Development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops

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Abstract

Shoot-tips and somatic embryos are the explants of choice for the *in vitro* long-term storage of *ex situ* plant genetic resources in liquid nitrogen. Cryopreservation of organized structures has significantly progressed, especially for species of tropical origin, with the development of several vitrification-based procedures such as encapsulation-dehydration, vitrification and droplet-vitrification approaches. They have allowed improvements in survival and recovery after cryopreservation compared with conventional crystallization-based protocols, proving their effectiveness for large scale application with embryos and shoot-tips of different plants. This review addresses the main physical and technological aspects involved in plant cryopreservation methods, illustrating the development of research with three cases: citrus, cassava and potato. These studies demonstrate how cryopreservation strategies are increasingly applied for their successful employment in the genebanks.

Key words: Cryopreservation; shoot-tips; somatic embryos; vitrification; genebank.

Introduction

Cryopreservation is the storage of biological specimens at ultra low temperature (-196°C) in a cryogenic medium such as liquid nitrogen (Withers and Engelmann, 1997). At this ultra-low temperature, all cellular divisions and metabolic processes are stopped, allowing conservation for a theoretically unlimited period of time (Engelmann, 2004).

Long-term conservation of plant genetic resources using cryopreservation relies on freezing embryos and/or shoot-tips. Embryos and shoot tips are complex structures with a heterogeneous cellular composition. They will thus require cryogenic protective treatments to ensure conservation of their structural integrity, and, when dealing with species of tropical origin, specific treatments are required to artificially induce cold tolerance, because tropical plants do not develop cold-tolerance mechanisms, and are thus highly sensitive to low temperatures (Engelmann 2000).

The state of water and the osmotic equilibrium related to movements of water into and out of the cells are parameters of particular importance for cryopreservation (Mazur, 2004). Water removal plays a central role in preventing freezing injury and in maintaining post-thaw viability.There are two types of cryopreservation protocols, which differ, based on their physical mechanisms. In the so called classical or conventional cryopreservation, freezing is performed in the presence of ice, while in the vitrification-based protocols; freezing normally takes place without ice formation.

Conventional protocols involve the pretreatment of samples with cryoprotective solutions composed by a single or a mixture of colligative chemical substances, for example dimethyl sulphoxide (DMSO), which are usually added progressively up to a final concentration that is lower by comparison than the concentration of cryoprotective solutions used in the vitrification-based procedures. This pretreatment removes some water from the cells. However, most of the water is removed during the first step of the cooling procedure, *i.e.* slow cooling to a given prefreezing temperature. During the second step of the freezing procedure, *i.e.* rapid immersion in liquid nitrogen, crystallization of the remaining water or vitrification of intracellular solutes takes place (Engelmann 2000).

By contrast, vitrification-based protocols involve some degree of dehydration and desiccation before freezing by exposure of samples to highly concentrated cryoprotective solutions and/or to physical drying conditions. As a result, most or all freezable water is removed during dehydration; subsequent cooling is generally performed rapidly by direct immersion in liquid nitrogen, thereby inducing vitrification of internal solutes (Engelmann 2000).

Cryopreservation of plant cells and organs has dramatically developed since the first successful report on freezing of flax cells (Quatrano, 1968). Over the last 15 years, significant improvements have been achieved in cryopreservation technology with the development of vitrification-based procedures. As a result, the number of plant species for which a cryopreservation protocol has been established is now well over 200 (Engelmann 2004), and the number of cases is increasing regularly, where cryopreservation can be routinely applied for long-term germplasm conservation.

This review consists of two main sections. The first section reviews the physical events which take place during freeze-induced and vitrification-based cryopreservation, and briefly describes the vitrification-based techniques available. In the second section, the development of cryopreservation techniques employed in relation to their large scale application is described using several examples.

Physical events occurring during cryopreservation

Freezing and ice formation

Crystallization during freezing is a complex process that comprises a number of critical steps: nucleation, growth of crystals and/or recrystallization, which are considered the main elements affecting survival of cells subjected to cryopreservation.

The first step in ice formation is nucleation, during which a template is formed that defines the characteristics of the crystal and upon which growth of the crystal is possible (Reid, 1994). Two types of nucleation are recognized: homogeneous and heterogeneous. Homogeneous nucleation is a process which occurs spontaneously during cooling at a temperature lower than the melting point of the pure crystalline phase (ice). For pure water, this temperature is approximately -40°C. However, the occurrence of homogeneous nucleation is a relatively uncontrollable event, and, in absence of heterogeneous nuclei, water remains supercooled (*i.e.* remains unfrozen below the freezing temperature of the liquid phase) until homogenous nucleation takes place (Panis, 1995). The sudden transition of extra- and intracellular supercooled water into ice generally produces cell injuries of physical and biochemical nature, which may result in loss of post-thaw viability. Therefore, to prevent the occurrence of intracellular ice formation, the interruption of supercooling (by induction of heterogeneous nucleation) is recommended.

Heterogeneous nucleation is the formation of ice nuclei (seeds) on a catalytic surface. The temperature, at which nuclei reach the required thermodynamic characteristics (critical size and energy) to become an ice crystal, is higher than that seen in homogeneous nucleation (Reid, 1986). Following a conventional cryogenic protocol, samples are slowly cooled in a cryoprotective solution, which usually is performed with the aid of a programmable freezing device. There is a direct relationship between the temperature at which heterogeneous nucleation should be induced by seeding ice and the concentration of the cryoprotective solution used. Seeding is usually performed 2-3°C below the freezing point of the cryoprotective solution, and the cooling device is either held at this temperature for a few minutes to equilibrate the thermal gradient, or the temperature may decrease continuously following the slow controlled freezing rate.

After seeding, ice formation takes place in the extracellular solution. This external crystallization promotes an efflux of water from the cytoplasm and vacuoles to the external space where it finally freezes (Farrant, 1980; Taylor, 1987). Intracellular water leaves the protoplast in order to restore the osmotic equilibrium produced by ice transition, when the external solution becomes more and more concentrated, and thus hypertonic to the cell (Panis, 1995). Depending on the cooling rate and on the slow cooling terminal prefreezing temperature, various amounts of water will be removed from the cells before the intracellular contents solidify when samples are immersed in liquid nitrogen (Pitt, 1992). However, freeze-dehydration is usually not sufficient to remove enough water from complex biological systems like embryos and shoot-tips, and a relatively homogeneous removal of intracellular water from such structures is difficult. Additionally, it is difficult to control dehydration during a freeze-dehydration based protocol.

The cooling regime mostly employed to guarantee an appropriate level of dehydration for successfully freezing different plant cells involves the reduction of temperature at 0.5°C to 1°C.min^{-1} down to around -40°C, followed by rapid immersion in liquid nitrogen (Panis, 1995). According to Toner (1993), the cooling rate at which the probability of cell survival drops to 50% also corresponds to the cooling rate at which 50% of cells are detected to form intracellular ice. Due to water efflux, the residual intracellular solution freezes at a lower temperature than its freezing point, or becomes sufficiently concentrated to form an amorphous glass (Engelmann, 2000). Therefore, depending on the amount of remaining intracellular water at the moment of immersing samples in liquid nitrogen, the following physical events may take place: large lethal crystals are formed if the water content is high, small, non-deleterious crystals if the water content is not high enough, or an amorphous glass is formed if cellular viscosity is so high that any remaining intracellular water becomes vitrified (Benson et al., 2006).

The formation of ice crystals detrimental to cellular integrity can also take place during warming procedure. A potential cause of injury is the use of a low warming rate to attain room temperature. Under slow warming conditions, there is a tendency for large crystals to grow at the expense of small ones, or for devitrification (ice crystal formation) to take place, when unstable glasses have been formed during cooling. Unstable glasses are obtained during rapid immersion in liquid nitrogen of little concentrated samples; such glasses are considered metastable, because devitrification can occur upon rewarming, returning to either a liquid or crystalline state (Benson et al., 2006).

To avoid the destabilization of the non-crystalline solid produced; thawing should be performed rapidly by placing the cryovials in a water bath at +40°C for a few minutes. If warming is carried out rapidly, there is insufficient time for devitrification to occur and post-thaw recovery is more likely to occur (Mazur, 2004). Based on Differential Scanning

Calorimetry (DSC) and cryomicroscopic studies (Rall et al., 1984; Rall and Polge, 1984), slow warming has been reported to be much more damaging than rapid warming, specially if following a slow freezing regime, where the pre-freezing terminal temperatures are around -30°C to -40°C prior to immersion in liquid nitrogen (Mazur, 2004).

Cryopreservation using classical protocols is based on the dynamics of freeze-dehydration. Optimization of the extracellular crystallization process is the key step during cooling, and implementing a high warming rate is the critical step to prevent recrystallization during thawing of samples.

Vitrification and non-crystalline solid formation

Vitrification is the freeze-avoidance mechanism that enables hydrated cells, tissues and organs to withstand exposure to the temperature of liquid nitrogen (Sakai, 2000). Vitrification *per se* is a physical process, defined as the transition of the liquid phase to an amorphous glassy solid at the glass transition (Tg) temperature (Fahy et al., 1984). The glass may contribute to preventing tissue collapse, solute concentration and pH alterations during dehydration. Because of the high viscosity of a glass, all chemical reactions that require molecular diffusion of water are virtually arrested, and biological material in this stable condition may be maintained for a long time (Burke 1986).

Vitrification is achieved by direct immersion in liquid nitrogen of samples which have been dehydrated at a non-freezing temperature. Therefore, the freeze-induced dehydration step characteristic of conventional procedures is eliminated. The vitrified state is achieved in systems that become sufficiently concentrated after a drastic desiccation process and that are cooled sufficiently rapidly so that the increase in cellular viscosity inhibits molecular rearrangement of water into a crystalline pattern (Taylor et al., 2004). As cooling progresses, the viscosity of intracellular solutes increases to the point where translational molecular motion is essentially halted and the solution becomes a glass. The resultant solid retains the random molecular arrangement of a liquid, but has the mechanical properties of a solid (MacFarlane, 1987; MacFarlane et al., 1992).

For plant cryopreservation, several efficient vitrification-based procedures have been developed by different research groups since 1989-1990 (Langis et al., 1989; 1990; Sakai et al., 1990; Towill, 1990; Uragami et al., 1989, Fabre and Dereuddre, 1990). This was significant progress, especially for cryopreserving complex organs like shoot-tips and somatic embryos that could not be effectively frozen following conventional protocols (Panis, 1995; Gonzalez-Arnao, 1996; Gonzalez-Arnao et al., 1998a; Gonzalez-Arnao et al., 2003). In addition, when a vitrification-based protocol is applied under well optimized conditions, the whole or most of the meristematic structure remains intact, which guarantees direct regrowth without transitory callus formation (Matsumoto et al., 1994; Hirai and Sakai, 1999a). Recovery without intermediate callus formation is essential for clonal conservation of germplasm (Takagi, 2000). This is an advantage in terms of avoiding genetic instability that may be produced when regrowth after cryopreservation starts (Sakai, 2004).

Existing vitrification-based cryopreservation techniques

Seven different vitrification-based procedures can be identified: (i) pregrowth; (ii) dehydration; (iii) pregrowth-dehydration; (iv) encapsulation-dehydration; (v) vitrification; (vi) encapsulation-vitrification and (vii) droplet-vitrification.

The pregrowth technique consists of cultivating samples in the presence of cryoprotectants, and then freezing them rapidly by direct immersion in liquid nitrogen. The pregrowth technique has been developed for *Musa* meristematic cultures (Panis et al*.,* 2002).

Dehydration is the simplest vitrification-based procedure**,** since it consists of dehydrating explants and 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 species (Engelmann, 2000). 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 Prof. Berjak's group in South Africa) allows freezing samples with relatively higher water content, thus reducing desiccation injury (Berjak et al., 1989). However, optimal survival is generally obtained when samples are frozen with water contents between 10 and 20% (fresh weight basis).

The pregrowth-dehydration procedure involves pregrowth of explants in the presence of cryoprotectants, dehydration under the laminar airflow cabinet or with silica gel, and then rapid freezing. This method has been applied notably to asparagus stem segments, oil palm polyembryonic cultures and coconut zygotic embryos (Uragami et al., 1990; Assy-Bah & Engelmann, 1992; Dumet et al., 1993).

Another significant advance in cryoprotective treatments of some vitrification-based protocols has been the encapsulation of tissues in calcium alginate beads (Fabre and Dereuddre, 1990). Encapsulation of explants allows the application of subsequent drastic dehydration processes prior to cryopreservation, which would otherwise be highly damaging or lethal to non-encapsulated samples (Gonzalez-Arnao and Engelmann, 2006). Cryopreservation using the encapsulation-dehydration procedure has been very effective for freezing apices of different plant species from temperate and tropical origin (Gonzalez-Arnao and Engelmann, 2006). The basic protocol comprises encapsulation, preculture of alginate coated samples in liquid medium with high sucrose concentration, desiccation, rapid cooling and slow rewarming. It is important to note that after such a drastic drying process (desiccation down to around 25% moisture content in alginate beads, fresh weight basis), survival of explants after thawing may become independent of the warming rate, as noted for example with carrot somatic embryos (Dereuddre et al., 1991), sugarcane apices (Gonzalez-Arnao, et al., 1993) and orchid seeds with fungal symbiont (Wood et al., 2000). For recovery, encapsulated samples are generally placed on standard culture medium without having to extract the explants from their alginate coating (Gonzalez-Arnao and Engelmann, 2006).

The freezing procedure referred to as vitrification comprises a pretreatment (loading treatment) at room temperature, followed by exposure to a vitrification solution at 25° or 0°C, rapid cooling and warming, and final removal of the vitrification solution by washing samples with an unloading solution consisting of liquid culture medium supplemented with 1.2 M sucrose (Withers and Engelmann, 1997). Different Plant Vitrification Solutions have been successfully developed by various research groups (Langis et al., 1990; Sakai et al., 1990). The most frequently used and efficient vitrification solution (Sakai et al., 1990) so far is PVS2 (30% glycerol (w/v) + 15% ethylene glycol (w/v) + 15% (w/v) DMSO in culture medium with 0.4 M sucrose). However, direct exposure of samples to any vitrification solution often leads to detrimental effects due the toxicity caused by their high concentration (over 7 M for PVS2). A pretreatment with cryoprotectants at a lower concentration has proved to significantly increase dehydration tolerance, and to mitigate the mechanical stress caused by the subsequent treatment with a highly concentrated PVS (Takagi, 2000; Thin and Takagi, 2000; Sakai, 2004). A mixture of 2 M glycerol + 0.4 M sucrose in liquid medium, termed "loading solution" (Nishizawa et al., 1993), applied for 20 min at room temperature is very effective to enhance osmotolerance (Sakai, 2004).

Encapsulation-vitrification is a combination of the encapsulation-dehydration and vitrification procedures, where samples are encapsulated in alginate beads, and then subjected to freezing following the vitrification approach. It has been applied to apices of an increasing number of species including yam (Hirai, 2001; Hirai and Sakai, 2001), pineapple (Gamez-Pastrana et al., 2004), sweet potato (Hirai and Sakai, 2003), cassava (Hirai, 2001; Charoensub et al., 2004) and other species (Sakai and Engelmann, 2007).

The combination of loading and PVS treatments with the droplet freezing method developed for cassava shoot tips (Kartha et al*.,* 1982**)**, has derived in a new and efficient technique called droplet-vitrification. This procedure has already been successfully applied to a number of crops (Ashmore et al., 2000; Leunufna and Keller, 2003; Panis et al., 2005; Kim et al., 2006; Sakai & Engelmann 2007). The droplet-vitrification technique is characterized by increased cooling and warming rates compared to other vitrification-based procedures, since samples are frozen in minute droplets of PVS placed on aluminium foil strips, which are plunged directly in liquid nitrogen. This protocol significantly increases the probability of obtaining a vitrified state during freezing, and of avoiding devitrification during warming (Panis et al., 2005).

Application of conventional and vitrification-based techniques to somatic embryos and shoot-tips

The first successful cryopreservation of somatic embryos and shoot-tips using a conventional, freeze-dehydration based protocol was achieved with carrot somatic embryos (Lecouteux et al. 1991) and pea shoot tips (Haskins & Kartha, 1980). Conventional protocols have been successfully applied to undifferentiated culture systems such as cell suspensions and calli, as well as to shoot tips of cold-tolerant species (Engelmann 2004). However, when conventional protocols are applied to shoot tips of tropical species, large zones of the apical dome are destroyed and plant regeneration is mediated by undesirable callus formation (Touchell and Dixon 1996; Bagniol et al.1992). Successful cryopreservation of cassava apices using a conventional cryogenic procedure is an exceptional example (Escobar et al., 1997; Engelmann, 2000).

The utilization of vitrification-based procedures such as encapsulation-dehydration, vitrification, dehydration, droplet freezing and their combinations, has allowed improving cryopreservation of differentiated explants, irrespective of their origin and genomic group (Schäfer-Menuhr et al., 1997a; Engelmann, 2000; Panis et al., 2005).

The first successful results using vitrification-based protocols for somatic embryos and shoot-tips were achieved with asparagus (Uragami et al., 1989), and pear (Dereuddre et al., 1990). These techniques have a broad spectrum of applicability, and usually ensure high recovery percentages after immersion in liquid nitrogen (Engelmann, 2003)**.** Vitrification-based protocols represent a significant contribution to the successful application of cryopreservation to complex structures. The cases of *Citrus* somatic embryos, and cassava and potato shoot tips illustrate the improvements in the applicability of cryopreservation in relation to changes in the cryogenic protocols employed.

Citrus somatic embryos

The first report of cryopreservation of citrus somatic embryos induced from *in vitro* cultured ovules of ´Washington navel´ sweet orange included a conventional cryogenic protocol (Marin and Duran-Vila, 1988; Marin et al., 1993). Careful selection of embryos at early developmental stages was required to achieve survival, which represented an initial drawback in the freezing procedure. The availability of sufficient material at the right physiological stage under tissue culture conditions was limited, since this is an uncontrolled

biological process. Moreover, the results after cryopreservation could not be improved either by selecting uniform embryo populations or by modifying the cooling rates during freezing. Survival was always low and erratic, ranging from 3.7% to a maximum of 30.5%. Recovery of cryopreserved cultures was in the form of secondary embryogenesis originating from the surviving zones of the embryos. Therefore, this first protocol set up was unsuitable for long-term conservation of *Citrus* germplasm (Duran-Vila, 1995).

Further studies focused on vitrification-based procedures aimed at developing a simple and reliable cryopreservation method for citrus somatic embryos. The encapsulation-dehydration, which had allowed successful freezing of citrus apices from juvenile plants (Gonzalez-Arnao et al., 1998b), also guaranteed highly successful results with somatic embryos derived from *in vitro* culture of ovules from six different sweet oranges, two mandarins and mandarin hybrids, two sour oranges and two other citrus species (Gonzalez-Arnao et al., 2003). Survival ranged between 75 and 95%, depending on the species/accession tested. In addition, embryos of several lemon, mandarin and grapefruit species obtained from *in vitro* culture of thin cut layer explants from stigma, style and ovaries were also cryopreserved. High survival percentages of 76, 95 and 100%, were obtained for explants derived from stigma, style and ovaries, respectively, following the same encapsulation-dehydration cryogenic protocol (Gonzalez-Arnao et al., 2003). Thin cut layer explants may represent an interesting additional source of material for producing citrus somatic embryos. Somatic embryos are alternative explants for cryopreservation of citrus germplasm, while freezing of adult tissues continues being unsuccessful (Gonzalez-Arnao et al., 2003).

The encapsulation-dehydration protocol successfully employed, irrespective of the tissues used for obtaining somatic embryos, involved the following optimal conditions: encapsulation of tissues in calcium alginate beads (3%), preculture in liquid medium containing 0.75M sucrose (24h), desiccation of beads with silica gel down to around 20% (fresh weight basis), rapid immersion in liquid nitrogen followed by slow rewarming. Recovery of encapsulated tissues takes place on solid standard medium, firstly in the dark for one week, then under standard culture conditions (Gonzalez-Arnao et al., 2003).

The advantage of this cryopreservation protocol over the previously developed conventional procedure was clearly demonstrated in significantly improved survival, and the starting culture source and citrus species were not a limiting factor. High and consistent recovery was obtained and the whole process was simplified, as the requirement for a determined developmental stage of embryos was not anymore necessary. A modification to the preculture treatment of this protocol had previously allowed freezing apices of juvenile citrus plants (Gonzalez-Arnao et al., 1998b).

According to the first reports on cryopreservation of citrus somatic embryos following a conventional protocol (Duran-Vila, 1995; Duran-Vila et al*.,* 1997), phenotypically normal plants were obtained from recovered embryos, even when the regenerated embryos originated from groups of surviving cells. By contrast, the encapsulation-dehydration technique ensured direct and rapid regrowth of whole embryos, as revealed by histological examination, which showed that most cells were not severely damaged (Gonzalez-Arnao et al., 2003).

Cryopreservation of *Citrus* somatic embryos shows the advantages of adapting a new cryogenic protocol to improve the effectiveness of freezing conditions for such complex structures.

Cassava shoot-tips

Cryopreservation research in cassava is one of the most illustrative examples of the application and adaptation of different cryopreservation techniques. Various authors have

successfully developed classical (Kartha et al., 1982; Escobar et al., 1997) and vitrification-based (Bajaj, 1983; Engelmann et al. 1995; Escobar and Roca, 1997; Charoensub et al., 1999, 2004) protocols for cryopreservation of cassava shoot tips. However, the most comprehensive work has been carried out and is still ongoing in CIAT (Centro Internacional de Agricultura Tropical), Cali, Colombia (Roca et al., 2000; CIAT 2006).

The first freezing protocol established for cassava shoot-tips (droplet freezing) involved a cryoprotective treatment with a solution containing 15% DMSO + 3% sucrose followed by slow controlled freezing (0.5ºC.min^{-1} down to -40ºC prior to direct immersion in liquid nitrogen) of shoot tips in droplets of cryoprotective medium (Kartha et al., 1982). Recovery took place both in the form of callusing and direct regrowth of cryopreserved shoot tips. Bajaj (1983) successfully cryopreserved cassava shoot tips by direct immersion in liquid nitrogen after treatment with a cryoprotectant medium containing 5% DMSO + 5% sucrose + 5% glycerol. Regrowth also included both callusing and direct development of shoot tips. A preliminary study aiming at testing the encapsulation-dehydration technique with cassava shoot tips was performed in France at the beginning of the nineties and reported by Engelmann et al*.*, (1995). It was shown with one cassava genotype that up to 60% survival could be achieved after a pregrowth period of 24 h in medium with 0.75 M sucrose or of 72 h in medium with 0.5 M sucrose, followed by desiccation to around 30% moisture content (fresh weight basis) and rapid freezing by direct immersion of samples in liquid nitrogen. However, some callusing was noted on the regrowth medium employed, which reduced the final number of apices which could directly regenerate whole plantlets. More recently, in the framework of collaborative research activities between Thai and Japanese scientists, it was shown that cassava shoot tips could be cryopreserved using the vitrification (Charoensub et al., 1999) and encapsulation-vitrification (Charoensub et al., 2004) techniques. In the vitrification protocol (Charoensub et al., 1999), shoot tips were precultured on medium with 0.3 M sucrose for 16 h, pretreated with a mixture of 2 M glycerol + 0.4 M sucrose for 20 min at 25°C, dehydrated with the PVS2 vitrification solution for 45 min at 25°C, and then rapidly frozen in liquid nitrogen. For encapsulation-vitrification (Charoensub et al., 2004), shoot tips were precultured on medium with 0.3 M sucrose for 16 h, encapsulated in 3% calcium alginate, osmoprotected for 90 min with a mixture of 2 M glycerol and 0.6 M sucrose at 25°C, and then dehydrated with the PVS2 solution for 4 h at 0°C before direct immersion in liquid nitrogen. In both studies, regrowth was rapid and direct, and the average survival percentage was very high (75-80%).

In CIAT, research for the development of a cryopreservation protocol for cassava shoot tips started at the end of the eighties (Roca et al., 2000). The first protocol developed was a classical protocol (Escobar et al., 1997). In this protocol, the growth conditions of donor cultures were modified by using a lower temperature (21-23ºC) and higher illumination (75µE m^{-2} s^{-1}) intensity than for standard propagation. Shoot-tips isolated from 3 to 4 month-old *in vitro* cultures were precultured on a modified MS solid medium (Roca, 1984) for 3 days and treated for an additional 3 days on medium supplemented with 1 M sorbitol + 0.1 M DMSO + 0.11 M sucrose. Shoot tips were then placed in a cryoprotectant solution containing 1 M sorbitol + 0.1 M DMSO + 0.11 M sucrose for 2 h in ice, and surface-dried for 1 h on filter paper at room temperature. Samples were cooled slowly using a Cryomed 1010 freezing device. The cooling program started at 5°C in chamber, lowering the temperature at 0.5°C/min down to -15°C, and then to -40ºC at 1°C/min prior to immersion of cryotubes in liquid nitrogen. After freezing and storage for at least 1 h at -196ºC, shoot-tips were thawed rapidly (+37°C) and cultured on a series of solid media with progressively decreasing sucrose concentration (Escobar et al., 2000a). This slow freezing procedure ensured over 50% survival with cultivar MCol 22. However, an experiment performed with 14 other cassava

cultivars, representing a wide geographic distribution showed that most cultivars tested displayed low survival (2-21%) and that some cultivars were recalcitrant to cryopreservation (Escobar et al., 2000b). A cryopreservation protocol using ultra-rapid freezing by direct immersion of samples in liquid nitrogen was developed (Escobar and Roca, 1997). It resulted in similar or higher recovery than with the slow programmed freezing protocol, but some cultivars still proved recalcitrant to cryopreservation whatever the technique employed (Escobar et al., 2000b). None of the two protocols tested was thus applicable for large scale, routine cryopreservation of the cassava germplasm collection maintained in the CIAT genebank.

The encapsulation-dehydration technique, which had produced potentially interesting results with cassava (Engelmann et al., 1995), was further examined. Shoot tips were encapsulated in 3% calcium alginate beads, precultured in medium with 0.75M sucrose for 3 days, desiccated with silica gel down to around 15-20% moisture content (fresh weight basis) and immersed directly in liquid nitrogen (Escobar et al., 2000c). This methodology was tested with five different cultivars, two of which had previously proved recalcitrant to cryopreservation. All five cultivars responded positively to encapsulation-dehydration, with shoot recovery ranging between 12 and 82%. Moreover, regrowth was rapid and direct, without callus formation.

Following these preliminary experiments, a pilot project was initiated in CIAT, which aimed at using encapsulation-dehydration for freezing the 630 accessions of the core collection established from the whole cassava germplasm collection, which includes 5940 accessions (Manrique, 2000; Escobar-Pérez, 2005). The viability within these 630 cryopreserved accessions was evaluated based on shoot formation according to three established categories: less than 30% (low recovery percentage), between 30 and 70% (medium percentage) and higher than 70% (high percentage of recovery). The minimal percentage of shoot formation considered as acceptable for the cryopreserved bank is 30%. Around 75% of the frozen collection has been classified within the groups with medium and high shoot formation. At present, new methodological adjustments are under investigation in order to improve the response of those clones that have a low recovery using the encapsulation-dehydration procedure. Current research is based on the use of the vitrification method as an alternative technique, and the preliminary results regarding survival, appear to be very promising (Escobar, personal communication).

Potato shoot tips

Potato is another good example of cryopreservation research and development, where both classical and vitrification-based freezing techniques have been tested with potato shoot tips for large scale application of cryopreservation in the genebanks.

The first report of successful cryopreservation of potato shoot tips involved a classical, two-step freezing procedure (Towill, 1981). Dimethylsulfoxide (DMSO) was employed as cryoprotectant and survival of frozen shoot tips was mainly in the form of callusing, even though some plantlets could be recovered. Modifying the growth regulator content of the recovery medium led to an improvement in shoot recovery (Towill, 1983). A similar protocol was developed by Manzhulin and Butenko (1983), which led to low recovery with the three varieties tested. Further experiments indicated that buds from the upper part of the *in vitro* mother-plants gave higher survival (Manzhulin, 1984). Henshaw et al. (1985) obtained highly variable survival percentages between experiments using a similar protocol. A more recent study (Zhao et al., 2005) confirmed that the application of a classical slow freezing procedure could not result in high recovery percentages of potato shoot tips.

The encapsulation-dehydration technique, which had been developed with pear shoot tips (Dereuddre et al., 1990), was then applied to potato shoot tips. Limited growth recovery was obtained from shoot tips which were encapsulated in calcium alginate beads and slowly frozen (Fabre and Dereuddre, 1990). Following modifications of the experimental conditions (preculture duration, sucrose concentration, bead moisture content after dehydration, recovery medium) high survival could be obtained after rapid freezing of potato shoot tips (Bouafia et al., 1995; 1996; Grospietch et al., 1999) demonstrated that the increase in survival was linked to sucrose accumulation in the encapsulated shoot tips.

The droplet freezing technique, which is based on the cryopreservation protocol established by Kartha et al. (1982) for cassava shoot tips, was published in Germany in 1996 (Schäfer-Menuhr et al., 1996). Germany holds a large collection of potato varieties which includes 2,825 accessions of *Solanum tuberosum* and some material of related wild species, which are conserved in the field. The technique developed consists of cryoprotecting shoot tips from *in vitro* plantlets with a 10% DMSO solution for 1-3 h and rapidly freezing the shoot tips placed in micro-droplets (2.5 µl) of 10% DMSO solution on aluminium foils by immersing the aluminium foils in liquid nitrogen. The number of cryopreserved varieties increased progressively to 219 (Schäfer-Menuhr et al., 1997b), 245 (Mix-Wagner, 1999), 519 (Mix-Wagner et al. 2003), to reach over 1000 today (Keller et al., 2005; 2006), with the same survival and recovery results as those obtained initially. No change in survival and recovery was noted with apices of 51 varieties which had been stored in LN for several years (Mix-Wagner et al., 2003). This technique was also successfully employed in Spain for freezing several potato varieties (Barandalla et al., 2003).

Other available vitrification-based techniques were tested using several potato varieties, including vitrification (Debabrata and Naik, 1998; Zhao et al., 2006; Kryzczuk et al., 2006), encapsulation-vitrification (Hirai and Sakai, 1999b) and droplet-vitrification (Halmagyi et al., 2005; Kim et al., 2006; Yoon et al., 2006). These techniques were generally more efficient than the droplet freezing method; however, the major advantage of the latter technique is its simplicity, which is of paramount importance for large scale application (Kryzczuk et al., 2006).

In the International Potato Center (CIP), where the world largest collection of potato landraces (4,432 accessions) is conserved *in vitro*, cryopreservation research began in 1995 using the vitrification method developed by Steponkus and collaborators (Steponkus et al., 1992; Golmirzaie and Panta, 1997). In this technique, shoot apices are cryoprotected using a vitrification solution containing ethylene glycol: sorbitol: bovine serum albumin (50:15:6 wt%), and frozen in 0.25 ml polypropylene straws containing 70 µl of vitrification solution. In a pilot project, 197 genotypes were tested; seventy-five percent withstood cryopreservation, with an average survival of 46% (Golmirzaie and Panta, 2000). Ultrastructural observation of the frozen shoot tips revealed that the changes most commonly observed were abnormal cytoplasm, cell plasmolysis at different stages and large numbers of small vesicles (Golmirzaie et al., 2000). Using this protocol, about 400 potato landraces comprising eight cultivated species of different ploidy levels (2n=2x, 3x and 4x) were tested; results showed great variability in response. From these diverse genotypes, approximately 30% did not survive and 30% showed survival lower than 15%. Results revealed the need for improving the protocol. In 2004, CIP in collaboration with Katholieke Universiteit Leuven (KUL), Belgium, adapted to potato the droplet-vitrification procedure originally designed for cryopreserving banana shoot tips (Panis et al., 2005). The resulting method uses 2-3 mm shoot tips from 3 week-old plantlets grown at 22°C, which are subjected to a 15-20 min exposure to a loading solution, 50 min exposure to PVS2 treatment at 0-4°C, and ultra-rapid freezing on aluminum foil strips. Shoot tips are thawed in liquid MS medium enriched with

1.2 M sucrose and kept for 20 min in this medium, then cultivated on MS meristem culture medium containing 0.3-0.1 M sucrose, with the sucrose concentration lowered of 0.1 M per day (Panta, et al., 2006). Applying this protocol, 46 accessions were successfully cryopreserved. The number of genotypes showing post-thaw recovery >5% increased when donor plantlets were cultured at 6°C. Currently, CIP is applying this technique routinely. Up to 2006, 446 accessions have been cryopreserved with recovery ranging between 5-85% (average of 30%), and 100 shoot tips per accession have been stored in liquid nitrogen. Research continues with the aim of assembling a cryo-collection for the long-term conservation of potato landraces. At least 30% (about 1,300 accessions) of the collection maintained by CIP will be cryopreserved.

Conclusion

In the context of development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures, the most significant advances have been associated with the adaptation of different vitrification-based procedures to germplasm of tropical species. The most successful methods so far employed with such complex structures have been vitrification, encapsulation-dehydration and more recently; droplet-vitrification has emerged as a very promising technique.

The main characteristic of vitrification-based protocols is the drastic dehydration process, which, compared with the freeze-dehydration step of classical procedures, increases the probability of obtaining a vitrified state during freezing due to the improved removal of intracellular water from a whole range of different cells forming an organized structure, resulting in higher recovery, irrespective of the origin and the genetic complexity of the germplasm subjected to cryopreservation.

The three examples reviewed in this paper clearly illustrate the technological evolution of cryogenic methodologies for shoot-tips and somatic embryos. In the case of citrus somatic embryos, it was demonstrated that encapsulation-dehydration overcame the ineffectiveness of the original classical protocol, and resulted in an increase (75%) in survival. In addition, this vitrification-based protocol guaranteed the direct regrowth of embryos obtained from different embryogenic sources and from several citrus species.

As regards potato and cassava, they are both species on which almost all plant cryopreservation strategies developed have been tested. They represent examples of the applicability of cryopreservation to genebanks. Successful cryopreservation of cassava and potato shoot-tips has been performed following classical, encapsulation-dehydration, vitrification, droplet and droplet-vitrification approaches. However, when comparing crystallization-based with vitrification-based protocols, the results revealed that the application of classical protocols to shoot-tips generally did not produce high survival and often led to low recovery when cryopreservation was extended to a broad range of different cultivars. By contrast, vitrification-based procedures proved to be more effective for large scale application, and the different methods developed can be employed in a complementary manner to achieve cryopreservation of germplasm, which are less tolerant to a specific protocol.

In conclusion, the number of cases where cryopreservation is becoming effective and can thus be routinely employed is increasing steadily, especially for vegetatively propagated crops. Indeed, cryopreservation is not seen as a replacement for conventional *ex situ* approaches. Cryopreservation offers genebank curators an additional tool to allow them to improve the conservation of germplasm collections placed under their responsibility.

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