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Cryopreservation of Embryos

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Introduction

For the last decade, plant cell cultures have become subjects of intensive research with emphasis on important biotechnological applications such as large scale production of clonal plants, genetic engineering, production and isolation of mutants, selection of variants, screening for resistance to various stresses and creation of new plant species through somatic cell hybridization. In this frame, somatic embryogenesis is widely used, due to its numerous advantages. However, this development leads to the production of an increasing number of strains. This creates problems for the management of laboratories. Moreover, many strains are faced with somaclonal variation and progressive loss of organogenic potentialities. Finally, genetically engineered lines are of extreme value, due to the amount of work necessary for their development. Therefore, safe and efficient techniques are needed for the long term conservation of this new germplasm.

On the other hand, the utilization of a restricted number of highly selected lines poses the problem of the conservation of the wild genotypes which often possess interesting agronomic characteristics. For this purpose, zygotic embryos represent a choice material, since they allow to conserve a good representation of the genetic variability of a given species with a limited number of samples.

Today, only cryopreservation (liquid nitrogen -196°C) can ensure the long term storage of germplasm. Resistance to freezing in liquid nitrogen has been shown for more than 70 plant species (Kartha, 1985 ; Dereuddre and Engelmann, 1987 ; Engelmann, in press) using various materials, such as cell suspensions, protoplasts, calluses, meristems and embryos.

This article presents a brief overview of the work carried out on cryopreservation of somatic and zygotic embryos and tries to identify the critical points for the successful freeze-preservation of such materials. Technical aspects, including the new cryopreservation techniques are dealt with by Dereuddre in the same volume.

Freezing characteristics of somatic and zygotic embryos

From a cryopreservation point of view, somatic and zygotic embryos have common characteristics. They are large and complex structures, formed of differentiated tissues, i.e. many different cellular types, depending on their developmental stage. However, somatic and zygotic embryos represent two different categories of material : somatic embryos are structures which have been neoformed *in vitro* and are thus highly hydrated. Somatic embryo cultures often comprize heterogenous structures in size and developmental stage, since in many embryogenic systems, synchronous development of embryos is difficult to achieve. They are generally of relatively smaller size than zygotic embryos (several hundred um to several mm).

On the contrary, zygotic embryos generally have lower water levels. They develop in the seed and are cryopreserved before being introduced *in vitro*. They are morphologically well defined structures (cotyledon(s), shoot and root pole, embryonic axis). Homogenous populations can be obtained by screening according to their developmental stage. Depending on their maturity, their size can be much larger than that of somatic embryos (up to more than 1 cm in the case of coconut).

For cryopreservation, the challenge is different with these two types of material. In most of the cases, when using somatic embryos, only the proliferation capacities (i.e. a sufficient amount of living cells to restart proliferation) and not necessarily the structural integrity of the embryos has to be preserved. On the contrary, with zygotic embryos (and somatic embryos cryopreserved at late developmental stages), the whole structure has to be preserved in order to regenerate directly a plant from each individual embryo.

Successive steps of a cryopreservation process

A standard cryopreservation technique comprizes successive steps (choice of starting material, pregrowth, freezing, thawing and post-treatment) for which optimal conditions have to be defined for each new material.

Choice of starting material

For cryopreservation, meristematic cells are the most likely to withstand freezing, due to their characteristics : they are of small size, have a very dense cytoplasm, few vacuoles (i.e. a low water level), their nucleo-cytoplasmic balance is very high. Therefore, in both cases, embryos comprizing the maximal amount of cells with such characteristics will be used preferentially. Young somatic embryos (globular stage) will be selected. With such structures, thermal gradients between external and internal cell layers are reduced and cryoprotective substances can act more efficiently and homogeneously. It may be sometimes necessary to set up a special medium in order to produce embryos of the right type. This is the case with oil palm : only a two-month culture on a medium enriched with sucrose will ensure the production of finger like, shiny white clumps of embryos which are the only ones likely to withstand freezing (Engelmann and Dereuddre, 1988a).

For the same reasons, immature zygotic embryos may appear as optimal candidates for cryopreservation due to their reduced size and their lower differentiation. However, one drawback for their utilization is that if *in vitro* culture of mature embryos can be easily achieved, it is not the case with immature ones, for which very complicated media formulations generally have to be set up. This is illustrated with coconut immature embryos, which can withstand freezing with high survival rates (Assy-Bah and Engelmann, in press) but for which *in vitro* culture conditions leading to the production of whole plants are not yet well defined (Engelmann and Assy-Bah, 1991). One will thus have to look for embryos at a stage where they are simultaneously most likely to survive freezing and easy to cultivate *in vitro*. The size and structural complexity of mature embryos can be reduced by cutting off the cotyledons and using embryonic axes instead of whole embryos.

Pregrowth

Before freezing in liquid nitrogen, the embryos have to be submitted to a pregrowth phase during which they will be partially dehydrated. Indeed, plant tissues contain high amounts of freezable water which will be converted to ice during freezing and will be harmful

for cell survival. They are therefore extremely sensitive to freezing injury. Protection against freezing injury has to be imposed artificially through various strategies, using cryoprotectants or other means.

Three types of pretreatments can be used with embryos : standard pretreatment using cryoprotective substances, desiccation an a combination of both treatments. The first method is mainly used with somatic embryos and the other two with zygotic embryos.

Use of cryoprotective substances

With this type of pretreatment, the embryos are cultured for various durations (several minutes to hours) in presence of cryoprotective substances. The following parameters have to be determined : the nature and concentration of cryoprotectants and the duration of the pretreatment. The cryoprotective substances used vary greatly one from the other by their molecular weight and their structure. The most commonly employed are dimethylsulfoxide (DMSO), sucrose, sorbitol, mannitol, polyethyleneglycol. The pretreatment may be carried out on solid medium only, in liquid medium only or successively with solid and liquid medium.

The cryoprotective substances may be used separately as for *Citrus sinensis* (Marin and Duran-Vila, 1988) or in combination, like sucrose + DMSO for coffee (Bertrand-Desbrunais *et al.*, 1988) or cassava (Sudarmonowati and Henshaw, 1990). The same technique is used with zygotic embryos of several species : solid medium containing high sucrose levels with *Capsella bursa pastoris* (Monnier and Leddet, 1980), or a mixture of glucose and glycerol with immature embryos of coconut (Assy-Bah and Engelmann, in press), liquid medium (propanediol and sucrose) in the case of *Juglans* and *Prunus* embryonic axes (de Boucaud *et al.*, 1991 ; de Boucaud and Brison, 1991).

Sucrose is very often employed in cryoprotective treatments and appears to play a central role in cryopreservation. It has an important osmotic effect but its absorption and its accumulation seems to be necessary for survival. Indeed, when pretreated on 0.75 M sucrose, the water level of *Capsella* embryos dropped within 24 hours down to a plateau. However, resistance to freezing in liquid nitrogen was obtained only after a minimal culture duration of 6 days (Monnier and Leddet, 1980). The same observation has been reported in the case of oil palm somatic embryos (Engelmann, 1986).

Desiccation

This technique is preferentially used with zygotic embryos or embryonic axes of various species. The explants are placed in the air current of a laminar flow cabinet in open Petri dishes and their dehydration regularly followed by weighing. However, these dehydration conditions cannot be very precisely controlled, due to the possible variations of the ambient

temperature or humidity and they are not necessarily reproducible. Other methods such as the use of silicagel, as recommended by Uragami *et al.* (1990) for *Asparagus* stem segment desiccation may allow more reproducible conditions. The dehydration duration varies according to the size of the embryos and their initial water content. Embryos must have a minimal size. Immature embryos of coconut were too small (5 to 15 mg) to be dehydrated using this technique and therefore had to be pretreated using the conventional way (Assy-Bah and Engelmann, in press). The optimal desiccation duration is generally comprized between 2 and 4 hours, depending on the species. The water content is thus drastically reduced from an initial average level of 50-60% down to 10-16%. Prolonged desiccation durations rapidly lead to a dramatic loss in the viability of unfrozen controls caused by dehydration injury.

Cryoprotective substances combined with desiccation

It is also possible to combine both techniques described above. Mycock *et al.* (1989) could freeze pea embryonic axes after a pretreatment with glycerol and DMSO followed by desiccation. Assy-Bah and Engelmann (in preparation) submitted mature embryos of coconut to a 4-hour desiccation under the laminar flow followed by a culture on a medium containing high levels of glucose and glycerol. In this case, the water content of the embryos could be decreased to an extremely low level (5 to 8%).

Freezing

Two different protocols can be used : a two-step freezing, comprizing a controlled slow freezing followed by the immersion of the samples in liquid nitrogen, or a one-step rapid freezing performed by plunging directly the samples in liquid nitrogen.

The two-step process is generally employed with somatic embryos. During slow freezing, crystallization takes place firstly in the external medium and water flows out of the cells to the external ice, thus continuing the dehydration of the samples. The two variables which allow to control the intensity of this dehydration are the cooling rate and the prefreezing temperature , i.e. the temperature at which the controlled freezing stops. These parameters may have to be very precisely determined. In the case of *Citrus sinensis*, embryos have to be frozen at 0.5°C.min^{-1} down to -42°C (Marin and Duran-Vila, 1988). On the contrary, oil palm embryos can withstand a wide range of freezing rates (from 0.1 to 200°C.min^{-1}, Engelmann and Dereuddre, 1988b). If the freezing conditions are not optimal, the survival rate of the material is lowered. The type of recovery can also be modified (direct regrowth versus adventive embryogenesis), as observed by Florin (1989) with carrot somatic embryos.

When rapid freezing is performed, the remaining internal water cristallizes in the form of microcrystals which are of a size unharmful to the cellular structures. However, the

samples have to be sufficiently dehydrated before freezing. Rapid freezing is commonly employed with zygotic embryos since their water content can be drastically reduced without damages during the pretreatment. It could be interesting to carry out thermal analysis experiments in order to determine if crystallization still takes place or if the remaining water vitrifies. Indeed, the conditions of this freezing procedure may be comparable to that of a vitrification process, that is low water content (obtained here by extensive dehydration instead of very concentrated cryoprotective solutions) and rapid freezing. This may explain for the high survival rates observed during cryopreservation of zygotic embryos.

Storage

No alteration to the material is known to take place during storage, provided that it remains at the temperature of liquid nitrogen. As concerns the effects of storage duration, no modification was observed in the recovery rate of oil palm somatic embryos after 15 months in liquid nitrogen (Engelmann, 1991). A similar result was obtained recently after 52 months in storage (Engelmann, unpublished).

Thawing

Thawing is generally rapid, using a water-bath thermostated at 40°C. This is made in order to avoid recrystallization and ice crystal growth which may occur during warming and produce intracellular damages. This is particularly important with material produced *in vitro* (somatic embryos) which still contains high levels of water. Carrot (Withers, 1979) and *Citrus sinensis* (Marin and Duran-Vila, 1988) are exceptions to this procedure. Oil palm somatic embryos are capable of reproliferation after slow or rapid thawing (Engelmann, unpublished).

Post-treatment

After the freeze-thaw cycle, culture conditions different from the standard procedure may have to be defined in order to stimulate the regrowth of the embryos. Indeed, regrowth of frozen somatic embryos is often delayed when compared with unfrozen controls. With *C. arabica* and *C. canephora* somatic embryos, a progressive reduction in the sucrose concentration of the medium and a culture in the dark allowed for a 20% improvement of the initial procedure (Bertrand-Desbrunais, 1991). With oil palm somatic embryos, auxin has to be added transitorily to the medium in order to stimulate reproliferation (Engelmann *et al.*, 1985). With zygotic embryos, initial regrowth is generally rapid. However, differences with unfrozen controls sometimes appear during the further development of the embryos. Chin *et al.* (1988) observed that the haustorium of cryopreserved *Howea* and *Veitchia* embryos did not develop.

The same observation has been made recently with coconut mature embryos (Assy-Bah and Engelmann, in preparation).

Conclusion

Cryopreservation has now been successfully applied to embryos of a large number of species from tropical and temperate origins (see Table 1). The techniques employed range from classical ones including cryoprotection and controlled freezing in the case of somatic embryos to simpler ones using desiccation and rapid freezing in the case of zygotic embryos. However, cryopreservation is still far from being routinely used for long-term storage of plant germplasm. Additional research is still needed in order to simplify, standardize and develop cryopreservation techniques. Another field of research concerns the development of techniques alternative to cryopreservation, such as storage at positive temperature of desiccated embryos. The current increasing amount of research dealing with germplasm conservation should lead in the near future to making available various efficient techniques for the safe long-term conservation of plant germplasm.

Table 1 : list of somatic and zygotic embryos successfully frozen in liquid nitrogen

Somatic embryos	
Citrus sinensis	Marin and Duran-Vila, 1988
Coffea arabica	Bertrand-Desbrunais *et al.*, 1988
Coffea canephora	Bertrand-Desbrunais, 1991
Daucus carota	Withers, 1979
Elaeis guineensis	Engelmann *et al.*, 1985
Manihot esculenta	Sudarmonowati and Henshaw, 1990
Zygotic embryos	
Aesculus hypocastanea	Pence and Dresser, 1988
Araucaria excelsa	Pritchard and Prendergast, 1986
Brassica napus	Withers, 1982
Capsella bursa pastoris	Monnier and Leddet, 1980
Carva	Pence and Dresser, 1988
Castanea	Pence and Dresser, 1988
Cocos nucifera	Chin *et al.*, 1989
Elaeis guineensis	Grout *et al.*, 1983
Fagus	Pence and Dresser, 1988
Hevea brasiliensis	Normah *et al.*, 1986
Hordeum vulgare	Withers, 1982
Howea fosteriana	Chin *et al.*, 1988
Juglans	Pence and Dresser, 1988
Manihot esculenta	Marin *et al.*, 1990
Musa	More *et al.*, 1991
Phaseolus vulgaris	Zavala and Sussex, 1986
Pisum	Mycock *et al.*, 1989
Prunus persica	de Boucaud and Brison, 1991
Quercus	Pence and Dresser, 1988
Triticum aestivum	Bajaj, 1983
Triticale	Bajaj, 1983
Veitchia merrillii	Chin *et al.*, 1988
Zea mays	Delvallée *et al.*, 1989

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