Importance of cryopreservation for the conservation of plant genetic resources

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Introduction

The two basic approaches to conservation of plant genetic resources are *ex situ* and *in situ* conservation. Article 2 of the Convention on Biological Diversity (UNCED 1992) provides the following definitions for these categories: *ex situ* conservation means the conservation of components of biological diversity outside their natural habitats. *In situ* conservation means the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species in their natural surroundings and, in the case of domesticates or cultivated species, in the surroundings where they have developed their distinctive properties. The two basic conservation strategies can be further subdivided into several specific techniques. *Ex situ* conservation includes seed storage, *in vitro* storage, DNA storage, pollen storage, field genebanks and botanical gardens, while the *in situ* approach encompasses genetic reserves, on-farm and home garden conservation (Maxted *et al.* 1997).

It is now well recognized that for any given genepool, a number of different complementary approaches and methods are necessary for a safe, efficient and cost-effective conservation. The appropriate strategy and the balance depend on factors such as the biological characteristics of the plants, their present management and use by humans, available infrastructure for conservation, number of accessions in a given collection and geographic sites, the purpose of conservation, the availability of germplasm, and political and administrative policies. The extent of the utilization of a particular method may differ from one genepool to another (Withers 1993).

For many plant species which produce orthodox seeds, i.e. which can be dehydrated extensively and stored dry at low temperature (Roberts 1973), the emphasis for genetic resource conservation will be on seed storage. However, three categories of crop present problems with regard to seed storage. First, there are those that do not produce seeds at all, and are propagated vegetatively, for example, banana and plantain (*Musa* spp.). Second, there are crops including potato (*Solanum tuberosum*), other root and tuber crops such as yams (*Dioscorea* spp.), cassava (*Manihot esculenta*) and sweet potato (*Ipomoea batatas*), and sugarcane (*Saccharum* spp.) that have some sterile genotypes and some that produce orthodox seed. However, like many temperate fruits, these seeds are highly heterozygous and therefore of limited utility for the conservation of gene combinations. These crops are usually propagated vegetatively to maintain clonal genotypes. Then, third, there are those crops that produce recalcitrant seeds (Roberts 1973) which include a large number of tropical fruits and timber species. Recalcitrant seeds cannot tolerate desiccation to moisture contents that

would permit exposure to low temperatures. They are often large with considerable quantities of fleshy endosperm. Finally, recent investigations have identified species displaying an intermediate form of seed storage behaviour (Ellis *et al.* 1990, 1991). These seeds can tolerate desiccation down to relatively low moisture content but the dry seeds are often injured by low temperature. Compared with truly recalcitrant seeds, the storage life of these seeds can be prolonged by some drying, but it is impossible to obtain the long-term conservation of orthodox seeds (Engelmann 1997a).

As regards the balance of techniques employed within complementary strategies developed for conserving the genetic resources of these problem species, the emphasis in the case of non-orthodox forest tree species will be on *in situ* conservation in genetic reserves, while for species which are propagated vegetatively the emphasis will be on *ex situ* conservation techniques, including field genebank and *in vitro* storage. However, it is essential to recognize that, owing to the various problems and limitations encountered with both genetic reserves and field genebanks (Withers and Engels 1990; Maxted *et al.* 1997), cryopreservation currently offers the only safe and cost-effective option for the long-term conservation of genetic resources of these problem species.

This paper presents the various cryopreservation techniques available, their current development and problems with their application to recalcitrant seed and vegetatively propagated species.

Cryopreservation techniques available

Most of the experimental systems employed in cryopreservation (cell suspensions, calluses, shoot-tips, embryos) contain high amounts of cellular water and are thus extremely sensitive to freezing injury since most of them are not inherently freezing-tolerant. Cells have thus to be dehydrated artificially to protect them from the damages caused by the crystallization of intracellular water into ice (Meryman and Williams 1985; Mazur 1984). The techniques employed and the physical mechanisms upon which they are based are different in classical and new cryopreservation techniques (Withers and Engelmann 1997). Classical techniques involve freeze-induced dehydration, whereas new techniques are based on vitrification. Vitrification can be defined as the transition of water directly from the liquid phase into an amorphous phase or glass, whilst avoiding the formation of crystalline ice (Fahy *et al.* 1984).

Classical cryopreservation techniques

Classical cryopreservation techniques involve slow cooling down to a defined prefreezing temperature, followed by rapid immersion in liquid nitrogen. With temperature reduction during slow cooling, the cells and the external medium initially supercool, followed by ice formation in the medium (Mazur 1984). The cell membrane acts as a physical barrier and prevents the ice from seeding the cell interior and the cells remain unfrozen but supercooled. As the temperature is further decreased, an increasing amount of the extracellular solution is converted into ice, thus resulting in the concentration of intracellular solutes. Since cells

remain supercooled and their aqueous vapour pressure exceeds that of the frozen external compartment, cells equilibrate by loss of water to external ice. Depending upon the rate of cooling and the prefreezing temperature, different amounts of water will leave the cell before the intracellular contents solidify. In optimal conditions, most or all intracellular freezable water is removed, thus reducing or avoiding detrimental intracellular ice formation upon subsequent immersion of the specimen in liquid nitrogen. However, too-intense freeze-induced dehydration can incur different damaging events owing to concentration of intracellular salts and changes in the cell membrane (Meryman *et al.* 1977). Rewarming should be as rapid as possible to avoid the phenomenon of recrystallization in which ice melts and reforms at a thermodynamically favourable, larger and more damaging crystal size (Mazur 1984).

Classical techniques are generally operationally complex since they require the use of sophisticated and expensive programmable freezers. In some cases, their use can be avoided by performing the freezing step with a domestic or laboratory freezer (Kartha and Engelmann 1994).

Classical cryopreservation techniques have been successfully applied to undifferentiated culture systems such as cell suspensions and calluses (Kartha and Engelmann 1994; Withers and Engelmann 1997). In the case of differentiated structures, these techniques can be employed for freezing apices of cold-tolerant species (Reed and Chang 1997). However, their successful utilization with tropical species is exceptional (Escobar *et al.* 1997).

New cryopreservation techniques

In vitrification-based procedures, cell dehydration is performed prior to freezing by exposure of samples to concentrated cryoprotective media and/or air desiccation. This is followed by rapid cooling. As a result, all factors which affect intracellular ice formation are avoided. Glass transitions (changes in the structural conformation of the glass) during cooling and rewarming have been recorded with various materials using thermal analysis (Sakai *et al.* 1990; Dereuddre *et al.* 1991; Tannoury *et al.* 1991; Niino *et al.* 1992). Dumet *et al.* (1993b) showed that increased survival rates for cryopreserved oil-palm somatic embryos were correlated with the progressive disappearance of ice crystallization peaks and their replacement by glass transitions.

Vitrification-based procedures offer practical advantages in comparison with classical freezing techniques (Steponkus *et al.* 1992). Like ultra-rapid freezing (above), they are more appropriate for complex organs (shoot-tips, embryos) which contain a variety of cell types, each with unique requirements under conditions of freeze-induced dehydration. By precluding ice formation in the system, vitrification-based procedures are operationally less complex than classical ones (e.g. they do not require the use of controlled freezers) and have greater potential for broad applicability, requiring only minor modifications for different cell types (Engelmann 1997b).

A common feature of all these new protocols is that the critical step to achieve survival is the dehydration step, and not the freezing step, as in classical protocols. Therefore, if samples to be frozen are amenable to desiccation down to sufficiently

low water contents (which vary depending on the procedure employed and the type and characteristics of the propagule to be frozen) with no or little decrease in survival in comparison with non-dehydrated controls, no or limited further drop in survival is generally observed after cryopreservation (Engelmann 1997b).

Seven different vitrification-based procedures can be identified: (i) encapsulation-dehydration; (ii) a procedure actually termed vitrification; (iii) encapsulation-vitrification; (iv) desiccation; (v) pregrowth; (vi) pregrowth-desiccation, and (vii) droplet freezing.

The **encapsulation-dehydration** procedure is based on the technology developed for the production of artificial seeds. Explants are encapsulated in alginate beads, pregrown in liquid medium enriched with sucrose for 1 to 7 days, partially desiccated in the air current of a laminar airflow cabinet or with silica gel down to a water content around 20% (fresh weight basis), then frozen rapidly. Survival rates are high and growth recovery of cryopreserved samples is generally rapid and direct, without callus formation. This technique has been applied to apices of numerous species of both temperate and tropical origins (Engelmann 1997b). Its applicability to cell suspensions and somatic embryos has also been demonstrated (Tessereau *et al.* 1994; Bachiri *et al.* 1995).

Vitrification involves treatment of samples with cryoprotective substances, dehydration with highly concentrated vitrification solutions, rapid freezing and thawing, removal of cryoprotectants and recovery. This procedure has been developed for apices, cell suspensions and somatic embryos of numerous different species (Sakai 1995, 1997).

Encapsulation-vitrification is a combination of encapsulation-dehydration and vitrification procedures, where samples are encapsulated in alginate beads, then subjected to freezing by vitrification. It has been applied to apices of carnation (Tannoury *et al.* 1991) and of lily, wasabi and *Armoracia* (Sakai 1997).

Desiccation is the simplest procedure since it consists of dehydrating explants, then freezing them rapidly by direct immersion in liquid nitrogen. This technique is mainly used with zygotic embryos or embryonic axes extracted from seeds. It has been applied to embryos of a large number of recalcitrant and intermediate seeds (Engelmann 1997a). Desiccation is usually performed in the air current of a laminar airflow cabinet, but more precise and reproducible dehydration conditions are achieved by using a flow of sterile compressed air or silica gel. Ultra-rapid drying in a stream of compressed dry air (a process called "flash drying" developed by Berjak's group in South Africa) allows freezing of samples with a relatively high water content, thus reducing the desiccation injury (Berjak *et al.* 1989a; Wesley-Smith *et al.* 1992). Optimal survival rates are generally obtained when samples are frozen with a water content of between 10 and 20% (fresh weight basis).

The **pregrowth** technique consists of cultivating samples in the presence of cryoprotectants, then freezing them rapidly by direct immersion in liquid nitrogen. The pregrowth technique has been developed for *Musa* apices (Panis 1995).

In a **pregrowth-desiccation** procedure, explants are pregrown in the presence of cryoprotectants, dehydrated under the laminar airflow cabinet or with silica gel, then frozen rapidly. This method has currently been applied only to asparagus

stem segments, oil-palm somatic embryos and coconut zygotic embryos (Uragami *et al.* 1990; Assy–Bah and Engelmann 1992; Dumet *et al.* 1993a).

The **droplet freezing** technique has presently been applied to potato apices only (Schäfer–Menuhr 1996). Apices are pretreated with liquid cryoprotective medium, then placed on aluminium foil in minute droplets of cryoprotectant and frozen directly by rapid immersion in liquid nitrogen.

Cryopreservation of vegetatively propagated and recalcitrant seed species

Vegetatively propagated species

Several review papers have been published recently, which provide lists of species which have been successfully cryopreserved (Bajaj 1995; Engelmann 1997a, 1997b). For vegetatively propagated species, cryopreservation has a wide applicability both in terms of species coverage – since protocols have been successfully established for roots and tubers, fruit trees, ornamentals and plantation crops of both temperate and tropical origin – and in terms of number of genotypes/varieties within a given species. The best example is potato, for which cryopreservation has been applied to 219 different accessions, with an average recovery rate of 40% (Schäfer–Menuhr 1996). With a few exceptions (e.g. potato, pear, mulberry), vitrification-based protocols have been employed. It is also interesting to note that in many cases, different protocols can be employed for a given species and produce comparable results. The only exception could be *Musa*, for which encapsulation-dehydration led to very low survival, owing to the extreme sensitivity of this material to high sucrose concentrations and air desiccation (Panis 1995). The survival rates obtained are generally high to very high and up to 100% survival could be achieved in some cases, e.g. *Allium*, yam and potato. Regeneration is rapid and direct, and callusing is observed only in cases where the technique is not optimized, such as with yam and cassava (Chabrillange *et al.* 1996; Mandal *et al.* 1996).

Different reasons can be mentioned to explain these positive results. The meristematic zone of apices, from which organized growth originates, is composed of a relatively homogeneous population of small, actively dividing cells, with few vacuoles and a high nucleo-cytoplasmic ratio. These characteristics make them more tolerant of desiccation than highly vacuolated and differentiated cells. As mentioned earlier, no ice formation takes place in vitrification-based procedures, thus avoiding the extensive damages caused by ice crystals which are formed during classical procedures. Histological examination revealed that the whole or most of the apex structure is generally preserved when vitrification-based techniques are employed, thus allowing direct, organized regrowth (Gonzalez Arnao *et al.* 1993). By contrast, classical procedures can lead to the destruction of large zones of the apices and callusing only or transitory callusing is often observed before organized regrowth starts (Haskins and Kartha 1980; Bagniol and Engelmann 1991).

Other reasons for the good results obtained are linked with tissue culture protocols. Many vegetatively propagated species successfully cryopreserved until now are cultivated crops, often of great commercial importance, for which cultural practices, including *in vitro* micropropagation, are well established. In addition, *in vitro* material is "synchronized" by the tissue culture and pregrowth procedures, and relatively homogeneous samples in terms of size, cellular composition, physiological state and growth response are employed for freezing, thus increasing the chances of positive and uniform response to treatments. Finally, vitrification-based procedures allow the use of samples of relatively large size (shoot-tips of 0.5 to 2–3 mm) which can regrow directly without any difficulty.

In many cases, whole plants have been regenerated from cryopreserved apices, transferred *in vivo* and observed for genetic stability. No modification which could be attributed to cryopreservation has yet been noted, which indicates that cryopreservation is safe as regards preservation of trueness to type (Engelmann 1997b). Even though potato is still the only example of large-scale, routine utilization of cryopreservation for long-term storage of a vegetatively propagated (and of any other) crop, freezing techniques are now operational for large-scale experimentation in an increasing number of cases. In view of the wide range of efficient and operationally simple techniques available, any vegetatively propagated species should be amenable to cryopreservation, provided that the tissue culture protocol is sufficiently operational for this species.

Recalcitrant seed species

Several review papers have been published in recent years which present extensive lists of plant species whose embryos and/or embryonic axes have been successfully cryopreserved (e.g. Kartha and Engelmann 1994; Pence 1995; Engelmann *et al.* 1995; Engelmann 1997a, 1997b). This might lead to the conclusion that freezing of embryos is a routine procedure applicable to numerous species, whatever their storage characteristics. However, careful examination of the species mentioned in these papers reveals that only a limited number of truly recalcitrant seed species are in fact included. This is partly because research in this area is recent and addressed by very few teams worldwide. Another reason is that recalcitrance is a dynamic concept (Berjak and Pammenter 1994), which evolves with research on the biology of species and improvement in classical storage procedures. For example, seeds of coffee and oil-palm were previously classified as recalcitrant (Chin and Roberts 1980) but recent research by Ellis *et al.* (1990, 1991) indicated that they display an intermediate storage behaviour.

In comparison with results obtained with vegetatively propagated species, it is obvious that research is still at a very preliminary stage for recalcitrant seeds. The desiccation technique is mainly employed for freezing embryos and embryonic axes. The survival rates achieved are extremely variable and generally low. Most importantly, survival is often limited and regeneration frequently restricted to callusing or incomplete development of plantlets. In only a limited number of cases have whole plants have been regenerated from cryopreserved material, e.g. *Howea*, *Veitchia* and coconut (Chin and Pritchard 1988; Assy Bah and Engelmann 1992).

There are a number of reasons to explain the current limited development of cryopreservation for recalcitrant seed species. First of all, there is a huge number of species with recalcitrant or suspected recalcitrant seeds and the majority are wild species. As a consequence, nothing or little is known on their biology, and even less on the seed storage behaviour of many of these species.

In cases where some information on seed storage behaviour is available, tissue culture protocols, including inoculation *in vitro*, germination and growth of plantlets, propagation and acclimatization which are needed for regrowth of embryos and embryonic axes after freezing, are often non-existent or not fully operational (Zakri *et al.* 1991; Dumet *et al.* 1996). This is particularly critical for tree species, which constitute a large part of recalcitrant seed species, especially in the tropics, and often pose great difficulties in tissue culture (Gupta 1988; Rao 1988; Harry and Thorpe 1994). Modifications – sometimes minor – in the *in vitro* culture conditions might lead to improvement in the recovery rates for some species. For example, modification in the hormonal balance of the culture medium could significantly improve the survival and recovery rates of cryopreserved coffee embryos (Abdelnour *et al.* 1992; Normah and Vengadasalam 1992).

Seeds and embryos of recalcitrant species also display various characteristics which make their cryopreservation difficult. One of the characteristics of recalcitrant seeds is that there is no arrest in their development, as with orthodox seeds (Berjak *et al.* 1989b). It is thus very difficult to select seeds at a precise developmental stage, even though this parameter is often of critical importance to achieve successful cryopreservation (Chandel *et al.* 1995; Engelmann *et al.* 1995). Very important variations in seed moisture content and maturity stage can be observed between provenances, between and among seed lots, as well as between successive harvests (Berjak *et al.* 1996). This might well explain contrasting results obtained by different laboratories working on the same species, and non-reproducible results with different seed lots.

Seeds of many species are too large to be frozen directly and embryos or embryonic axes have to be employed. However, embryos are often of very complex tissue composition which display differential sensitivity to desiccation and freezing, the root pole seeming more resistant than the shoot pole (Pritchard and Prendergast 1986; Pence 1992, 1995; Dussert, pers. comm.). In some species, embryos are extremely sensitive to desiccation and even minor reduction in their moisture content – down to levels much too high to obtain survival after freezing – leads to irreparable structural damage, as observed notably with cacao (Chandel *et al.* 1995). Finally, embryos of some species are too large to envisage using them for cryopreservation, and seeds of some species (e.g. *Symphonia globulifera, Barringtonia racemosa*) do not contain well-defined embryos (Berjak *et al.* 1996).

There are various options to consider for improving storage of non-orthodox seeds. With some species such as tea, mahogany and neem, seeds are relatively small and tolerant to desiccation, and can thus be cryopreserved directly after partial desiccation under the laminar flow (Hu *et al.* 1994; Marzalina 1995; Berjak and Dumet 1996). With other species which are more desiccation-sensitive, very precisely controlled desiccation (e.g. using saturated salt solutions) and cooling

conditions may allow freezing of whole seeds, as demonstrated recently with various coffee species (Dussert *et al.* 1997, 1998).

As mentioned earlier, desiccation has been employed preferentially for freezing embryos and embryonic axes of recalcitrant seed species and there is scope for various technical improvements in the current cryopreservation protocols. Pregrowth of embryos on media containing cryoprotective substances may give the tissues increased tolerance to further desiccation and reduce the heterogeneity of the material. Berjak and co-workers have demonstrated that flash drying, followed by ultra-rapid freezing, has been very effective for cryopreservation of several species (Berjak *et al.* 1989a; Wesley–Smith *et al.* 1992). Their hypothesis is that very rapid dehydration imposes a stasis on metabolism and precludes the deleterious reactions that would take place under lower desiccation rates and that ultra-rapid freezing induces vitrification of internal solutes or the formation of ice crystals too small to disrupt cellular integrity. Even though some species have proven far too desiccation-sensitive to be cryopreserved this way (Pammenter *et al.* 1993), this is a potentially interesting approach which deserves further research and experimentation with additional species. Other cryopreservation techniques including pregrowth-desiccation, encapsulation-dehydration and vitrification, which have been seldom employed so far should be experimented with (Engelmann 1992; Pence 1995). Finally, it should be emphasized that selecting embryos at the right developmental stage is of critical importance for the success of any cryopreservation experiment (Chandel *et al.* 1995; Engelmann *et al.* 1995).

However, in these cases, basic protocols for disinfection, inoculation *in vitro*, germination of embryos or embryonic axes, plantlet development, and possibly limited propagation will have to be established prior to any cryopreservation experiment. *In vitro* culture of mature embryos is usually achieved with simple culture media (including mineral salts and carbon source) but more complex media have to be formulated to obtain growth of immature material (Raghavan 1994).

With species for which attempts to freeze whole embryos or embryonic axes have proven unsuccessful, various authors have suggested using shoot apices sampled on the embryos, adventitious buds or somatic embryos induced from the embryonic tissues (Pence 1995; Berjak *et al.* 1996). This might be the only solution for species which do not have well-defined embryos but this will require that more sophisticated tissue culture procedures be developed and mastered. In addition to these technical difficulties, this would reduce the range of genetic variability captured (Pence 1995; Berjak *et al.* 1996), especially when using somatic embryogenesis, since response to inducing treatments is generally highly genotype-specific and somatic embryo cultures might be obtained from a limited number of genotypes only (e.g. Bozkov 1995; Gana *et al.* 1995). It might be advisable to use this approach with species for which relevant tissue culture procedures are already available, such as rubber tree or cacao. In case apices are to be employed, it might be more practical and efficient to sample them on *in vitro* plantlets rather than on embryos to reduce the risks of contamination, and to use more homogeneous material.

Conclusion

Significant progress has been made during the past 10 years in the area of plant cryopreservation, with the development of various efficient vitrification-based freezing protocols. An important advantage of these new techniques is their operational simplicity, since they will be applied mainly in developing tropical countries where the largest part of genetic resources of problem species is located.

For many vegetatively propagated species, cryopreservation techniques are sufficiently advanced to envisage their immediate utilization for large-scale experimentation in genebanks. Research is much less advanced for recalcitrant seed species. This is because of the large number of mainly wild species (with very different characteristics) which fall within this category, and the comparatively limited level of research activities aiming at improving the conservation of these species. However, various technical approaches can be explored to improve the efficiency and increase the applicability of cryopreservation techniques to recalcitrant species. In addition, research is actively performed by various groups worldwide to improve knowledge of biological mechanisms underlying seed recalcitrance. It is hoped that new findings on critical issues such as understanding and control of desiccation sensitivity will contribute significantly to the development of improved cryopreservation techniques for recalcitrant seed species.

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