Cryopreservation of plant species : progress and prospects

F. ENGELMANN*

RÉSUMÉ : Un procédé de cryoconservation comprend une série d'étapes successives, pour lesquelles des conditions précises doivent être définies pour chaque matériel végétal. De nouvelles techniques sont expérimentées (encapsulation, vitrification, utilisation d'un congélateur ménager), qui visent à simplifier les procédés classiques. La résistance à la congélation dans l'azote liquide a été obtenue pour plus de 70 espèces végétales. Cependant, l'utilisation en routine de la cryoconservation reste encore exceptionnelle.

SUMMARY : A cryopreservation process comprizes successive steps, for which precise conditions have to be defined for each plant species.
New techniques are tested (encapsulation/dehydration, vitrification, use of domestic freezer), which aim at simplifying the standard processes.
Resistance to freezing in liquid nitrogen has been obtained for more than 70 different plant species. However, the routine use of cryopreservation still remains exceptional.

Introduction

As regards preservation possibilities, plant species have been divided into 2 categories :

— orthodox seeds, which can withstand dehydration to 5 % or less (dryweight basis) without damage. When dry, the viability of these seeds can be prolonged by keeping them at the lowest temperature and moisture possible,

— recalcitrant seeds, which are high in moisture and are unable to whithstand much desiccation. They are predominantly seeds from tropical or subtropical species. They can be stored only in wet medium in order to avoid dehydration injury and in relatively warm conditions because chilling injury is very common among these species. They remain viable only for a short time (several weeks or months), even if kept in the required moisture conditions. This group comprizes many crop species of great economic importance such as oil palm, coconut, cocoa, coffee, etc.

Moreover, the conservation of plants which are vegetatively propagated, such as cassava, potato, yam, etc, poses also considerable problems.

During the last years *in vitro* culture techniques have been extensively developed and applied to more than 1,000 species. The use of *in vitro* tissue culture techniques can be of great interest for germplasm storage and multiplication of recalcitrant and vegetatively propagated species due to their numerous advantages.

Indeed, very high multiplication rates can be obtained (up to one million plants in one year, starting from one single plant). The plants are placed in an aseptic system, free from contamination. Moreover, the space requirements are considerably decreased and the expenses in terms of labour and finances reduced.

The methods employed are different depending on the storage duration requested. For

* *ORSTOM (French Scientific Research Institute for Development through Cooperation) - BP 5045, 34032 Montpellier Cedex France*

short and medium term storage the aim is to reduce the growth and to increase the intervals between subcultures. This is achieved by modifying the culture conditionsmainly by lowering the culture temperature.

Medium term storage techniques are now routinely employed in many laboratories and International Germplasm Conservation Centers. However, the management of large numbers poses considerable problems, even if the intervals between transfers are greatly extended. Moreover, the risks of genetic variation, which lead to the loss of trueness to type, increase with the *in vitro* storage duration. Finally, prolonged storage periods in more or less detrimental conditions may induce the selection of particular genotypes, thus leading to the loss of a part of the germplasm conserved.

For long term storage, cryopreservation, i.e. storage at very low temperatures, usually that of liquid nitrogen (– 196 °C), is the only current method. At this temperature, all cellular divisions and metabolic events are stopped. The plant material can be stored without alterations or modifications for a theoretically unlimited period of time. Moreover, the cultures are stored in a small space sheltered from contamination, with very limited maintenance.

Cryopreservation

Methodology

A cryopreservation process comprizes the following successive steps which have to be defined for every species : choice and obtainment of material, pretreatment, freezing, storage, thawing and post-treatment. The main parameters which have to be determined for each of these steps are summarized on table 1.

Tableau 1 : principaux paramètres à définir pour les étapes successives d'un protocole standard de cryoconservation.

Table 1 : Main parameters to set up for the successive steps of a standard cryopreservation protocol.

Successive steps	Main parameters to set up
1) choice of starting material	type and physiological stage of explants (special obtaining medium)
2) pretreatment	duration, nature and concentration of cryoprotective substances
3) freezing	freezing rate (ultra-rapid/rapid, slow) prefreezing temperature
4) storage	– 196 °C
5) thawing	rapid, slow (water-bath temperature)
6) post-treatment	elimination of cryoprotective substances (rinsing, dilution, diffusion) reduction of osmotic shock (sugar concentration) regrowth (growth regulators, medium modifications, light)

Choice and obtainment of material

As a general rule, the material will be chosen as young and as meristematic as possible. Indeed, the cells of this type of material are the most likely to withstand freezing : they are small, contain only a few vacuoles, i.e. only a small amount of water, their cytoplasm is dense and their nucleo-cytoplasmic balance is high.

The material can be sampled on *in vivo* or *in vitro* plants. *In vitro* material is generally preferable since the explants are already miniaturised and free of contamination.

The physiological stage of the material is very important. In the case of cell suspensions, only material which is in the exponential growth stage can successfully withstand freezing. With carnation meristems, survival decreases progressively with their rank on the shoot axis, starting from the terminal meristem.

It is sometimes necessary to set up a special culture medium in order to obtain starting material in sufficient quantities. It is the case with oil palm somatic embryos : only a special type of embryos, shiny white, finger-like shaped, which are foten grouped into clumps, are likely to withstand freezing. These particular embryos are very rarely observed in standard culture conditions. Their frequency is increased by a two-month culture on a medium containing 0.3M sucrose instead of 0.1M, which is used in the standard culture medium.

It was indicated recently that a long-term period of tissue culture before cryopreservation significantly reduced the ability of potato shoot-tips to survive freezing.

Pretreatment

The pretreatment corresponds to the culture of the material during a certain period of time (several minutes to a few days) in conditions which prepare it for the freezing process. It is carried out using various cryoprotective substances like sucrose, sorbitol, mannitol, dimethylsulfoxide, polyethylene glycol, etc, which differ greatly

from one another by their molecular weight and their structure. The exact functionning of these substances is unclear : they have an osmotic role thus dehydrating the cells ; they may however act also by protecting membranes and enzymatic binding sites from freezing injury. They are sometimes classified in penetrating and non-penetrating compounds, the former having both above mentioned effects, the latter acting only as osmoticums.

For every species, one will have to determine the cryoprotectant, its concentration as well as the duration of the pretreatment. In some cases, the pretreatment will have to be adapted for different clones or varieties of the same material.

Freezing

Different types of freezing processes can be carried out : ultra-rapid, rapid or slow freezing. In the latter case, a programmable freezing apparatus is needed in order to obtain precise and reproducible freezing conditions. The different freezing profiles are presented on figure 1.

At the cellular level, the different freezing processes mentioned above correspond to different mechanisms regarding water flux and crystallization : during slow freezing, crystallization occurs first in the external medium. The water flows out of the cells to the external ice. The cells will have to be sufficiently dehydrated so as crystallisation of the residual water will cause no damage but at the same time not too dehydrated in order to avoid toxicity due to the concentration of the internal solutes, which increases with dehydration. During rapid freezing, intracellular ice crystallizes in microcrystals of a size which is not harmful to the integrity of the cell components.

For each material, the following criteria must be determined :

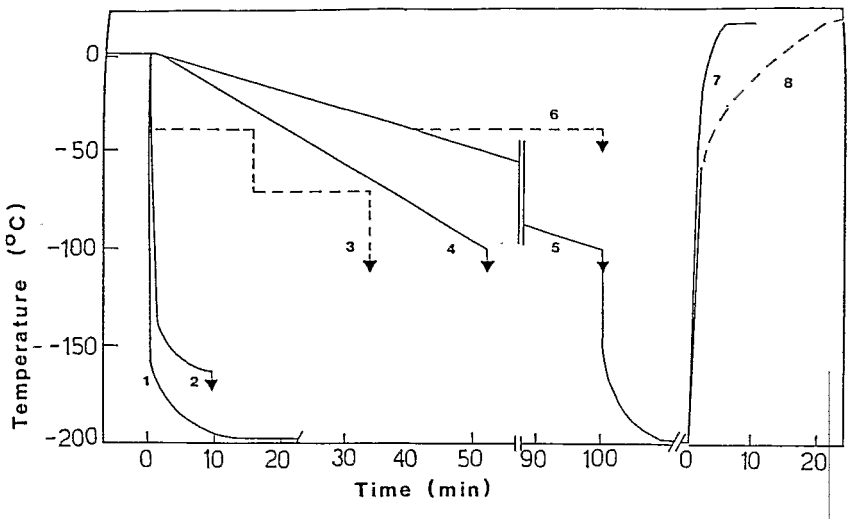

Figure 1 : profils caractéristiques de congélation et réchauffement (d'après Withys 1980).

1 - Refroidissement rapide, obtenu en immergeant l'échantillon ou l'ampoule dans l'azote liquide.

2 - Refroidissement intermédiaire, obtenu en suspendant l'échantillon au-dessus de l'azote liquide.

3 - Refroidissement par paliers ; le passage d'un palier à l'autre peut être rapide, comme sur cette figure, ou plus progressif, dans le cas d'un échantillon de taille plus importante.

4 - Refroidissement lent à 2 °C.min^{-1}.

5 - Refroidissement lent à 1 °C.min^{-1}.

6 - Refroidissement lent interrompu par un plateau à une température donnée.

7 - Réchauffement rapide dans de l'eau à 35-40 °C.

8 - Réchauffement lent dans l'air à température ambiante.

Les flèches indiquent le moment de l'immersion des échantillons dans l'azote liquide.

Figure 1 : Typical freeze-thaw profiles (after Withers, 1980)

1. Rapid freezing obtained 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.

3. Step-wise or prefreezing ; transition from one step to next may be steep as shown here or more gentle in the case of 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 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 cooling by transfer to liquid nitrogen.

— freezing rate : in some cases it must be very precise, as for strawberry meristems. Indeed, for a freezing rate of 0.84 °C.min^{-1}, 90 % survival can be obtained. If the cooling rate is increased to 1 °C.min^{-1}, the survival drops to less than 20 %. On the contrary, oil palm somatic embryos can be successfully frozen using a much wider range of cooling rates, between 0.5 and 200 °C.min^{-1},

— starting and prefreezing temperature : i.e. the temperatures at the start and the end of programmed freezing. These parameters can be very important : in the case of cassava meristems, a prefreezing temperature of – 20 °C ensures 91 % of survival ; only 3.3 % are observed if the controlled freezing stops at – 40 °C. However with grape embryogenic cell suspensions, regrowth is possible only for a prefreezing temperature of – 40 °C, even if satisfactory viability rates are obtained for different prefreezing temperatures.

Storage

The storage duration is theoretically unlimited, provided that the samples are permanently kept at the temperature of liquid nitrogen. The material remains exposed to natural radiation. It was calculated with animal cells that the level of mutations caused by natural radiation during storage may reach an irreparable level after thawing only after an extended storage period.

Thawing

In the majority of the cases, thawing is carried out rapidly by immersing the cryotubes containing the samples in a water-bath with a thermostatically controlled temperature of approximately + 40 °C. The aim of a quick thawing is to prevent the ice microcrystals formed during freezing from growing to larger crystals of a size which would be harmful to cellular integrity.

Post-treatment

Post-treatment consists of cultivating the material in conditions ensuring its best possible recovery. Cryoprotective substances are progressively eliminated by rinsing, dilution and diffusion, for they are toxic if kept too long in contact with the material.

It is sometimes necessary to attenuate the osmotic shock caused by an immediate transfer on a medium with low osmotic potential by successive transfers of the material on progressively less concentrated media. In some cases, the nature of the medium must be changed (solid versus liquid, and vice versa), in order to improve the regrowth. With cell suspensions, a transitory culture phase on solid medium is commonly used before returning to liquid conditions.

Recovery can eventually take place in the dark, in order to avoid protooxidation phenomena, which can be harmful for the recovery of the material.

Finally, the composition of the culture medium can be provisionally modified. With cell suspensions of rice, the NH_4 ions are suppressed for a few days until regrowth has started. In the case of oil palm somatic embryos, the hormonal content of the medium is transitorily modified : an auxin must be added during a few days after thawing, in order to stimulate the recovery of the proliferation.

Viability assessment

The only definitive assessment of viability is the growth of the material after thawing. Regrowth, in many cases, however can be very slow and it is very important to know as soon as possible after thawing if the material is still living. Two main tests exist in order to measure the viability of the material, which can be applied very rapidly after thawing. These tests are :

— FDA (fluorescein diacetate) : FDA is absorbed by the living cells and transformed into fluorescein, whose fluorescence is measured in UV. This test is quantitative ;

— TTC (triphenyl tetrazolium chloride) : TTC is reduced into formazan, colored in red, in the mitochondria of the living cells. This test is quantitative for cell suspensions but is only qualitative for large tissues and organs.

Their major disadvantage is that they are destructive ; Moreover, it was observed recently that the FDA test is very precise for estimating the viability, but it gives no information on the capacities of the cells to proliferate. Non destructive methods for estimating the viability of the material are sought, such as chromatographic analysis of volatile hydrocarbon production (ethylene, ethane) by cryopreserved tissues.

Results

Various types of cultures

Today, cryopreservation has been applied to more than 70 different species which were in the form of cell suspensions, calluses, meristems and embryos.

For cell suspensions, routine techniques adapted to a large number of species have been used for several years. Freezing of cell suspensions usually comprizes a pretreatment with cryoprotective substances, followed by a slow cooling (0.1 to 2-3 °C.min^{-1}), to temperatures ranging between – 30 to – 50 °C. After thawing, a transitory culture on solid medium is generally performed, before returning to liquid medium.

Callus cryopreservation is generally performed using similar techniques. It is interesting to note that, in the case of *Picea abies* calluses, it was observed that frozen material showed a better regrowth than the nonfrozen controls. This is certainly due to the selection between different cell types imposed by cryopreservation : all the differenciated cells of the callus are killed during the freeze-thaw cycle and all the meristematic cells remain viable. The possibility of regenerating actively growing suspensions could prove a further advantage of cryopreservation applied to cell suspensions or calluses.

In the case of meristems, the aim is to preserve the whole structure, which is of macroscopic size, and to obtain its direct regrowth without adventious organogenesis. The number of species on which cryopreservation has been performed using meristems is limited. With this type of explants, rapid as well as slow freezing can be employed.

Concerning embryos, the main characteristics of this type of material is its size, which is generally large, according to cryopreservation standards. The embryos comprize differentiated structures and tissues. Thus, embryos as young and as immature as possible (e.g. globular stage) will be preferentially used. As

concerns embryo cryopreservation, two different categories of material can be considered : zygotic embryos, which are harvested on *in vivo* material and placed in *in vitro* conditions only after cryopreservation, and embryos which are already cultivated *in vitro*. The challenge is different for these two categories : for gigotic embryos, the whole structure has to be preserved in order to give rise to a whole plant, whereas with somatic embryos, only the proliferation capacities of the material must be preserved and not necessarily their structural integrity. For this latter group, standard cryopreservation techniques are used.

In the case of zygotic embryos, which are excised from the seed and frozen immediately, the cryopreservation process is generally different. The cotyledons are often removed and only the embryonic axes used. The partial dehydration, usually provided by the contact with the cryoprotective solution, is obtained by placing the explants under the laminar flow and letting them dehydrate in the air current. The intensity of this dehydration is adapted to the desiccation tolerance/sensitivity of the species. For example, *Hevea* freshly excised embryonic axes have a moisture content of 55 % and 100 % viability without any treatment, but do not resist freezing in liquid nitrogen. After 3 hours of desiccation, the water content drops to 16 %, the viability is 87 % and the survival after freezing reaches 67 %. Rapid dry freezing is usually employed, but controlled slow cooling ($2\ ^{\circ}C.min^{-1}$) can also to be successful as with cassava embryonic axes.

New cryopreservation techniques

The aim of these new freezing techniques is to look for eventual simplification of the standard cryopreservation process.

Encapsulation/dehydration

This technique is based on the technology developed for the production of synthetic seeds, in which the embryos are encapsulated in a bead of alginate, to which various substances can be added and represents a sort of synthetic albumen. It was developed by a French research team, using potato meristems. It is based ont the fact that encapsulation protects the embedded structure and makes it resistant to treatments which otherwise would be lethal. The alginate beads containing the explants are cultivated for several hours/days in a liquid medium with high sucrose level, then partially desiccated under the laminar flow and frozen either slowly or rapidly. After slow thawing, the beads are transferred to a standard medium. Regrowth of the explants is satisfactory. In the case of potato, up to 26 % of the frozen apices could develop into plants.

Vitrification

Vitrification is the transformation of water from the liquid phase directly into an amorphous phase, or glass, at low temperature, whilst avoiding the formation of crystalline ice. This technique was developed recently by various authors using cell suspensions, protoplasts, somatic embryos and meristems of various species. In a vitrification process, the material is frozen ultrarapidly, in the presence of very high cryoprotectant concentrations. A very precisely timed pretreatment is necessary, as the high concentration in cryoprotectants is rapidly toxic. Dilution of the cryoprotective medium, after thawing, must also be very precise. It seems that, at least for cell suspensions, the complexity of the freezing phase in the standard procedure is transferred to the pretreatment phase, making this technique far from being extensively usable in the near future.

Use of a domestic freezer

This technique was developed recently using coffee embryogenic cell suspensions and somatic embryos. After a standard pretreatment with high sucrose concentrations, the cryotubes containing the suspension are placed in a commercial freezer at $-20\ ^{\circ}C$ for 24 hours, then immersed in liquid nitrogen. A 50 % viability rate (measured by TTC reduction) could be obtained immediately after thawing. After 42 days in culture, the performance of the cryopreserved line, as regards the final cell density, the productivity of embryos and the percentage of embryos at the torpedo stage were similar to that of the non-frozen control.

Potential use for these new techniques

These new techniques, vitrification, encapsulation/dehydration, use of domestic freezer, will certainly not replace the conventional processes, but they present two main advantages. Firstly, they may offer additional possibilities for plant materials which are recalcitrant to conventional freezing procedures. Second, they may offer the possibility of avoiding the use of a programmable freezer ; this would simplify the process, thus facilitating its utilization.

Conformance to type, storage duration

The possible variations of the material due to cryopreservation have been principally checked with respect to the production of particular compounds by cell strains. Until now, no modifications, after thawing, of the properties of the stored material have been observed. As concerns organized structures, plants obtained from frozen meristems or embryos of several species were phenotypically normal (table 2). However, in a recent work with *Triticum aestivum*, the authors could select freezing tolerant calluses by re-

Tableau 2 : Durées maximales de conservation dans l'azote liquide expérimentée pour différentes espèces végétales cultivées in vitro.

Table 2 : Maximal storage durations in LN experimented for plant species cultured in vitro.

Species	Material	storage duration (years)	reference
Digitalis lanata	cell suspension	3	Diettrich et al., 1985
Fragaria ananassa	meristems	2 (normal plants)	Kartha et al., 1980
Arachis hypogea	"	2 (normal plants+seeds)	Bajaj, 1983
Manihot esculenta	"	4	Bajaj, 1985
Solanum tuberosum	"	4	Bajaj, 1985
Elaeis guineensis	somatic embryos	1,5 (normal plants)	Engelmann et al., 1986

peated exposures to LN.The cryoselected calluses regenerated plants with enhanced cold hardiness. The seed progeny of some of the lines tested exhibited significantly enhanced tolerance to $-12^{\circ}C$, thus indicating that cryoselection appears to involve, at least in part, selection for genetic rather than epigenetic variants. The implications of this work may be of great importance, particularly in the case of tropical species, which are cold sensitive. Indeed, the possibility of producing plants with increased cold resistance is very interesting.

As concerns storage duration, the experience is very limited plant material. Indeed, the maximal storage duration experimented is 4 years, in the case of cassava and potato meristems. To date, all storage experiments led to the obtainment of true-to-type material (table 2).

Present development of cryopreservation

If resistance to freezing at the temperature of liquid nitrogen has been demonstrated for a large number of species, the current applications of cryopreservation as a routine technique are exceptional. The first two examples concern oil palm and cassava.

Research for the development of a cryopreservation process for oil palm somatic embryos started in France in 1984 in order to solve the problems encountered with the industrial development of the *in vitro* micropropagation process set up by ORSTOM/IRHO. Indeed, the continuous creation of clones poses laboratory management problems. Moreover, the risks of producing abnormal material increase with the *in vitro* culture duration.

The process set up is characterized by its simplicity : freezing can be carried out by direct immersion of the embryos in liquid nitrogen, thus avoiding the use of a programmable freezing apparatus.

In a first step, the process was tested in the laboratory in France with 27 clones. Plants originating from cryopreserved cultures were grown in the field at the La Mé IRHO experimental station in Côte d'Ivoire. No difference was observed when compared with the controls. In a second step, starting in 1989, the technique was successfully transferred to the overseas laboratories (1 in Côte d'Ivoire, 1 in Malaysia, 2 in Indonesia). It has been applied to nearly 80 clones in these laboratories. This increase in scale shows that slight modifications in some steps of the current process could have been necessary in order to adapt it to the different conditions of each laboratory.

Research is still progressing in Montpellier in order to improve the performance of the technique. A more fundamental approach concerning the physiology of the embryos related to cryopreservation is being developed simultaneously.

With cassava, a technique has recently been developed in CIAT (Centro International de Agricultura Tropical, Cali, Colombie), which is in charge of a very large *in vitro* collection, including more than 5,000 genotypes. It was been tested on approximately 20 different genotypes and will be extented in the near future.

Conclusion

In conclusion, tissue culture techniques, together with cryopreservation techniques, are of great interest for the medium and long-term conservation of plant germplasm.

If routine techniques can be easily set up for medium term storage of plant collections, it is not the case for cryopreservation. The resistance to liquid nitrogen temperature has been proved for a large number of species. However, the use of cryopreservation is limited to small laboratory collections and its use on a large scale is currently exceptional. Indeed, the successful freezing

of plant material, which implies the setting up of very precise conditions, requires extensive research using sophisticated equipement. In this context, the search for less sophisticated freezing techniques, such as encapsulation/dehydration, vitrification or the use of a domestic freezer is of great interest. However, the resort to conventional freezing techniques may still remain obligatory in the majority of the cases.

In recent years, national and international bodies, public research institutes and private firms have shown increasing interest in germplasm storage and cryopreservation. This encourages us to feel optimistic about the development of routine techniques for the safe storage of tropical germplasm.

References

DEREUDDRE J. and ENGELMANN F. 1987 - The use of cryopreservation for setting up banks of plant germplasm. *In* Proc. Coll. Franco-Britannique IAPTC, Angers, France, 8-9 oct. 1987, 48-78.

ENGELMANN F., 1990 - Oil palm somatic embryo cryopreservation : progress and prospects. Bull. Soc. Bot. Fr., 137, Actual. Bot., 93-98.

ENGELMANN F. 1986 - Cryoconservation des embryons somatiques de palmier à huile (*Elaeis guineensis* Jacq.) : mise au point des conditions de survie et de reprise. Thèse de Doctorat d'Université, Univ. Paris 6, 226 p.

ENGELMANN F. and DEREUDDRE J. 1988b - Cryopreservation of oil palm somatic embryos : importance of the freezing process. Cryo-Lett., 7 : 220-235.

FABRE J. and DEREUDDRE J. 1990 - Encapsulation dehydration : a new approach to cryopreservation of *Solanum* shoot-tips. Cryo-Lett., 11 : 413-426.

KARTHA K.K. 1985 - Cryopreservation of Plant Cells and Organs. Kartha K.K. Ed., Boca Raton, CRC Press, 276 p.

KARTHA K.K., LEUNG N.L. and MROGINSKI L.A. 1982 - *In vitro* growth responses and plant regeneration from cryopreserved meristems of cassava (*Manihot esculenta* Crantz). Z. Pflanzenphysiol., 107 : 133-140.

KENDALL E.J., QURESHI J.A., KARTHA K.K., LEUNG N., CHEVRIER N., CASWELL K. and CHEN T.H.H., 1990 - Regeneration of freezing-tolerant spring wheat (*Triticum aestivum* L.) plants from cryoselected callus. Plant Physiol., 94 : 1756-1762.

NORMAH M.N., CHIN H.F. and HOR Y.L. 1986 - Desiccation and cryopreservation of embryonic axes of *Hevea brasiliensis* Muell. Arg. Pertanika, 9 : 299-303.

SEITZ U. 1987 - Cryopreservation of plant cell cultures. Planta medica, 4 : 311-314.

TESSEREAU H., LECOUTEUX C., FLORIN B., SCHLIENGER C. and PETLARD V. 1990 - Use of a simplified freezing process and dehydration for the storage of embryogenic cell lines and somatic embryos. *In* Seeds : Genesis of Natural and Artificial Forms, Amiens, France, Nov. 12-13, 1990, Le Biopole Végétal, Ed., 123-133.

WITHERS L.A. and KING P.J. 1980 - A simple freezing unit and routine cryopreservation method for plant cell cultures. Cryo-Lett., 1 : 213-220.