryos and clonal plantlets of carrot (Daucus carota L.) Plant Physiol., 63, 460-467.

Withers, L.A. 1980a Low temperature storage of plant tissue cultures. In, Advances in Biochemical Engineering. Volume 18. ed. A. Fiechter, Springer, Berlin pp. 102-150.

Withers, L.A. 1980b Preservation of germplasm. In, Perspectives in plant cell and tissue culture. International Review of Cytology. Suppl. IIB. ed. I.K. Vasil and D.G. Murphy, Academic Press, New York, pp. 101-136.

Withers, L.A., 1980c Cryopreservation of plant cell and tissue cultures. In, Tissue Culture for Plant Pathologists. ed. D. Ingram and J. Helgeson, Blackwell, Oxford. (in press)

Withers, L.A. 1980d Cryopreservation of plant tissue cultures. In, Memoirs, XX Anniversary of the Postgraduate College, Chapingo, Mexico. ed. V.M. Villalobos. (in press).

Withers, L.A. 1980e The cryopreservation of higher plant tissue and cell cultures - an overview with some current observations and future thoughts. Cryo-letters, 1, 239-250.

Withers, L.A. 1980f Unpublished observations.

Withers, L.A. and Batchelor, S. 1980 Unpublished observations.

Withers, L.A. and Cocking, E.C.D. 1972 Fine structural studies on spontaneous and induced fusion of higher plant protoplasts. Protoplasma, 11, 59-75.

Withers, L.A. and Davey, M.R. 1978 A fine-structural study of the freeze-preservation of plant tissue cultures. I. The frozen state. Protoplasma, 94, 207-219.

Withers, L.A. and King, P.J. 1979a Proline - A novel cryoprotectant for the freeze-preservation of cultured cells of Zea mays L. Plant Physiology, 64, 675-678.

Withers, L.A. and King, P.J. 1979b The freeze-preservation of cultured plant cells. (Abstract). Experientia, 35, 984.

Withers, L.A. and King, P.J. 1980a A simple freezing-unit and routine cryopreservation method for plant cell cultures. Cryo-letters, 1, 213-220.

Withers, L.A. and King, P.J. 1980b Unpublished observations.

Withers, L.A. and Street, H.E. 1977a The freeze-preservation of cultured plant cells: III The pregrowth phase. Physiol.Plant., 39, 171-178.

Withers, L.A. and Street, H.E. 1977b Freeze-preservation of plant cell cultures. In, Plant Tissue Culture and its Bio-Technological Application. eds. W. Barz, E. Reinhard and M-H, Zenk, pp. 226-244. Springer, Berlin.

Wolstenholme, C.E.W. and O'Connor, M. 1970 (eds.) The Frozen Cell. Churchill, London.

Wood, T.H. and Taylor, A.L. 1957 X-Ray inactivation of yeast at freezing temperatures. Radiation Res., 7, 99-106.

Wu, L. and Li, H.W. 1971 Induction of callus tissue initiation from different somatic tissue organs of rice plant by various concentrations of 2,4-dichlorophenoxyacetic acid. Cytologia, 36, 411-416.

Yamaguchi, T. and Nakajima, T. 1973 Hormonal regulation of organ formation in cultured tissue derived from root tuber of sweet potato. In, Proc. 8th Intern. Conf. Plant Growth Substances, Tokyo, pp. 1121-1127.

Yeoman, M.M. and MacLeod, A.J. 1977 Tissue (callus) cultures - Techniques. In, Plant Cell and Tissue Culture, ed. H.E. Street, pp. 31-58. Blackwell, Oxford.

Yeoman, M.M. and Street, H.E. 1977 General cytology of cultured cells. In, Plant Tissue and Cell Culture. ed. H.E. Street, pp. 137-176. Blackwell, Oxford.

Yoshida, S. and Sakai, A. 1974 Phospholipid degradation in frozen plant cells associated with freezing injury. Plant Physiol., 53, 509-511.

Zenk, M-H. 1978 The impact of plant cell culture on industry. In, Frontiers of Plant Tissue Culture, 1978 ed. T.A. Thorpe, pp. 1-4. International Association for Plant Tissue Culture.