Recent Progress in Cryopreservation of Plant Genetic Resources

Masaya ISHIKAWA*

Abstract

Cryopreservation, if successful, may enable to achieve long-term storage of various types of plant genetic resources, including seeds, both short-lived and recalcitrant types, vegetatively propagated plants *in vitro*, cultured tissues and cells, etc. In the last couple of years, significant progress has been made in the field of cryopreservation techniques. Basically, three methods are currently applied, namely desiccation, vitrification and conventional slow prefreezing. Here some successful cases of cryopreservation and problems encountered using these methods will be introduced. Seeds of *Eutrema wasabi* and *Poncirus trifoliata*, both of which have been classified as recalcitrant seeds, could withstand liquid nitrogen temperature either after slow desiccation of the entire mature seeds or after excision of the embryo axis, respectively. Cultured shoot primordia of melon were successfully cryopreserved by the application of the slow prefreezing method. Somatic embryos of melon survived cryogenic storage after controlled desiccation in the presence of abscisic acid or by the application of the slow prefreezing method. Cryopreservation using the vitrification method for meristems and buds of relatively cold-hardy plants has been successful. However, the application of these methods to plants sensitive to freezing such as potatoes remains difficult, mainly due to the low survival rate.

Introduction

There is a growing need for cryopreservation which enables to achieve stable long-term storage of various types of plant genetic resources. Cryopreservation could provide the ideal condition for base collection of vegetatively propagated plants, recalcitrant seeds and even orthodox seeds as well. As the number of accessions in the seed bank increases, periodical germination check-up and regeneration become actually difficult with the budget or staff allocated. For orthodox seeds or pollen with shorter life, storage at lower temperatures is preferable. The remarkable progress in the field of plant tissue culture in the last decades has introduced another category of genetic resources which require cryopreservation for long-term storage, including protoplasts, cells, meristems, somatic embryos, embryogenic calli, cultured shoot primordia, etc. They are important either as a tool for clonal propagation, production of virus-free plants, transformation, regeneration, or as an experimental system for basic research. These cultures or their specific characters can be easily lost due to prolonged subcultures, contamination, limitation in labor, personnel changes, etc. Cryopreservation could enable to safely preserve these cultures and provide them when necessary with the original characters.

In the last couple of years, significant progress has been made in the field of cryopreservation techniques, especially for cultured materials. The present report aims at introducing and reviewing these developments including some of the studies carried out in our laboratory.

Presented at the 27th International Symposium on "Plant Genetic Resource Management in the Tropics", Tsukuba, Ibaraki, Japan, 25-26 August 1993, held by Tropical Agriculture Research Center (TARC).

* Department of Genetic Resources II, National Institute of Agrobiological Resources, Kan'nondai, Tsukuba, Japan 305

Principles of cryopreservation techniques

The most important factors controlling the survival of plant cells at liquid nitrogen temperature are the reduction in the amount of free water and the increase in the amount of bound water. Free water freezes at extremely low temperatures. In contrast, bound water, which comprises about 15 to 20% of the fresh weight of a tissue and which is bound to proteins, saccharides, etc, cannot be frozen or becomes readily vitrified even at extremely low temperatures. Currently, three cryopreservation methods are available, namely desiccation, slow prefreezing and vitrification. They differ in the way the amount of free water is being reduced (Fig. 1). Desiccation method removes free water by transferring into the air. In the slow prefreezing method, free water inside the cells is transferred to extracellular ice during the slow freezing process (extracellular freezing) (Fig. 1). Vitrification method employs extensive plasmolysis to remove free water by soaking the specimens in high osmotic solutions. With any of these methods, preculture is often employed to precondition the specimens prior to cryopreservation. Preculture is designated to reduce the amount of free water and obtain cells with a less vacuolated state. Addition of cryoprotectants such as DMSO, sucrose, etc, also reduces the amount of free water and increases that of bound water (Chen *et al*., 1984). In the following paragraphs, each method is described with some case studies and advantages and problems are discussed.

1 Desiccation method

The principles of this method are to induce an intrinsic tolerance to desiccation by triggering the genes responsible for desiccation tolerance and to reduce the water content to a sufficiently low level where there is little free water. Orthodox seeds undergo these processes spontaneously during the course of their maturation. Most higher plants seem to have the genes for tolerance to a totally desiccated state, since they tolerate such a state in a part of their life cycle, either at the seed stage or pollen stage. It remained to be determined whether it is possible to induce similar processes in recalcitrant seeds, buds, meristems, somatic embryos, etc, to preserve them in liquid nitrogen. The answer is positive, in some cases. Typical procedures are summarized in Table 1. Here some successful cases will be described in the following paragraphs.

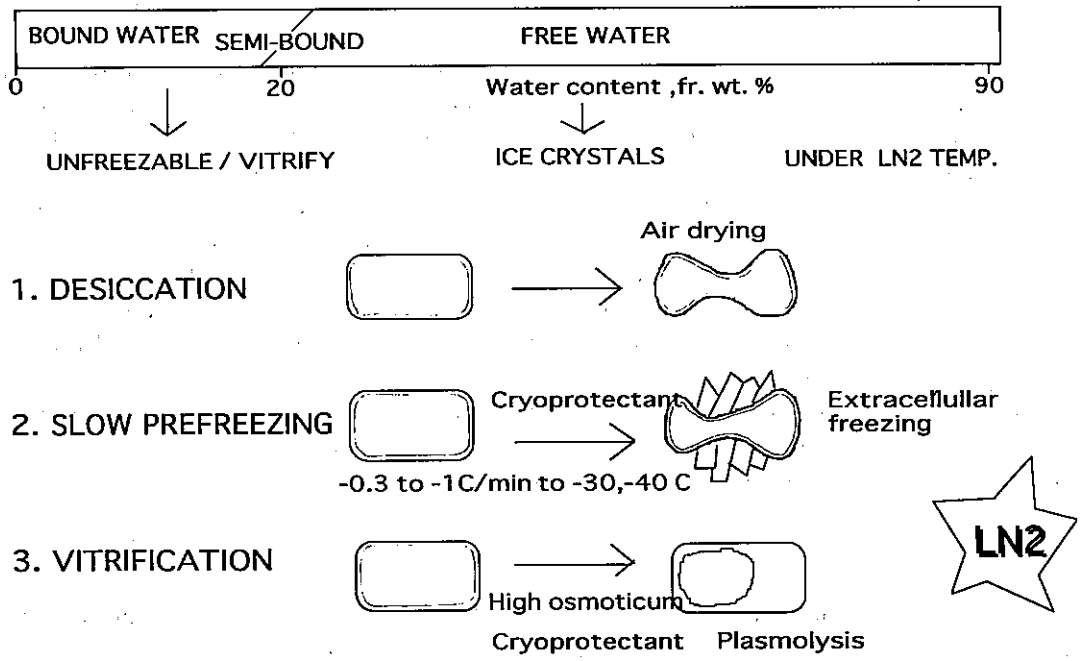

Fig. 1 Principles of methods of cryopreservation, illustrating desiccation, slow prefreezing and vitrification with emphasis placed on the way of reducing the amount of free water

Table 1 Desiccation method

Principles :	1. Induce intrinsic tolerance to desiccation. 2. Reduce water content to reach a low level of free water
Step 0 :	Selection of suitable organs or tissues and stages for storage
Step 1 :	Preculture (trigger genes responsible for desiccation tolerance) ABA/ high osmoticum 0.5-1.2 M sucrose/ proline, etc
Step 2 :	Drying
Slow :	1. controlled desiccation under a constant relative humidity 2. encapsulation in alginate beads
Rapid :	1. in a laminar flow chamber 2. under silica gel
Step 3 :	Direct storage in LN_2 / -80°C/ -20°C
Applicability :	Buds, meristems, calli, somatic embryos, seeds, embryo axis
Advantages :	1. Handling and storage after desiccation are easy as in the case of orthodox seeds. 2. If successful with meristems or buds, the entire dome or bud tends to survive, thus eliminating possible variant formation.
Problems :	1. Longevity of the specimen under dry state is unknown due to the lack of protection barriers like seed coats. Lower storage temperatures preferable. 2. Cultivar differences in desiccation tolerance

1. Recalcitrant seeds

Eutrema wasabi is a plant growing in chilly water and seeds usually drop into water at maturity and have been considered to be recalcitrant seeds. However, it appeared that the seed storage behavior depended on the rate of desiccation and relative humidity (RH) during the storage (Fig. 2) (Nakano *et al*., 1989). When the whole seeds were exposed to RH 0% at room temperature, the water content was reduced to 6.7% (fr. wt.) in 12 days and their germinability was lost. When the seeds were placed at RH 85%, the water content reached an equilibrium at 14%, and they retained their germinability (80%). However, they totally lost their germinability in one month of storage. Seed germinability and longevity were best retained when seeds were placed at RH 50% : the water content reached an equilibrium of 9% and the seeds still showed high germination rate (over 90%) (Fig. 2). The seeds dried to a 8 % water content at RH 50% for 30 days were subjected to cold storage at 0, −20, −80 and −196°C. The seeds stored in the liquid nitrogen vapor phase retained their germinability even after 1 year. These results indicate that wasabi seeds tolerate a water content as low as 8% like orthodox seeds only when they were desiccated mildly and that for desiccated seeds, storage at cryogenic temperatures was preferable due to the shorter longevity.

2. Recalcitrant seeds

Seeds of *Poncirus trifoliata* are typical recalcitrant seeds as shown in Fig. 3 (Hemachandra, 1990) and also in a literature (Honjo and Nakagawa, 1978). When the embryo axis (3 to 4 mm) was excised, 20 to 40% of the excised axis tolerated a lower water content (13 to 20% on fr. wt. basis). The desiccated axis survived liquid nitrogen exposure (20 to 25%) (Fig. 3) (Yamaguishi-Ciampi, 1990), indicating that tissues show differences in the desiccation tolerance and that cryopreservation of recalcitrant *Poncirus trifoliata* seeds is possible. In the foregoing two cases, the seeds or embryo axis survived desiccation without any preculture. However, for more sensitive tissues, preculture is a prerequisite.

3. Somatic embryos of melon

Desiccation and cryopreservation were carried out in somatic embryos induced from hypocotyls of mature melon seeds (Shimonishi *et al*., 1991). Induced embryos were precultured on MS medium with 10 ppm abscisic acid (ABA) and 3% sucrose for 3 days. In the absence of this preculture with ABA, the embryos did not tolerate desiccation (data not shown). The embryos were classified into L (3 mm), M (2 mm), S (1 mm) according to their size. Then, they were desiccated aseptically in chambers at constant RH (50,

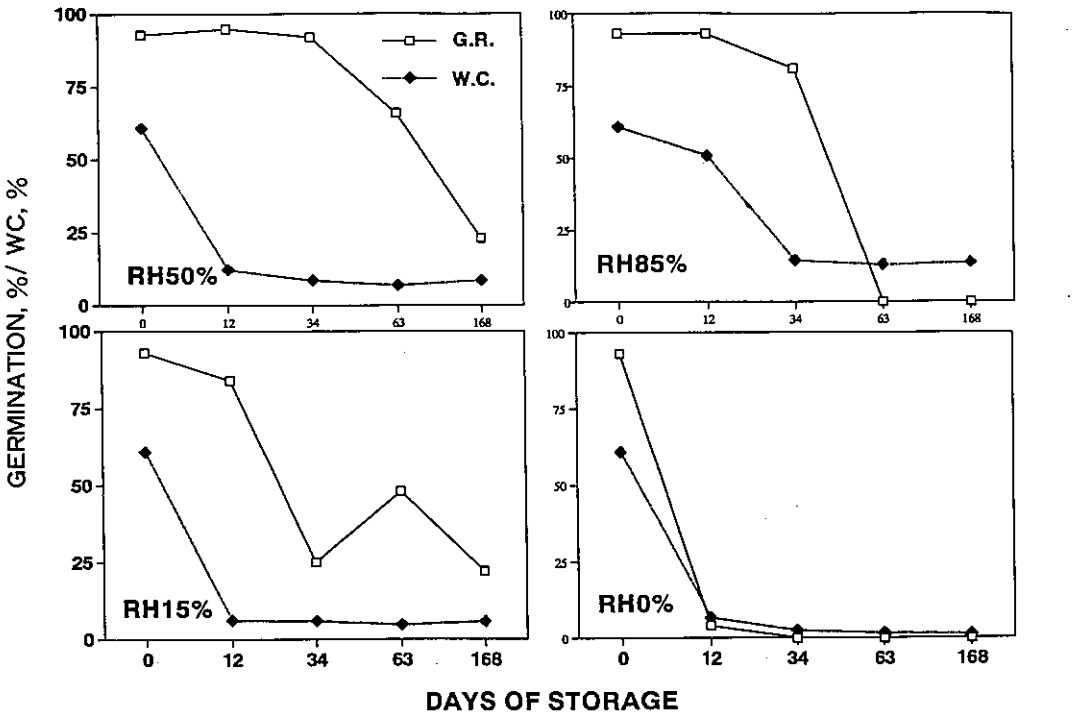

Fig. 2 Longevity and water content of wasabi (*Eutrema wasabi*) seeds stored in chambers at different relative humidities and at room temperature. G. R.: germination rate, %. W. C.: water content on fresh weight basis, %. (Modified from Nakano *et al.*, 1989)

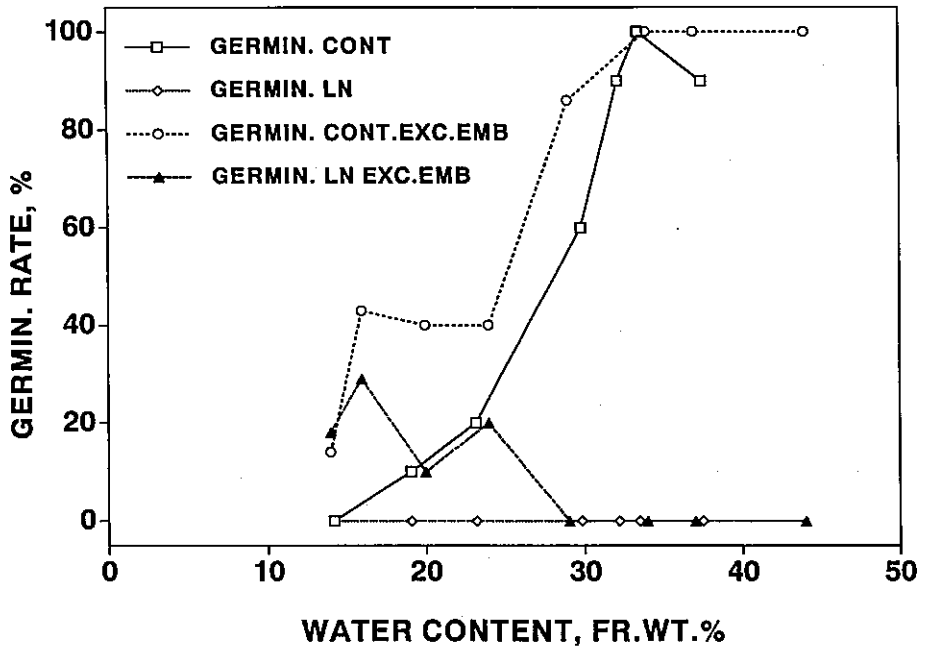

Fig. 3 Survival of entire seeds and excised embryo axes (EXC. EMB) of *Poncirus trifoliata* desiccated to various water contents (CONT) and those desiccated and exposed to liquid nitrogen temperature (LN). (Modified from Hemachandra, 1990, Yamaguishi, 1990)

60, 65%) by using special containers with membrane filters (for details, see Shimonishi *et al.*, 1991 or Ishikawa, 1992). Fig. 4 shows the time course of embryo desiccation in these containers. The water content reached an equilibrium within 3 to 4 days. Following desiccation, the embryos were transferred to glass vials, directly plunged into liquid nitrogen and stored for 1 day. Then they were rewarmed rapidly in water at 40°C and recultured. Survival rate of melon embryos after desiccation and after cryopreservation was similar regardless of the treatments (Fig. 5), indicating that the survival was mainly restricted by the desiccation tolerance. The desiccation tolerance was affected by the size of the embryo and RH. M, L (2 to 3 mm) sizes led to better survival than S (1 mm) regardless of RH (Fig. 5). Desiccation of the em-

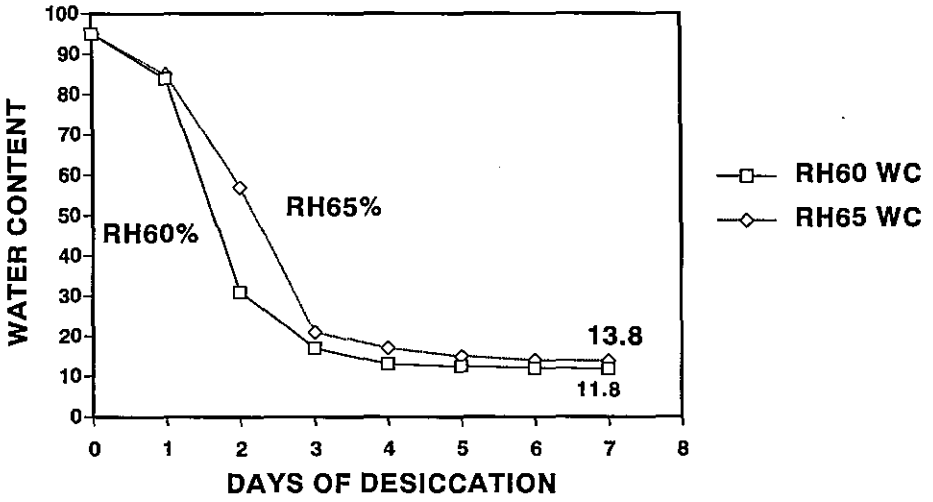

Fig. 4 Time course of desiccation of melon somatic embryos under constant relative humidities. Somatic embryos were placed on a filter paper containing 3% sucrose and 10 ppm abscisic acid in a small culture bottle with two holes covered with Miliseal@. The culture bottle was placed in a chamber set at a constant relative humidity for controlled desiccation. Water content was expressed as the percentage of the fresh weight. (Shimonishi *et al.*, 1991)

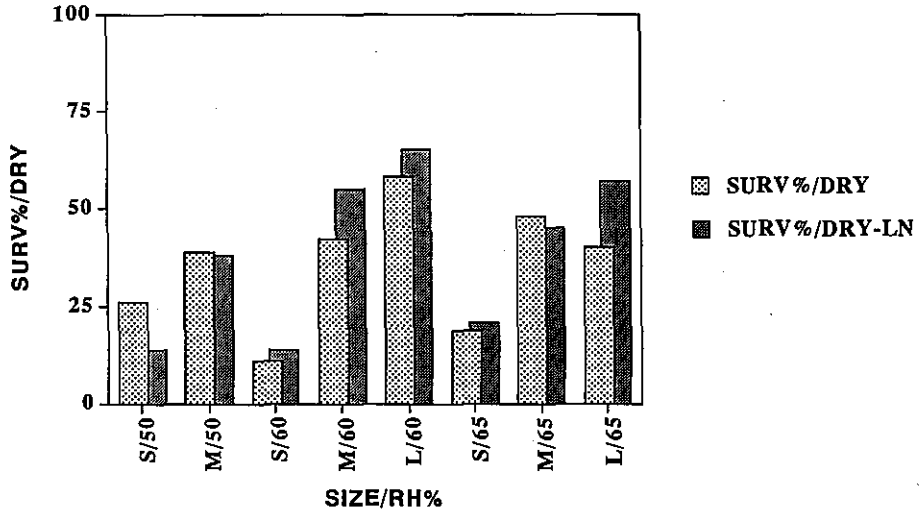

Fig. 5 Effect of embryo size (S, M, L) and relative humidity (50, 60, 65% RH) employed on the survival of melon somatic embryos after controlled equilibrium desiccation (DRY) and liquid nitrogen exposure (DRY-LN). For details, refer to the text. (Modified from Shimonishi *et al.*, 1991)

bryos at RH 60 or 65% produced better survival than that at RH 50% (Fig. 5). Most of the somatic embryos which survived cryopreservation germinated on the reculture medium and were able to grow into plants.

4. Potato meristems

Cryopreservation of potato meristems has been attempted by applying the slow prefreezing method with limited success (Grout and Henshaw, 1978; Bajaji, 1981; Towill, 1988). As most of the apices which survived cryopreservation developed calli before regenerating into plants, partial survival of apical cells was inferred, indicating that a particular cell can be selected in addition to variant formation due to callus development. To avoid this phenomenon the entire meristem should survive through the use of desiccation or vitrification method. In our laboratory, we attempted to desiccate lateral buds of *in vitro* grown plantlets from commercially important cultivars of potato (*Solanum tuberosus*). A typical protocol is as follows. Excised lateral buds (with 3 to 4 mm stems) at various stages and positions on the nodes were either encapsulated in alginate beads or processed without encapsulation. Then, they were precultured on increasing osmoticum (0.3 to 0.7 M sucrose at 2 day intervals) before being dried in a laminar flow chamber. The beads dried to various water contents (7 to 25%) were processed for cryopreservation. We have observed that most of the buds survived desiccation to about 10% water content and retained a high regeneration rate with marginal callus formation, but that the dried buds hardly survived liquid nitrogen exposure. Fabre and Dereuddre (1990), studying on *Solanum phureja*, recorded a survival rate of about 30% in the cryopreservation of desiccated lateral buds. To my knowledge, cryopreservation of buds of commercial potato varieties by the application of the desiccation method has been unsuccessful.

5. Problems and advantages

Since the desiccated materials contain a negligible amount of free water, handling and storage after desiccation are easy like in the case of orthodox seeds. They are more resistant to accidental temperature rise during storage than the materials cryopreserved with other methods. Another advantage is that this method does not require any special equipment, although the preculture process is rather time-consuming. This method utilizes intrinsic mechanisms, thus avoiding the use of toxic chemicals such as DMSO. When this method is applied to meristems or buds, the entire dome or tissues tends to survive, which could eliminate possible selection and variant formation.

Seeds have testa or seed coats, which act as a protection barrier against stresses from oxygen, mechanical damage, etc. during storage. However, artificially desiccated materials shown here do not have such a barrier and the longevity of such materials could be shorter than that of natural seeds. Therefore, storage at lower temperatures is recommended.

One problem encountered in the application of the desiccation method to the preservation of a germplasm is that cultivars usually show various degrees of desiccation tolerance even after the induction process. This phenomenon is partly due to genetic differences in sensitivity to desiccation and to insufficient optimization of desiccation tolerance induction. Solution to this problem requires more research into the mechanism of desiccation tolerance of plants, especially the induction mechanism.

As could be seen from the examples presented here, some materials show a better survival with slow drying under controlled relative humidity or in alginate beads and others with rapid drying in a laminar flow chamber. To induce desiccation tolerance, for some materials ABA treatments are preferable, while for others, treatment with gradual increase of osmoticum such as sucrose is more suitable. With either of these combinations, the genes responsible for the desiccation tolerance are switched on, resulting in cellular changes and during drying, sucrose (exogenous and/ or endogenous) seems to be required as a desiccation protectant to membranes and cellular components. With desiccation method, various cultured materials have been cryopreserved: calli of carrot (Nitzsche, 1980) and rice (Shin *et al*., 1991), embryos of alfalfa (Senaratna *et al*., 1990), oil palm (Dumet *et al*., 1993), meristems of sugarcane (Paulet *et al*., 1993) and *Primula* (Kato and Ishikawa, unpublished), buds of asparagus (Uragami *et al*., 1990), apple, pear and mulberry (Niino and Sakai, 1992), and embryo axes of tea (Chaudhury *et al*., 1991).

2 Slow prefreezing method (2-step method)

In this method, freezing tolerance is conferred by the addition of cryoprotectants and dehydration of cells, by extracellular freezing (Fig. 1, Table 2), which is initiated by ice-inoculation and pursued during

slow (−0.3 to −1°C/ min) cooling to −30 to −40°C. This method is more conventional and has been reviewed elsewhere (Kartha, 1985, Withers, 1985). Recent efforts have been made to simplify the procedures. The most time-consuming steps are the gradual dropwise addition of cryoprotectant and the dilution of the cryoprotectant, often carried out on ice at 0°C. Chen *et al*. (1984) developed a method to circumvent the latter step by plating the cryopreserved cells on filter papers and later transferring the filters onto fresh media. In our laboratory, we coincidentally simplified the "addition" part by developing a cryoprotectant (Fig. 6). We observed that the cryoprotectant leading to the highest survival rate (Sucrose: DMSO: Glycerol=10 : 10 : 5% w/ w) can be added directly to the specimen at room temperature without affecting the survival after cryopreservation (Ishikawa *et al*., 1991). However, the cryoprotectant must be gradually diluted. With this cryoprotectant, we could successfully cryopreserve somatic embryos (Niwata *et al*., 1991) and shoot primordia of melon (Ogawa *et al*., 1991) and embryogenic calli of Taro (Shimonishi *et al*., 1993).

Many people bileve that slow prefeezing method requires an expensive program freezer. However, it

Table 2 Slow prefreezing method

Principles:	1. Confer freezing tolerance by the addition of cryoprotectants 2. Dehydration by extracellular freezing
Step 0:	Selection of the appropriate stage
Step 1:	Preculture
Step 2:	Addition of cryoprotectants: slow/ rapid
Step 3:	Ice inoculation (-8 to -10°C)
Step 4:	Slow freezing or stepwise freezing 0.3-1°C/ min to -30 to -40°C
Step 5:	Plunge into LN_2 and storage.
Step 6:	Rapid thawing
Step 7:	Removal of cryoprotectants
Applicability:	Suspension culture, calli, somatic embryos, multiple shoots, meristems
Advantages:	suited to fine specimens
Problems:	some steps are time-consuming storage should be conducted at strictly less than -135°C

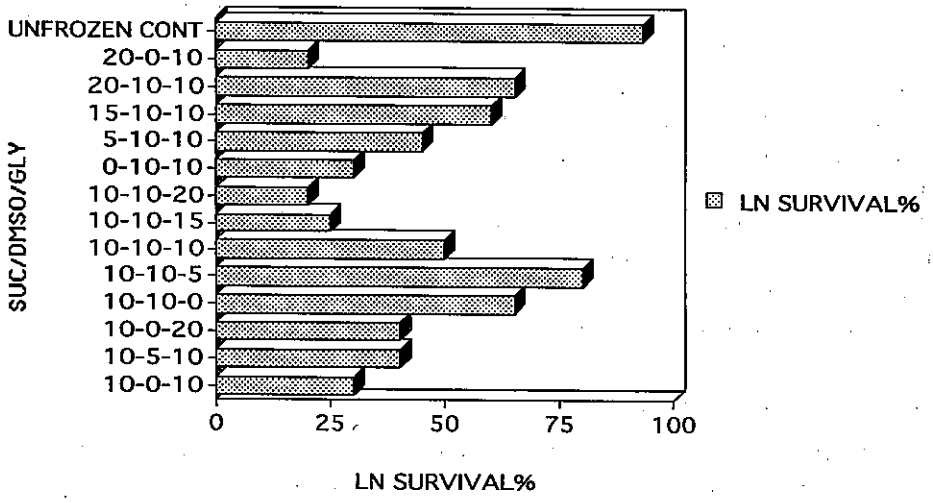

Fig. 6 Effect of various cryoprotectants (combination of sucrose, DMSO, glycerol) on the survival of bromegrass cultured cells exposed to liquid nitrogen with slow prefreezing to -30°C at -0.3°C/ min. (Ishikawa *et al*., 1991)

should be remind that an ethanol bath cooled by manual addition of sliquid nitrogen or dry ice is sufficient to produce 0.3 to 1°C/ min of cooling rates for smaller number of samples.

Slow prefreezing method seems to give better results with minute materials and materials in liquid cultures such as suspension cultured cells. When it is applied to complex tissues or larger materials such as meristems, it leads to a partial survival of the tissues as mentioned in the case of potato shoot tip.

3 Vitrification method

This method has been developed very recently, although vitrification of aqueous solutions had been studied since the 1930 s (Luyet, 1937). Possibility of vitrification of plant cell water in extremely cold-hardy species surviving liquid nitrogen temperatures was examined in the 1960 s (Sakai, 1966). Since then, little progress had been made in this area. However, after cryopreservation of animal cells by the vitrification method was reported (Fahy *et al*., 1984, Takahashi *et al*., 1986), the application of vitrification to plant tissue cryopreservation has been actively promoted. Several vitrification solutions have been developed (Uragami *et al*., 1989; Sakai *et al*., 1990; Langis and Steponkus, 1990; Towill, 1990; Ishikawa and Tandon, unpublished) and many plant species have been successfully cryopreserved (Table 4).

As summarized in Table 3 and Fig. 1, the principles of the vitrification method are as follows: dehydrate a specimen by exposing it to the vitrification solution containing a high osmoticum and alter the condition of intracellular water so as to vitrify by the penetration of antifreeze substances in the vitrification solution. Since the specimen must tolerate extreme dehydration, preculture is usually required to induce the tolerance. The vitrification solutions contain a high concentration of either glycerol, ethylene glycol or DMSO, which are toxic to plant cells. Thus, it is important for successful vitrification to identify the minimum exposure time required for the vitrification of the tissues in avoiding toxic effects on the tissues (Sakai *et al*., 1990; Yamada *et al*., 1991) and in promoting the acclimation the cells to this solution. The simplest procedure is to immerse the specimens directly in the vitrification solution at room temperature for various period of time (30 sec to 2 h depending on the materials) (Table 3). To attenuate the effect, the immersion is sometimes performed at colder temperatures or after gradual or stepwise increase in the concentration of the vitrifying substances (Towill, 1990; Langis *et al*., 1989).

This method has been found to be successful with complex tissues such as meristems (Table 4) rather than single cells, presumably due to the strong penetration capacity of the vitrification solution and to the

Table 3 Vitrification method

Principles:	1. Induce desiccation tolerance by preculture 2. Dehydrate specimen by exposure to high osmoticum (vitrification solution) and promote vitrification of intracellular water through the penetration of antifreeze substances
Step 0:	Select an appropriate tissue or stage for vitrification
Step 1:	Preculture with osmoticum (0.3-1.2 M sucrose, sorbitol)
Step 2:	Expose to vitrification solution for 30 sec to 2 hours. 0/ 25°C direct/ gradual increase/ pretreatment
Step 3:	Directly plunge into LN_2 and store
Step 4:	Rapid rewarming
Step 5:	Removal of vitrification solution
Applicability:	meristems, buds, calli, multiple shoots, etc.
Advantages:	1. If successful, whole meristems tend to survive, avoiding callus formation. It is approproate for genetic resources preservation. 2. No expensive facility is needed.
Problems:	1. Toxicity and permeability of the vitrification solution 2. Storage should be conducted strictly under −135°C or lower.

Table 4 Successful cryopreservation of cultured cells and meristems by vitrification method

Materials	Vitrification solution	Authors
Cultured cells		
Brassica campestris	Steponkus	Langis *et al*., 1989
Asparaqus	PVS 1	Uragami *et al*., 1989
	PVS 2	Nishizawa *et al*.,1993
Citrus sinensis	PVS 2	Sakai *et al*., 1990
Citrus	PVS 2	Sakai *et al*., 1991
Tobacco	PVS 2	Takano and Tamura, 1992
Catharanthus	PVS 2	Van Iren (unpublished)
Bromegrass	L	Our lab. (unpublished)
Maize	L, L+,	Upadhyay and Ishikawa, 1993
Meristems		
Carnation	Steponkus	Langis *et al*., 1990
Mint	Towill	Towill 1990
White clover	PVS 2	Yamada *et al*., 1991
Lolium perenne	PVS 2	Yamada (unpublished)
Mulberry	PVS 2	Niino *et al*., 1992
Pear	PVS 2	Niino *et al*., 1992
Apple	PVS 2	Niino *et al*., 1992
Potato	L, L+	Our lab. (unpublished)
Chrysanthemum	Steponkus	Schnabel-Preikstas *et al*., 1992
Sweet potato	Steponkus	Schnabel-Preikstas *et al*., 1992
	PVS 2	Towill and Jarret 1992
Ribes	PVS 2	Reed 1992
Tea	PVS 2	Kuranuki and Sakai 1992
Garlic	PVS 2	Niwata 1992
Melon	PVS 2	Ogawa *et al*., 1993
Asparagus	PVS 2	Kohmura *et al*., 1992

Modified from Sakai (1993)

relative surface area of the specimen. In the case of single cells, the penetration of the solution is very rapid and makes the cells leaky simultaneously. The cells lose vital components which defuse to the solution and can not be readily reabsorbed. In the case of more complex tissues, the penetration of the vitrification solution is more gradual and leaked substances into the intercellular space, if any, are easily reabsorbed. More recently, by exposing the cells to pretreatment, it has become possible to overcome this tendency (Upadhyay *et al*., 1993) and a wider application to cultured cells is anticipated.

The advantage of the vitrification method is that it does not require any special machine or equipment. The optimization of the conditions (preculture, pretreatment, imbibition time) is rather time-consuming, but once determined, the procedure can be simplified. The disadvantages of this method are the toxicity of the vitrification solution and sensitivity to a temperature rise during cryogenic storage. The development of a less toxic vitrification solution and/ or a method to induce enhanced tolerance to the vitrification solution are required to promote wider applications of this method.

A major advantage of the vitrification method is that the entire regenerable part of the tissues tend to survive cryopreservation when the method is successfully applied (Yamada *et al*., 1991), which would eliminate the possibility of selection and variant formation as mentioned earlier in the case of the desiccation method.

Table 5 Cross-applicability of cryopreservation methods

Crop	Slow Prefreezing	Desiccation	Vitrification
Melon			
Somatic embryos	+++	+++	?
shoot primordia	+++	−	++ (2 steps)
Bromegrass			
suspension culture	+++	−	++ (2 steps)
Maize			
embryogenic callus	++	++	+
Potato lateral buds	−	±	±
Primula winter buds	+	+++	−
Kiwi fruits buds	−	+++	−
Garlic meristems	++	?	+++
Clover meristems	+++	?	+++
Apple shoot	+++	+++	+++
Citrus nucellar cells	++	?	+++

4 Cross-applicability of cryopreservation methods

It is difficult to predict which cryopreservation method will give successful results. It is known only after try and error. Table 5 shows the results of various attempts made in our laboratory and others. As the desiccation method requires a high tolerance to dehydration stress, the materials which are suited to the desiccation method should theoretically be suited to the vitrification method or vice-versa. However, this assumption was not verified as indicated in Table 5. It could be partly due to insufficient optimization of the method applied or to unknown differences in the mechanism of cryoprotection. There is also a tendency that hardy (resistant) materials can be preserved with any methods whereas tender ones scarcely survive cryogenic storage with any of these methods. For publication purposes, hardy species or materials of a uniform developmental stage are more promising as a material of cryopreservation. However, more attempts should be made for successful cryopreservation of delicate materials including major vegetatively propagated plants such as potato, which is more challenging and may require another breakthrough in the cryopreservation technique.

Conclusion and perspectives for the future

Due to the recent progress in the cryopreservation techniques, a variety of methods has become available. Plants or tissues which have been successfully cryopreserved exceed 70. In some cold-hardy species, a range of cultivars have been covered by the application of a method for meristems and cryopreservation has become more practical (Yamada, 1991, Niino *et al*., 1992, Niino and Sakai, 1992). However, the nature of cryopreservation works remains unchanged: the methods applicable depend on crops and/ or tissues and require trial and error. Also for the majority of the vegetatively propagated plants that are more often sensitive to cold and/or to drought, cryopreservation is still difficult. Therefore, future research themes should cover the following aspects:

1. Further extension of the recently developed techniques to a wider range of plants, especially, less hardy plants, tropical and subtropical plants should be considered. For each crop species, it is important to find appropriate organs or tissues and an appropriate developmental stage for cryopreservation where desiccation tolerance could be highly expressed. Quality control of cultured materials is also vital so as to produce uniform specimens for cryopreservation.
2. New methods with a wider applicability and with higher survival rates should be developed. In the case of genetic resources, the survival of the entire regenerable tissues (ex. dome in the apical meristem) is preferable to avoid the production of variants. Methods that enable to overcome cultivar differences, that are more convenient and standardized, should be developed.

3. Basic studies on the mechanisms of desiccation and freezing tolerances as well as cryoprotection should be promoted.
4. Possible selection and genetic changes induced by cryopreservation, especially at the DNA level should be investigated (Harding, 1991).

To extend the newly developed cryopreservation techniques to a wider range of plants, especially tropical and subtropical plants, training programs will be required. As the cryopreservation work shifts from experimental to practical stages, central banks with large liquid nitrogen tanks should be developed to enable to store a large number of cell lines and genetic resource accessions.

Acknowledgements :

The author would like to express sincere thanks to all the collaborators; Ms. A. Yamaguishi-Ciampi, Ms. E. Niwata, Ms. R. Ogawa, Mr. Y. Honma, Mr. K. Shimonishi, Mr. S. Suzuki, Mr. P. V. Hemachandra, Mr. T. Kato, Mr. M. Suzuki, Dr. M. P. Upadhyay and Dr. P. Tandon.

References

1) Bajaj, Y. P. S. (1981): Regeneration of plants from potato meristems freeze-preserved for 24 months. Euphytica, 30, 141-145.
2) Chaudhury, R., Radhamani, J. and Chandel, K. P. S. (1991): Preliminary observations on the cryopreservation of desiccated embryonic axes of tea (*Camellia sinensis* (L.) O. Kuntze) seeds for genetic conservation. Cryo-Letters, 12, 31-36.
3) Chen, T. H. H., Kartha, K. K., Constabel, F. and Gusta, L. V. (1984): Freezing characteristics of cultured *Catharanthus roseus* (L). G. Don cells treated with dimethylsulfoxide and sorbitol in relation to cryopreservation. Plant Physiology, 75, 720-725.
4) Chen, T. H. H., Kartha, K. K., Leung, N. L., Kurz, W. G. M., Chatson, K. B. and Constabel, F. (1984): Cryopreservation of alkaloid-producing cell cultures of periwinkle (*Catharanthus roseus*). Plant Physiology, 75, 726-731.
5) Fabre, J. and Dereuddre, J. (1990): Encapsulation-dehydration: a new approach to cryopreservation of *Solanum* shoot-tips. Cryo-Letters, 11, 413-426.
6) Fahy, G. M., MacFarlane, D. R., Angell, C. A. and Meryman, H. T. (1984): Vitrificationas an approach to cryopreservation. Cryobiology, 21, 407-426.
7) Grout, B. W. W. and Henshaw, G. G. (1978): Freeze preservation of potato shoot-tip cultures. Annals of Botany, 42, 1227-1229.
8) Harding, K. (1991): Molecular stability of the ribosomal RNA genes in *Solanum tuberosum* plants recovered from slow growth and cryopreservation. Euphytica, 55, 141-146.
9) Hemachandra, P. V. (1990): JICA Plant Genetic Resources Group Training Course Reports.
10) Honjo, H. and Nakagawa, Y. (1978): Suitable temperature and seed moisture content for maintaining the germinability of citrus seed for long term storage. *In*: Long Term Preservation of Favorable Germplasm in Arboreal Crops. Edited by T. Akihama and K. Nakajima. pp. 31-35.
11) Ishikawa, M., Tandon, P., Yamaguishi, A. C. and Miyazaki, S. (1991): Cryopreservation of bromegrass cells with slow prefreezing and rapid prefreezing methods. Annual Meeting of Japanese Plant Physiologists. 96.
12) Kartha, K. K. (1985): Meristem culture and germplasm preservation. *In*: Cryopreservation of Plant Cell and Organs (K. K. Kartha, ed.). CRC Press, Florida, pp. 115-133.
13) Kohmura, H., Sakai, A., Chokyu, S. and Yakuwa, T. (1992): Cryopreservation of invitro-cultured multiple bud clusters of asparagus (*Asparagus officinalis* L. cv Hiroshimagreen (2 n=30)) by the techniques of vitrification. Plant Cell Reports, 11, 433-437.
14) Kuranuki, Y. and Sakai, A. (1992): Cryopreservation of shoot tips of *in vitro* tea plant by vitrification method. Japanese Journal of Breeding. 42 (suppl. 1), 330-331, (in Japanese).
15) Langis, R., Schnabel, B., Earle, E. D., and Steponkus, P. L. (1989): Cryopreservation of *Brassica campestris* L. cell suspensions by vitrification. Cryo-Letters, 10, 421-428.

16) Langis, R., Schnabel-Preikstas, B. J., Earle, E. D. and Steponkus, P. L. (1990): Cryopreservation of carnation shoot tips by vitrification. Cryobiology. 27, 657-658.
17) Luyet, B. J. (1937): The vitrification of organic colloids and of protoplasm. Biodinamica, 29, 1-15.
18) Nakano, T., Honma, Y., Toda, M., Hoshiya, Y. and Ishikawa, M. (1989) Germination and freeze preservation of dried seeds in wasabi (*Eutrema wasabi* Maxim). Japanese Journal of Breeding. 39 (Suppl. 1), 168-169.
19) Niino, T., Sakai, A and Nojiri, K. (1992): Cryopreservation of *in vitro*-grown shoottips of apple and pear by vitrification. Plant Cell, Tissue. Organ. Culture, 28, 261-266.
20) Niino, T., Sakai, A., Enomoto, S., Magoshi, J. and Kato, S. (1992): Cryopreservation of *in vitro*-grown shoot tips of mulberry by vitrification. Cryo-Letters, 13, 303-312.
21) Niino, T. and Sakai, A. (1992): Cryopreservation of alginate-coated *in vitro*-grown shoot tips of apple, pear and mulberry. Plant Science, 87, 199-206.
22) Nishizawa, S., Sakai, A., Amano, Y. and Matsuzawa, T. (1993): Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification method. Plant Science, 91, 67-73.
23) Nitzsche, W. (1980): One year storage of dried carrot callus. Z. Planzenphysiol., 100, 269-271.
24) Niwata, E., Ogawa, R., Ishikawa, M. and Oosawa, K. (1991): Cryopreservation by prefreezing method for somatic embryos of melon. Japanese Journal of Breeding, 41 (suppl. 1), 188-189.
25) Niwata, E. (1992): Cryopreservation of shoot tips of garlic by vitrification. Japanese Journal of Breeding, 42 (suppl. 1), 326, (in Japanese).
26) Ogawa, R., Niwata, E., Ishikawa, M. and Oosawa, K. (1991): Cryopreservation by prefreezing method for shoot primordium of melon. Japanese Journal of Breeding, 41 (suppl. 1), 190-191.
27) Paulet, F., Engelmann, F. and Glaszmann, J. C. (1993): Cryopreservation of apices of *in vitro* plantlets of sugarcane (*Saccharum* sp. hybrids) using encapsulation/dehydration. Plant Cell Reports, 12, 525-529.
28) Reed, B. (1992): Cryopreservation of *Ribes* apical meristems. Cryobiology, 29, 740.
29) Sakai, A. (1966): Survival of plant tissue at super-low temperature IV. Cell survival with rapid cooling and rewarming. Plant Physiol., 41, 1050-1054.
30) Sakai, A. (1993): Cryogenic strategies for survival of plant cells and meristems cooled to −196°C. In JICA Genetic Resources Projects REF No. 6, pp. 5-26.
31) Sakai, A., Kobayashi, S. and Oiyama, I. (1990): Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. Plant Cell Reports, 9, 30-33.
32) Sakai, A., Kobayashi, S. and Oiyama, I. (1991): Survival by vitrification of nucellar cells of navel orange (*Citrus sinensis* var. *brasiliensis* Tanaka) cooled to −196°C. Journal of Plant Physiology, 137, 465-470.
33) Schnabel-Preikstas, B., Earle, E. D. and Steponkus, P. L. (1992): Cryopreservation of chrysanthemum shoot-tips by vitrification. Cryobiology, 29, 747.
34) Schnabel-Preikstas, B., Earle, E. D. and Steponkus, P. L. (1992): Cryopreservation of sweet potato shoot-tips by vitrification. Cryobiology, 29, 738-739.
35) Senaratna, T., Mackersie, B. and Bowley, S. T. (1990): Artificial seeds of alfalfa (*Medicago sativa* L.) induction of desiccation tolerance in somatic embryos. *In Vitro* Cell Dev. Biol., 26, 85-90.
36) Shimonishi, K., Ishikawa, M., Suzuki, S. and Oosawa, K. (1991): Cryopreservation of melon somatic embryos by desiccation method. Japanese Journal of Breeding, 41, 347-351.
37) Shimonishi, K., Karube, M. and Ishikawa, M. (1993) Cryopreservation of Taro (*Colocasia esculenta*) embryogenic callus by slow prefreezing. Japanese Journal of Breeding 43 (suppl. 2), 187.
38) Shin, D. H., Virigool, S., Shinozaki, K.-Y. and Oono, K. (1991): Survival mechanism of dried calli and regeneration of plants in rice. Japanese Journal of Genetics, 66, 13-25.
39) Takahashi, T., Hirsh, A., Erbe, E. F., Bross, J. B., Steere, R. L. and Williams, R. J. (1986): Vitrification of human monocytes. Cryobiology, 23, 103-115.
40) Takano, M. and Tamura, S. (1992): Vitrification of cultured cell of *Nicotiana tabacum*. Cryobiology, 29, 739.
41) Towill, L. E. (1988): Improved survival after cryogenic exposure of shoot tips from in vitro plantlet

culture of potato. Cryobiology, 20, 567-573.
42) Towill, L. E. (1990): Cryopreservation of isolated mint shoot tips by vitrification. Plant Cell Reports, 9, 178-180.
43) Towill, L. E. and Jarett, L. (1992): Cryopreservation of sweet potato (*Ipomoea batatas* (L.) Lam.) shoot tips by vitrification. Plant Cell Reports, 11, 175-178.
44) Upadhyay, M. P., Ishikawa, M., Tandon, P., Kunihiro, Y., Ozawa, K. and Nakajima, S. (1993): Cryopreservation of maize embryogenic callus by vitrification. Japanese J. of Breeding, 43 (supple 1), 198.
45) Uragami, A., Sakai, A., Nagai, M. and Takahashi, T. (1989): Survival of cultured cells and somatic embryos of *Asparagus officinalis* cryopreserved by vitrification. Plant Cell Reports., 8, 418-421.
46) Uragami, A., Sakai, A. and Nagai, M. (1990): Cryopreservation of dried axillary buds from plantlets of *Asparagus officinalis* L. grown *in vitro*. Plant Cell Reports, 9, 328-331.
47) Withers, L. A. (1985): Cryopreservation of cultured cells and meristems. *In*: Cell Culture and Somatic Cell Genetics of Plants. Vol. 2 (I. Vasil, ed.). Academic Press, pp. 253-316.
48) Yamada, T., Sakai, A., Matsumura T. and Higuchi, S. (1991): Cryopreservation of apical meristems of white clover (*Trifolium repens* L.) by vitrification. Plant Science, 78, 81-87.
49) Yamaguishi-Ciampi, A. (1990): JICA Plant Genetic Resources GroupTraining Course Report.