In Vitro Conservation Methods

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6.1. Introduction

The most widely used method of conserving plant genetic resources depends on seed. Many species produce seeds that can be dried to low moisture contents and stored at low temperature. Their longevity can be extended by reducing their moisture content and decreasing their storage temperature. Seeds that can tolerate extensive desiccation and can be stored in this way are termed orthodox (Roberts, 1973).

However, several categories of crops present problems with regard to seed storage. A number of species, predominantly tropical or subtropical, such as coconut (*Cocos nucifera*), cacao (*Theobroma cacao*) and many tree and shrub species, have seeds which do not undergo maturation drying and are shed at relatively high moisture contents (Chin and Pritchard, 1988). These seeds are unable to withstand much desiccation and are often sensitive to chilling, and therefore, cannot be stored dry at low temperature. These so-called recalcitrant seeds (Roberts, 1973) have to be kept in moist, relatively warm conditions, and even when stored in an optimal manner, their longevity is limited to weeks, occasionally months. Recent investigations have identified species which exhibit an intermediate form of seed storage behaviour (Ellis *et al.*, 1990). These seeds can tolerate desiccation to fairly low moisture content but the dry seeds are injured by low temperature. In comparison to truly recalcitrant seeds, the storage life of these seeds can be prolonged by some drying, but long-term conservation, i.e. comparable to orthodox seeds, remains unattainable. This category of seeds includes economically important

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species such as coffee (*Coffea* spp.) and oil palm (*Elaeis guineensis)* (Ellis *et al.*, 1990, 1991).

Some crop species have genotypes which do not produce seeds and others, such as potato (*Solanum tuberosum*), yam (*Dioscorea* spp.), cassava (*Manihot* spp.), sweet potato (*Ipomea batatas*) and sugar-cane (*Saccharum* spp.), have either sterile genotypes or produce orthodox seeds which are highly heterozygous and are therefore of limited interest for the conservation of particular gene combinations. These species are mainly propagated vegetatively to maintain clonal genotypes. Other crop species such as banana and plantain (*Musa* spp.) do not produce seeds and are thus multiplied vegetatively.

At present, the most common method to preserve the genetic resources of these problem crop species is as whole plants in the field. There are, however, several serious problems with field genebanks (Withers and Engels, 1990). The collections are exposed to natural disasters and attacks by pests and pathogens; moreover, labour costs and the requirement for technical personnel are very high. In addition, distribution and exchange from field genebanks is difficult because of the vegetative nature of the material and the greater risks of disease transfer.

Until now, most plant genetic resources conservation has focused on crop species. However, the conservation of rare and endangered plant species has also become an issue of concern (Fay, 1994).

Finally, the development of biotechnology has led to the production of a new category of germplasm, including clones obtained from elite genotypes, cell lines with special attributes, and genetically transformed material (Engelmann, 1994). This new germplasm is often of high added value and very difficult to produce. The development of efficient techniques to ensure its safe conservation is therefore of paramount importance.

During the last 20 years, *in vitro* culture techniques have been extensively developed and applied to more than 1000 different species (Bigot, 1987). Tissue culture techniques are of great interest for the collecting, multiplication and storage of plant germplasm (Engelmann, 1991a). Tissue culture systems allow propagation of plant material with high multiplication rates in an aseptic environment. Virus-free plants can be obtained through meristem culture in combination with thermotherapy, thus ensuring the production of disease-free stocks and simplifying quarantine procedures for the international exchange of germplasm. The miniaturization of explants allows reduction in space requirements and consequently labour costs for the maintenance of germplasm collections.

However, the high multiplication rates which can be achieved using *in vitro* culture procedures lead to the regular production of large amounts of plant material. This creates problems for the management of large *in vitro* collections. In addition, risks of losing material through contamination or human error are present at each subculture. More importantly, the risks of

losing the genetic integrity of the plant material through somaclonal variation increase with time in culture (Scowcroft, 1984). Storage techniques which reduce the burden placed upon all *in vitro*-based procedures and preserve the genetic integrity of the plant material are urgently needed.

Different *in vitro* conservation methods are employed, depending on the storage duration required (Engelmann, 1991a). For short- and medium-term storage, the aim is to reduce growth and to increase the intervals between subcultures. For long-term storage, cryopreservation – i.e. storage at ultra-low temperature, usually that of liquid nitrogen (−196°C) – is the only current method. At this temperature, all cellular divisions and metabolic processes are stopped. The plant material can thus be stored without alteration or modification for a theoretically unlimited period of time. Moreover, cultures are stored in a small volume, protected from contamination, requiring a very limited maintenance. *In vitro* collecting, slow growth and cryopreservation techniques are described and analysed in the following sections. The sections describing *in vitro* and slow growth techniques have been adapted from a recent review by Withers and Engelmann (1997).

6.2. *In vitro* Techniques for Collection and Exchange of Germplasm

6.2.1. In vitro *collecting*

Collectors are faced with various problems when collecting germplasm of recalcitrant seed and vegetatively propagated plant species. Collecting missions often require travelling for relatively long periods in remote areas. It is thus necessary to keep the material collected in good state for some days/weeks before it can be placed in optimal growth or storage conditions. There are thus great risks that recalcitrant seeds either germinate or deteriorate before they are brought back to the genebank (Allen and Lass, 1983). In addition, many recalcitrant seeds have considerable weight and bulk, which increases the volume of material to handle and induces additional costs, if an adequate sample of the population is to be collected. With vegetatively propagated species, the material collected will consist of stakes, pieces of budwood, tubers, corms or suckers. Not only will most of these explants not be adapted to survival once excised from the parent plant, but they will also present health risks due to their vegetative nature and contamination with soil-borne pathogens (Peacock, 1987; Withers, 1987).

Difficulties can also be encountered when collecting germplasm of orthodox seed-producing species. Even with careful planning of the time

of a collecting mission, there might be no or little seed available for all or part of the germplasm to be collected, or seeds might not be at the optimal developmental stage, or might be shed from the plant or eaten by grazing animals (IBPGR, 1984; Guarino *et al.*, 1995; Withers, 1995).

The problems described previously can be overcome if it is realized that the seed is not the only material which can be collected: zygotic embryos, or vegetative tissues such as pieces of budwood, shoots or apices can be sampled, transported and grown successfully if placed under adequate conditions.

Following an expert meeting organized by IBPGR in 1984 and sponsorship of various research programmes, *in vitro* collecting techniques have been developed for different materials including embryos of coconut (Assy-Bah *et al.*, 1987, 1989), cacao, avocado, *Citrus*, vegetative tissues of cacao (Yidana *et al.*, 1987), *Musa*, coffee (CATIE, 1997), *Prunus*, grape (Elias, 1988), cotton (Altman *et al.*, 1990) and several forage grasses (Ruredzo, 1989).

The critical points to consider for the development of *in vitro* collecting techniques have been synthesized and analysed by Withers (1995). The techniques developed are very simple and flexible, as illustrated below with coconut and cacao.

In the case of coconut, the *in vitro* field collecting technique developed by Assy-Bah *et al.* (1987, 1989) consisted of extracting from the nut with a corkborer a plug of endosperm containing the embryo. After surface sterilization with calcium hypochlorite or commercial bleach, the embryos were dissected on the spot under the shelter of a wooden box and inoculated onto semisolid medium, or the endosperm plugs were transported to the laboratory where the embryos were dissected and inoculated onto semisolid medium in aseptic conditions. This technique is very efficient since after approximately 6 to 9 months in culture in the laboratory under standard conditions, an average of 75% of the embryos collected developed into plantlets which could be successfully transferred to the nursery and then to the field. In addition, embryos inoculated *in vitro* in the field could be kept in the open for two months before being grown in the laboratory, without influencing their further development.

This technique has been modified by other researchers towards higher (Sossou *et al.*, 1987) or lower sophistication (Rillo and Paloma, 1991). In its more sophisticated version, inoculation of the embryos onto sterile medium is performed in an inflatable glove box. The simpler procedure consists of sterilizing the endosperm plugs containing the embryos and of placing them in a cool box in sterile plastic bags for transportation. Upon arrival in the laboratory, a second disinfection is performed and the embryos are dissected and inoculated onto culture medium inside the laminar airflow cabinet.

In the case of cacao, an *in vitro* collecting method was developed for

budwood (Yidana *et al.*, 1987). Considering that absolute sterility would be difficult to achieve in the field and would not necessarily be essential for robust, woody material, the aim was to place the samples collected in conditions which would suppress or delay deterioration. Stem nodal cuttings were disinfected with boiled water in which drinking-water sterilizing tablets had been dissolved and containing fungicides, then inoculated onto semisolid medium supplemented with fungicide and antibiotics. Explants could be maintained in a relatively clean (though not necessarily completely sterile) condition for up to 6 weeks.

6.2.2. In vitro *germplasm exchange*

In vitro culture techniques have been used extensively for the international exchange of germplasm because of their obvious advantages over *in vivo* material, notably their reduced weight and volume, and phytosanitary condition. Indeed, meristem culture, alone or employed in combination with thermotherapy, can eliminate viral pathogens (Kartha, 1986). Plant material can thus be multiplied, stored and exchanged in a disease-free state. In particular, because aseptic culture conditions are used and provided that the plant material has been cleansed of internal contaminants, problems with the international movement of germplasm are considerably reduced. Most countries will accept batches of plants *in vitro* with a phytosanitary certificate, without requiring a rigorous quarantine period (Fay, 1994). However, an important caution in this area is that transfer to *in vitro* culture does not confer disease-free status (Withers and Engelmann, 1997). Indexing, using one or a combination of the various techniques available, which include symptomatology, grafting/inoculation on indicator plants, ELISA and molecular techniques such as dsRNA detection, is the only sure way of making this judgement.

Routine procedures for the international exchange of *in vitro* cultures have been developed, notably for potato, cassava, yam and *Musa* (e.g. Espinoza *et al.*, 1992). Samples are usually placed in glass, or preferably plastic, test tubes on the standard culture medium, possibly with the gelling agent modified to increase its firmness and a reduction in the carbon source to limit growth. Heat-sealable polythene bags are sometimes employed instead of more fragile plastic or glass test tubes (Reed, 1991). With species such as potato and sweet potato, where it is possible to induce their formation *in vitro*, tubers are also used for germplasm exchange, since they are often easier to transport and to handle than plantlets (Dodds, 1992; Espinoza *et al.*, 1992).

Encapsulation of plant material in alginate beads has been suggested recently as a possible way of germplasm exchange in the case of banana (Rao *et al.*, 1993). Using encapsulated apices instead of *in vitro* plantlets

would result in a further volume reduction. In addition, encapsulated apices might be either grown *in vitro* after their receipt, or directly sown in the soil as seeds where they would develop into plantlets (Mathur *et al.*, 1989). The encapsulation technique has also been employed with nodal segments of yam (Hasan and Takagi, 1995). In a germplasm exchange simulation experiment, encapsulated nodal segments were conserved for two weeks in the dark in cryotubes containing culture medium, without any detrimental effect on their further growth.

6.3. Slow-growth Storage

6.3.1. Classical techniques

Standard culture conditions can be used for medium-term storage with species which have a naturally slow-growing habit only. A wide range of *Coffea* species are thus conserved on standard medium at 27°C without subculturing for durations varying from 6 to 12 months, depending on the species (Bertrand-Desbrunais, 1991). However, for most species, growth reduction is achieved by modifying the environmental conditions and/or the culture medium. The most widely applied technique is temperature reduction, which can be combined with a decrease in light intensity or culture in the dark.

Temperatures in the range of 0–5°C are employed with cold-tolerant species. Strawberry (*Fragaria* × *ananassa*) plantlets have been stored at 4°C in the dark and kept viable for 6 years with the regular addition of a few drops of liquid medium (Mullin and Schlegel, 1976). Apple (*Malus domestica*) and *Prunus* shoots survived 52 weeks at 2°C (Druart, 1985).

Tropical species are often cold-sensitive and have to be stored at higher temperatures, which depend on the cold sensitivity of the species. Kiwi fruit shoots can be conserved at 8°C (Monette, 1986) and taro (*Colocasia esculenta*) tolerates 3 years of storage at 9°C (Staritsky *et al.*, 1986). *Musa in vitro* plantlets can be stored at 15°C without transfer for up to 15 months (Banerjee and De Langhe, 1985). Other tropical species such as oil palm and cassava are much more cold-sensitive: oil palm somatic embryos and plantlets do not withstand even a short exposure to temperatures lower than 18°C (Corbineau *et al.*, 1990). Cassava shoot cultures have to be conserved at temperatures higher than 20°C (Roca *et al.*, 1984).

Various modifications can be made to the culture medium in order to reduce growth. Embryogenic cultures of carrot could be conserved on a medium without sucrose for two years, and reproliferated if a sucrose solution was supplied (Jones, 1974). Kartha *et al.* (1981) could conserve coffee plantlets on a medium devoid of sugar and with only half of the

mineral elements of the standard medium. Replacement of sucrose by ribose allowed the conservation of banana plantlets for 24 months (Ko *et al.*, 1991). The addition of osmotic growth inhibitors (e.g. mannitol) or hormonal growth inhibitors (e.g. abscisic acid) is also employed successfully to reduce growth (Westcott, 1981a,b; Staritsky *et al.*, 1986; Ng and Ng, 1991; Viterbo and Rabinowitch, 1994; Vysotskaya, 1994).

The type of explant as well as its physiological state when entering storage can influence the duration of storage achieved. Roxas *et al.* (1995) indicate that, in the case of chrysanthemum, nodal segments showed higher survival rates than apical buds. The presence of a root system generally increases the storage capacities, as observed by Kartha *et al.* (1981) with coffee plantlets. Microtubers can be successfully employed as storage propagules, as demonstrated with potato (Kwiatowski *et al.*, 1988). Preconditioning the explants by exposing them briefly to temperature and light conditions intermediate between standard and storage conditions was favourable for *Nephrolepsis* and *Cordyline* cultures (Hvoslef-Heide, 1992). Higher survivals were obtained with shoot cultures of wild cherry, chestnut and oak if they were kept for 10 days under standard conditions after the last subculture before their transfer to the cold storage chamber (Janeiro *et al.*, 1995).

The type of culture vessel, its volume as well as the type of closure of the culture vessel can greatly influence the survival of stored cultures (Engelmann, 1991a; Withers, 1992). Roca *et al.* (1984) indicate that cassava shoot cultures could be stored for longer periods in a better condition by increasing the size of the storage containers. White spruce embryogenic tissues withstood a one-year storage period in hermetically sealed serum-capped flasks (Joy *et al.*, 1991). Replacing cotton plugs by polypropylene caps, thus reducing the evaporation of the culture medium, increased the survival rate of *Rauvolfia serpentina* during storage (Sharma and Chandel, 1992). The use of heat-sealable polypropylene bags instead of glass test tubes or plastic boxes was beneficial for the storage of several strawberry varieties (Reed, 1991, 1992).

At the end of a storage period, cultures are transferred onto fresh medium and usually placed for a short period in optimal conditions to stimulate regrowth before entering the next storage cycle.

6.3.2. Alternative techniques

Alternative techniques include modification of the gaseous environment of cultures, and desiccation and/or encapsulation of explants. Growth reduction can be achieved by reducing the quantity of oxygen available to the cultures. The simplest method consists of covering the explants with paraffin, mineral oil or liquid medium. This technique was first developed

by Caplin (1959), who could store carrot calluses for 5 months under a layer of paraffin oil. Augereau *et al.* (1986) and Moriguchi *et al.* (1988) applied this technique to calluses of *Catharanthus* and grape, respectively. Florin (1989) showed that 50% of a collection of 313 callus lines survived after storage under mineral oil for 12 months. However, Mannonen *et al.* (1990) reported that conservation under mineral oil did not preserve the productivity of *Catharanthus* and *Panax ginseng* callus cultures for more than 6 months.

Similar experiments performed with shoot cultures of various species led to contradictory results (Withers and Engelmann, 1997). After 4 months of storage under mineral oil, regrowth of surviving coffee shoot cultures was very slow (Jouve *et al.*, 1991) and pear microcuttings did not survive (Chatti-Dridi, 1988). In contrast, several ginger species could be conserved for up to two years under mineral oil with high viability (Dekkers *et al.*, 1991).

Reduction of the quantity of oxygen can also be achieved by decreasing the atmospheric pressure of the culture chamber or by using a controlled atmosphere. Tobacco and chrysanthemum plantlets could be stored under low atmospheric pressure (with 1.3% oxygen) for 6 weeks (Bridgen and Staby, 1981). Oil palm polyembryogenic cultures were conserved for 4 months at room temperature in a controlled atmosphere with 1% oxygen (Engelmann, 1990). Dorion *et al.* (1994) showed that hypoxic regimes at standard or intermediate temperature could replace low-temperature storage for shoot cultures of peach. Rice calluses could be stored for 12 weeks in a CO_2 or N_2 saturated atmosphere (Watanabe *et al.*, 1991).

Desiccation of cultures as a means of achieving medium-term conservation was first reported by Nitzsche (1980), who stored dehydrated carrot callus for one year at 15 °C under 25% relative humidity. Increasing interest has been paid recently to this technique, with the development of so-called synthetic seeds for various plant species (Gray and Purohit, 1991; Attree and Fowke, 1993). Synthetic seed is a generic term for a somatic embryo delivery system used as a means of clonal propagation (Janick *et al.*, 1993). The aim is to use such somatic embryos as true seeds: embryos, encapsulated or not in alginate gel, could be stored after partial dehydration and sown directly *in vivo*. After progressive dehydration using saturated salt solutions, naked alfalfa somatic embryos could be conserved with 10–15% moisture content at room temperature for one year and showed only a 5% decrease in their conversion rate at the end of the storage period (Senaratna *et al.*, 1990). Carrot somatic embryos were stored for 8 months at 4 °C without viability loss (Lecouteux *et al.*, 1992). Shorter storage durations were achieved with encapsulated material. Encapsulated carrot somatic embryos survived after 3 months in liquid medium at low temperature (Shigeta *et al.*, 1993) and encapsulated shoot tips of *Valeriana wallichii* could be conserved for over 6 months at 4–6 °C (Mathur *et al.*,

1989). According to Redenbaugh *et al.* (1991), the rapid dehydration of the encapsulating matrix limits the respiration of the encapsulated material, which is the cause for the rapid survival loss generally observed.

6.4. Cryopreservation

6.4.1. History

The development of cryopreservation for plant cells and organs has followed the advances made with mammalian species, albeit several decades later. The first report on survival of plant tissues exposed to ultra-low temperatures was made by Sakai in 1960 when he demonstrated that very hardy mulberry twigs could withstand freezing in liquid nitrogen after dehydration mediated by extra-organ freezing. More than a decade later it was shown that cultured cells of flax could resist freezing to −50 °C after pretreatment with dimethylsulphoxide (DMSO) (Quatrano, 1968). This study was followed by a similar one in which cell cultures of carrot were shown to survive after freezing in liquid nitrogen (Latta, 1971). The methodology employed in the above experiments followed the classical procedures which had been successful with other living systems (Ashwood-Smith and Farrant, 1980; Grout and Morris, 1987): chemical cryoprotection, slow dehydrative cooling followed by rapid immersion in liquid nitrogen, storage in liquid nitrogen, rapid thawing, washing and recovery.

The development for plant tissue cultures of so-called classical techniques which took place in the 1970s and 1980s is based on the above sequence of treatments (Kartha, 1985). In recent years, several new cryopreservation techniques have been developed which allow cryopreservation to be applied to a larger range of tissues and organs, in different technical environments (Kartha and Engelmann, 1994; Withers and Engelmann, 1997).

6.4.2. Dehydration and freezing injury

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 damage caused by the crystallization of intracellular water into ice (Meryman, 1966; Mazur, 1969, 1970, 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 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 undergo supercooling; this is followed by ice formation in the medium (Mazur, 1969). The cell membrane acts as a physical barrier and prevents the ice from seeding the cell interior, so 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 extracellular 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. 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. Rewarming should be as rapid as possible to avoid the phenomenon of recrystallization, in which ice melts and re-forms at a thermodynamically favourable, larger and more damaging crystal size (Meryman and Williams, 1985).

New techniques are vitrification-based procedures. In such procedures, the freezable intracellular water is removed prior to freezing by exposing the samples to highly concentrated cryoprotective media and/or air desiccation. Dehydration is followed in most cases by rapid cooling. As a result, the internal solutes vitrify and deleterious intracellular ice formation is avoided. Glass transitions (i.e. 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.*, 1991a; Tannoury *et al.*, 1991; Niino *et al.*, 1992a).

6.4.3. Classical procedures

For each new material to be cryopreserved, optimal conditions have to be defined for each of the successive steps of the protocol.

The physiological state of the material can affect its survival. Cell suspensions are more likely to withstand freezing when they are employed during their exponential growth phase, i.e. when they are small and have a relatively low water content (Withers and Street, 1977). Survival of carnation shoot tips after freezing decreased progressively with their rank on

the shoot apex, starting from the terminal meristem, thus reflecting physiological and developmental differences found in explants sampled on the same plant (Dereuddre *et al.*, 1988). Harding *et al.* (1991) indicated that a long-term period in tissue culture before cryopreservation significantly reduced the ability of potato apices to survive freezing. A pregrowth period before the cryoprotective treatment on medium with osmotically active compounds such as mannitol or sorbitol can increase freeze-tolerance (Seitz, 1987).

Samples are submitted to a cryoprotective treatment before freezing. It is carried out using various cryoprotective substances such as dimethylsulphoxide, sorbitol, mannitol, sucrose or polyethylene glycol. Additional information on the role and mechanisms of action of cryoprotectants can be found in several reviews (e.g. Finkle *et al.*, 1985; Meryman and Williams, 1985; Kartha and Engelmann, 1994). Cryoprotectants are employed alone or in binary or ternary mixtures. Mixtures of cryoprotectants have proved especially effective with cell suspensions (Withers, 1985).

Classical cryopreservation procedures include a two-step freezing: slow, controlled cooling to a defined prefreezing temperature followed by rapid immersion of samples in liquid nitrogen. Freezing rates of between 0.5 and 2°C min^{-1} down to prefreezing temperatures around −40°C generally give satisfactory results in most cases (Dereuddre and Engelmann, 1987). However, while for some materials, such as oil palm somatic embryos, a wide range of freezing rates can be employed without modification of the recovery rate (Engelmann and Dereuddre, 1988), other materials (e.g. grape cell suspensions) require very precise freezing parameters to achieve survival (Dussert *et al.*, 1991). Most classical freezing procedures require the use of expensive programmable freezing devices which achieve precise freezing conditions. However, sophisticated apparatus is not always necessary to obtain high survival. Withers and King (1980) have successfully cryopreserved various cell suspensions with an improvised and simple apparatus that can offer reproducible but nonlinear slow cooling. More recently, several authors have successfully employed domestic or laboratory deep-freezers to perform the slow cooling step (Lecouteux *et al.*, 1991; Sakai *et al.*, 1991; Nishizawa *et al.*, 1992; Engelmann *et al.*, 1994a; Tessereau *et al.*, 1994).

Removal of cryoprotectants by washing after thawing, which was widely adopted in the past, was found to be deleterious for a number of cell cultures. A much less stressing technique for removing cryoprotectants, developed by Chen *et al.* (1984), consists of moving cultures on a filter paper through a series of Petri dishes of solid culture medium. This technique was successfully applied to various cell suspensions and calluses (Kartha and Engelmann, 1994). In some cases, standard culture conditions have to be modified to improve recovery of cryopreserved cultures. Recovery of cell suspensions is generally improved if they are

cultured for some days on solid medium before being transferred to liquid medium (Dussert *et al.*, 1992). Cultures are often placed in the dark or under reduced light intensity to avoid photooxidation phenomena which are harmful to the material (Benson, 1990). Finally, the culture medium can be transitorily altered by modifying its hormonal balance, mineral composition or by incorporating activated charcoal (Engelmann *et al.*, 1985; Kuriyama *et al.*, 1989).

Classical freezing techniques have been assessed with different materials including cell suspensions, calluses, apices and embryos. They have proven to be highly successful with most cell suspensions and calluses, i.e. culture systems which consist of small units of relatively uniform morphology. However, apart from exceptions like carnation apices (Dereuddre *et al.*, 1988), these techniques are not suitable for the cryopreservation of larger units comprising a mixture of cell sizes and types, such as apices and zygotic and somatic embryos.

6.4.4. New techniques

New cryopreservation techniques offer practical advantages in comparison to classical ones (Steponkus *et al.*, 1992; Sakai, 1995). Vitrification-based procedures are operationally less complex than classical ones since they do not require the use of a programmable freezer. In addition, since ice formation is avoided during freezing, they are more adapted for freezing complex organs such as apices or embryos which contain a variety of cell types, each with unique requirements under conditions of freeze-induced dehydration (Withers and Engelmann, 1997). Finally, they have greater potential for broader applicability than classical techniques since only minor modifications are required for various cell types.

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. Table 6.1 presents a list of species to which these new freezing techniques have been applied.

Encapsulation-dehydration

The encapsulation-dehydration technique is based on the technology developed for the production of synthetic seeds where somatic embryos are encapsulated in a bead of hydrosoluble gel (K denbaugh *et al.*, 1991). This technique has been applied mostly to apices of more than ten species of temperate and tropical origin, and to somatic embryos of several crop species as well as to a *Catharanthus* cell suspension (Table 6.1).

Before cryopreservation, specimens can be submitted to conditioning treatments which increase their survival potential. In the case of

Table 6.1. List of plant species for which new cryopreservation techniques (encapsulation-dehydration, vitrification, encapsulation-vitrification, pregrowth-desiccation, pregrowth, desiccation) have been developed using different types of specimens.

Species	*Specimen*	*No. of accessions*	*Technique*	*Reference*
Aesculu hypocastanea	Zygotic embryo	1	Desiccation	Pence, 1990
Allium sativum	Apex	12	Vitrification	Niwata, 1995
Allium wakegi	Apex	7	Vitrification	Kohmura *et al.*, 1994
Arachis hypogaea	Zygotic embryo	6	Desiccation	Runthala *et al.*, 1993
Araucaria excelsa	Zygotic embryo	1	Desiccation	Pritchard and Prendergast, 1986
Armoracia rusticana	Hairy root culture	1	Encaps./dehydr.	Phunchindawan *et al.*, 1994
		1		Hirata *et al.*, 1995
Artocarpus heterophyllus	Zygotic embryo	?	Desiccation	Krishnapillay, 1989
Asparagus officinalis	Stem segment	1	Pregrowth/desicc.	Uragami *et al.*, 1990
	Somatic embryo	1	Vitrification	Uragami *et al.*, 1989
	Cell suspension	1	Vitrification	Uragami *et al.*, 1989
		1		Nishizawa *et al.*, 1993
Baccaurea motleyana	Zygotic embryo	1	Desiccation	Normah and Marzalina, 1995
Baccaurea polyneura	Zygotic embryo	1	Desiccation	Normah and Marzalina, 1995
Beta vulgaris	Apex	2	Encaps./dehydr.	Vandenbussche and De Proft, 1995
Brassica campestris	Cell suspension	1	Vitrification	Langis *et al.*, 1989
Brassica napus	Microspore Embryo	1	Pregrowth/desicc.	Uragami *et al.*, 1993
		1	Encaps/dehyd.r	Uragami *et al.*, 1993
Bromus inermis	Cell suspension	1	Vitrification	Ishikawa *et al.*, 1994
Calamus manan	Zygotic embryo	1	Desiccation	Marzalina *et al.*, 1992

Continued

Table 6.1. Continued

Species	*Specimen*	*No. of accessions*	*Technique*	*Reference*
Camellia sinensis	Apex	12	Vitrification	Kuranuki and Sakai, 1994
	Zygotic embryo	ns	Desiccation	Chaudhury *et al.*, 1991
		1		Wesley-Smith *et al.*, 1992
Capsella bursa-pastoris	Zygotic embryo	1	Pregrowth	Monnier and Leddet, 1978
Carva	Zygotic embryo	1	Desiccation	Pence, 1990
Castanea	Zygotic embryo	1	Desiccation	Pence, 1990
Catharanthus	Cell suspension	1	Encaps./dehydr.	Phunchindawan *et al.*, 1994
Centaurium rigualii	Nodal segment	1	Vitrification	Gonzalez-Benito and Perez, 1994a
Chicorium intybus	Apex	1	Encaps./dehydr.	Vandenbussche *et al.*, 1993
Chrysanthemum morifolium	Apex	1	Vitrification	Schneibel-Preikstas *et al.*, 1992a
Citrus aurantifolia	Zygotic embryo	1	Desiccation	Normah and Nordaini, 1994
Citrus halimii	Zygotic embryo	1	Desiccation	Normah and Hamida, 1992
Citrus mitis	Zygotic embryo	1	Desiccation	Normah and Nordaini, 1994
Citrus sinensis	Nucellar cell suspension	1	Vitrification	Sakai *et al.*, 1990
		4		Sakai *et al.*, 1991
Cocos nucifera	Zygotic embryo	4	Pregrowth/desicc.	Assy-Bah and Engelmann, 1992a,b
Coffea spp.	Zygotic embryo	1	Desiccation	Normah and Vengadasalam, 1992
		3		Abdelnour-Esquivel *et al.*, 1992
		1		Hor *et al.*, 1993
	Apex	2	Encaps./dehydr.	Engelmann *et al.*, 1994b
	Somatic embryo	1		Hatanaka *et al.*, 1994
	Somatic embryo	1	Desiccation; pregrowth/desicc.	Mycock *et al.*, 1995
Corylus avellana	Zygotic embryo	2	Desiccation	Gonzalez-Benito and Perez, 1994b
		1		Normah *et al.*, 1995
		1		Reed *et al.*, 1994

Continued

Table 6.1. Continued

Species	*Specimen*	*No. of accessions*	*Technique*	*Reference*
Cucumis melo	Somatic embryo	1	Pregrowth/desicc.	Shimonishi *et al.*, 1991
Daucus carota	Somatic embryo	1	Encaps./dehydr.	Dereuddre *et al.*, 1991b
Dianthus caryophyllus	Apex	1	Encaps./dehydr.	Tannoury, 1993
	Nodal segment	1	Encaps./dehydr.	Fukai *et al.*, 1994a
		1	Encaps./vitrif.	Tannoury *et al.*, 1991
		1	Vitrification	Langis *et al.*, 1990
Durio zibenthinus	Zygotic embryo	1	Pregrowth/desicc.	Hor *et al.*, 1990
Elaeis guineensis	Polyembryogenic culture	1	Pregrowth/desicc.	Engelmann *et al.*, 1985
		7		Dumet *et al.*, 1993
		75		Dumet, 1994
	Zygotic embryo	1	Desiccation	Grout *et al.*, 1983
		1		Engelmann *et al.*, 1995a
		1	Encaps./dehydr.	Engelmann *et al.*, 1995b
Eucalyptus gunnii	Apex	1	Encaps./dehydr.	Poissonier *et al.*, 1992
Euphoria longan	Zygotic embryo	1	Desiccation	Fu *et al.*, 1990
Fagus	Zygotic embryo	1	Desiccation	Pence, 1990
Fraxinus excelsior	Zygotic embryo	1	Desiccation	Brearley *et al.*, 1995
Hevea brasiliensis	Zygotic embryo	1	Desiccation	Normah *et al.*, 1986
Hopea odorata	Zygotic embryo	1	Desicc./slow cooling	Chai *et al.*, 1994
Howea fosteriana	Zygotic embryo	1	Desiccation	Chin *et al.*, 1988
Ipomea batatas	Apex	1	Vitrification	Schneibel-Preikstas *et al.*, 1992c
	Apex	2		Towill and Jarret, 1992
Juglans regia	Zygotic embryo	1	Desiccation	Pence, 1990
	Somatic embryo	1	Encaps./dehydr.	de Boucaud *et al.*, 1994
Landolphia kirkii	Zygotic embryo	1	Desiccation	Vertucci *et al.*, 1991
Lansium domesticum	Zygotic embryo	1	Desiccation	Normah and Jamilah, 1992
Lilium	Apex	4	Encaps./vitrif.	Sakai and Matsumoto, 1995
		6	Vitrification	Matsumoto *et al.*, 1994a
Livistonia chinensis	Zygotic embryo	1	Desiccation	Normah and Marzalina, 1995

Continued

Table 6.1. Continued

Species	Specimen	No. of accessions	Technique	Reference
Malus spp.	Apex	3	Encaps./dehydr.	Niino and Sakai, 1992
		5		Paul, 1994
		5	Vitrification	Niino *et al.*, 1992a
Manihot esculenta	Zygotic embryo	1	Desiccation	Marin *et al.*, 1990
	Apex	2	Encaps./dehydr.	Benson *et al.*, 1992
		3		Engelmann *et al.*, 1994b
	Somatic embryo	1	Desiccation; pregrowth/desicc.	Mycock *et al.*, 1995
Menta	Apex	1	Vitrification	Towill, 1990
Morus	Apex	1	Encaps./dehydr.	Niino and Sakai, 1992
		13	Vitrification	Niino *et al.*, 1992b
Musa spp.	Zygotic embryo	2	Desiccation	Abdelnour-Esquivel *et al.*, 1992
	Apex	1	Encaps./dehydr.	Panis, 1995
	Meristematic clump	5	Pregrowth	Panis, 1995
Nephelium lappaceum	Zygotic embryo	1	Desiccation	Hor *et al.*, 1990
Nicotiana tabacum	Cell suspension	1	Vitrification	Takano and Tamura, 1992
		1		Reinhoud *et al.*, 1994
Olea europaea	Zygotic embryo	1	Desiccation	Gonzalez-Rio *et al.*, 1994
Oryza sativa	Cell suspension	1	Vitrification	Watanabe and Steponkus, 1994
Panax ginseng	Hairy root culture	1	Vitrification	Yoshimatsu *et al.*, 1994
Phaseolus vulgaris	Zygotic embryo	1	Pregrowth	Zavala and Sussex, 1986
Phoenix dactylifera	Somatic embryo	1	Desiccation; pregrowth/desicc	Mycock *et al.*, 1995
Pisum sativum	Zygotic embryo	1	Pregrowth/desicc.	Mycock *et al.*, 1989
	Somatic embryo	1	Desiccation; pregrowth/desicc.	Mycock *et al.*, 1995
Poncyrus trifoliata	Zygotic embryo	1	Desiccation	Radhamani and Chandel, 1992
Prunus amygdalus	Zygotic embryo	1	Desiccation	Chaudhury and Chandel, 1995
Prunus persica	Zygotic embryo	1	Desiccation	De Boucault and Brison, 1991
Prunus spp.	Apex	2	Vitrification	Brison *et al.*, 1995
Ptychosperma macarthurii	Zygotic embryo	1	Desiccation	Normah and Marzalina, 1995

Continued

Table 6.1. Continued

Species	*Specimen*	*No. of accessions*	*Technique*	*Reference*
Pyrus spp.	Apex	1	Encaps./dehydr.	Dereuddre *et al.*, 1990
		1		Niino and Sakai, 1992
		1		Scottez *et al.*, 1992
		11		Scottez, 1993
		8	Vitrification	Niino *et al.*, 1992a
Quercus	Zygotic embryo	1	Desiccation	Pence, 1990
Ribes	Apex	3	Encaps./dehydr.	Reed and Yu, 1995
		?	Vitrification	Reed, 1992
Saccharum	Apex	7?	Encaps./dehydr.	Paulet *et al.*, 1993
		14		Engelmann *et al.*, 1994b
Secale cereale	Protoplast	1	Vitrification	Langis and Steponkus, 1990
Shorea leprosula	Zygotic embryo	1	Desicc., slow cooling	Chai *et al.*, 1994
		1	Encaps./dehydr.	Engelmann *et al.*, 1995c
Shorea macrophylla	Zygotic embryo	1	Desicc./slow cooling	Marzalina, 1995
Shorea ovalis	Zygotic embryo	1	Desicc./slow cooling	Normah and Marzalina, 1995
Shorea parvifolia	Zygotic embryo	1	Desicc./slow cooling	Normah and Marzalina, 1995
Solanum spp.	Apex	1	Encaps./dehydr.	Fabre and Dereuddre, 1990
		6		Benson *et al.*, 1995
		1	Vitrification	Schneibel-Preikstas *et al.*, 1992c
		5		Lu and Steponkus, 1994
		100	Droplet freezing	Schäfer-Menuhr, 1994, 1995
Swietenia macrophylla	Zygotic embryo	1	Encaps./dehydr.	Marzalina, 1995
Theobroma cacao	Zygotic embryo	1	Pregrowth/desicc.	Pence, 1991
Trifolium repens	Aapex	3	Vitrification	Yamada *et al.*, 1991
Triticum aestivum	Zygotic embryo	1	Pregrowth	Zavala and Sussex, 1986
Veitchia merrillii	Zygotic embryo	1	Desiccation	Chin *et al.*, 1988
Vitis vinifera	Apex	1	Encaps./dehydr.	Plessis *et al.*, 1991
		6		Plessis *et al.*, 1993
Wasabia japonica	Apex	4	Vitrification	Matsumoto *et al.*, 1994b
		5	Encaps./vitrif.	Matsumoto *et al.*, 1995
Zea mays	Zygotic embryo	1	Pregrowth	Delvallée *et al.*, 1989

cold-tolerant species, *in vitro* mother plants or apices can be placed at low temperature for several weeks (Niino and Sakai, 1992; Scottez *et al.*, 1992; Paul, 1994). Mulberry apices are transferred daily on media with progressively increasing sucrose concentrations to initiate dehydration (Niino and Sakai, 1992).

Specimens are usually encapsulated in 3% calcium alginate gel. They are then submitted to the following successive steps: pregrowth, dehydration, freezing, thawing and recovery. Pregrowth is performed in liquid medium enriched with sucrose (0.3 to 1.5 M) for periods varying between 16 hours (Niino and Sakai, 1992) and 10 days (Fabre and Dereuddre, 1990). Replacement of sucrose with other sugars did not improve survival of grape apices (Plessis *et al.*, 1993). Progressive increase in sucrose concentration generally overcomes sensitivity to direct exposure to high sucrose levels which is encountered with some species such as eucalyptus, grape and coffee (Plessis *et al.*, 1991; Poissonier *et al.*, 1992; Engelmann *et al.*, 1994b).

Desiccation is performed using either the air current of a laminar airflow cabinet or silica gel. The latter method is generally preferred because it ensures more precise and reproducible desiccation rates (Paulet *et al.*, 1993). The water content of the beads allowing optimal survival rates is around 20% (fresh weight basis). If pregrowth conditions have been well defined, only limited loss is observed after desiccation with most species. However, banana apices were found to be highly sensitive to both sucrose pregrowth treatment and to even limited desiccation, which induced a drastic survival drop (Panis, 1995).

Desiccated samples are usually frozen by direct immersion in liquid nitrogen. However, modifications in the freezing rate can have different consequences on the survival of different materials. Survival of encapsulated grape apices was higher after slow cooling down to −100°C (Plessis *et al.*, 1991). In contrast, survival of sugar-cane apices was higher after rapid freezing than after slow, controlled cooling (Gonzalez-Arnao *et al.*, 1993a,b). Freezing encapsulated carnation apices at cooling rates of between 0.5 and 200°C min^{-1} had no effect on survival (Tannoury *et al.*, 1994).

Samples are usually stored in liquid nitrogen at −196°C. Scottez (1993) and Tannoury (1993) showed that survival of pear and carnation apices, respectively, was not modified after 2 and 3 years of storage at −196°C. Several authors have demonstrated that samples can be frozen and conserved in deep-freezers provided that the storage temperature is below that of ice recrystallization (−50 to −70°C). Pear apices were conserved at −75°C for one year (Scottez, 1993) and apple, pear and mulberry apices were stored for 5 months at −135°C (Niino and Sakai, 1992).

Samples are usually placed directly under standard conditions for recovery. However, transitorily modified conditions can enhance recovery

in some cases. Sugar-cane apices are placed in the dark for one week on a medium supplemented with growth hormones (Paulet *et al.*, 1993). Addition of the antioxidant ascorbic acid significantly improved the recovery of sugar-beet apices, which are extremely sensitive to oxidation (Vandenbussche and De Proft, 1995). Extraction of apices from the beads was necessary to allow regrowth of pear and grape apices (Plessis *et al.*, 1991; Scottez *et al.*, 1992). Regrowth of material frozen using the encapsulation-dehydration technique is usually direct and rapid, without callus formation. This is due to the fact that, contrary to what is observed after classical freezing, where many cells are destroyed and callusing is frequently observed during recovery, encapsulation-dehydration preserves the structural integrity of most cells. Therefore, regrowth usually originates from the whole meristematic zone, as observed notably in the case of sugar-cane (Gonzalez-Arnao *et al.*, 1993a).

There are presently four crops (pear, apple, sugar-cane and potato) on which the encapsulation-dehydration technique has been successfully extended to several genotypes or varieties (Table 6.1). In all cases, even though genotypic variations were noted, results were sufficiently high to envisage routine application of the cryopreservation protocols developed.

Vitrification

Vitrification involves treatment ('loading') of samples with cryoprotective substances, dehydration with highly concentrated vitrification solutions, rapid freezing and thawing, removal of cryoprotectants ('unloading') and recovery. Vitrification procedures have been developed for more than 20 different species using protoplasts, cell suspensions, apices and somatic embryos (Table 6.1).

The physiological state of the explants can influence their survival potential. In some cases, the plant material is thus submitted to various treatments before the cryopreservation procedure itself. *In vitro* mother plants can be cultured at low temperature for several weeks (Niino *et al.*, 1992a,b). Explants can be placed on a medium with cryoprotectants (Towill, 1990; Brison *et al.*, 1995) and/or cultured at low temperature for a short period (Yamada *et al.*, 1991). Pepó (1994) indicated that axillary shoot tips are much more sensitive to vitrification than apical shoots.

Loading consists of placing the explants in liquid medium containing cryoprotective substances (sucrose, glycerol, ethylene glycol) for a short period, varying between 5 and 90 min, depending on the material. This reduces the sensitivity of the material to the highly concentrated vitrification solutions.

Vitrification solutions are complex mixtures of cryoprotectants which have been formulated for their high ability to vitrify (i.e. form an amorphous glassy structure). Most solutions employed are derived from ones elaborated by either of two groups: that of Sakai's group (Sakai *et al.*, 1990),

which consists of 22% glycerol, 15% ethylene glycol, 15% polypropylene glycol, 7% DMSO and 0.5 M sorbitol; and that of Steponkus' group (Langis *et al.*, 1989), which comprises 40% ethylene glycol, 15% sorbitol and 6% bovine serum albumin. The duration of contact between explants and the vitrification solutions is a critical parameter, in view of their high toxicity. The dehydration period generally increases with the size of the explants used. Rye protoplasts are dehydrated for 60 s only (Langis and Steponkus, 1990), whereas the optimal dehydration duration of apices of pear and apple is 80 min (Niino *et al.*, 1992b).

Performing the dehydration step at 0°C instead of room temperature reduces the toxicity of vitrification solutions and thus broadens the window of exposure durations ensuring survival of samples. This also allows the manipulation of a large number of samples at the same time. Survival of asparagus embryogenic cell suspensions dropped rapidly after 5 min of dehydration at 25°C but high survival was obtained for dehydration periods between 5 and 60 min if it was performed at 0°C (Nishizawa *et al.*, 1993).

Specimens are then cooled rapidly by direct immersion in liquid nitrogen in order to achieve vitrification of internal solutes. Reduction in the volume of cryoprotectants and the use of plastic straws (500 µl–1 ml) which have a small diameter and a large surface area of contact with the exterior allow an increase in the cooling rate, reaching 990°C min^{-1} in the case of asparagus cell suspensions frozen in 50 µl of medium in a 500 µl straw (Uragami *et al.*, 1989). Apices of mint and sweet potato were frozen without cryoprotective medium, thus reaching a cooling rate of 4800°C min^{-1} (Towill, 1990; Towill and Jarret, 1992).

Rewarming of samples is performed as rapidly as possible to avoid devitrification, which would lead to the formation of ice crystals detrimental to cellular integrity. Samples are immersed in a water-bath or liquid medium held at 20–40°C.

Unloading aims at removing progressively the vitrification solution in order to reduce the osmotic shock. Liquid medium containing 1.2 M sucrose or sorbitol is added progressively to dilute the vitrification solution. Explants are then transferred to standard conditions.

Vitrification procedures generally achieve high survival rates. Recovery is usually direct and rapid, even though a few authors have reported callusing and/or abnormal plant development (Towill, 1990; Gonzalez-Benito and Perez, 1994a). Vitrification experiments involving a large range of genotypes have been performed with several species including *Allium*, tea, *Citrus*, apple, mulberry, grape, potato and wasabi (Table 6.1).

Encapsulation-vitrification

This technique is a combination of encapsulation-dehydration and vitrification procedures. Samples are encapsulated in alginate beads, then

subjected to freezing by vitrification. It has been developed by Tannoury *et al.* (1991) with carnation apices, and applied recently by Sakai's group to apices of *Armoracia*, lily and wasabi (Phunchindawan *et al.*, 1994; Matsumoto *et al.*, 1995; Sakai and Matsumoto, 1995).

In the case of carnation, encapsulated apices were pregrown for 16 h with progressively more concentrated sucrose solutions, then incubated for 6 h in a vitrification solution containing ethylene glycol and sucrose, and frozen either rapidly or slowly. Maximum survival was 100% and 92% after rapid and slow cooling, respectively (Tannoury *et al.*, 1991).

The technique employed with wasabi apices was slightly different. Apices were encapsulated in beads containing various loading solutions (glycerol or ethylene glycol and sucrose), then placed in the vitrification solution either at 25°C for 30 min or at 0°C for 70–100 min before rapid freezing. Ninety-five percent of frozen apices recovered growth within 3 days.

Even though this technique has been used with a limited number of species only, it possesses great potential both in terms of efficiency and practicality. Matsumoto *et al.* (1995) mention that the recovery rate of apices frozen using the encapsulation-vitrification technique was 30% higher than with the encapsulation-dehydration technique. A reason for this might be that the alginate capsule reduces the toxicity of the vitrification solution. From a practical point of view, the number of manipulations is reduced as well as the total duration of the procedure.

Desiccation

Desiccation is a very simple procedure since it consists of dehydrating the plant material before rapid freezing by direct immersion in liquid nitrogen. This technique has been applied mainly to zygotic embryos or embryonic axes of various species, including numerous tropical forest trees (see Normah and Marzalina, 1995, for a review), as well as to somatic embryos of several species and to shoot tips of mulberry (Table 6.1).

Various parameters, such as the developmental stage of the embryos at the time of harvest, can greatly influence survival. Mature coffee embryos displayed a higher survival rate than immature ones (Abdelnour-Esquivel *et al.*, 1992). High variability was observed in the survival of embryos of several recalcitrant-seed-producing tree species harvested at different periods (Pence, 1992).

Desiccation is usually performed by placing the embryos in the air current of a laminar airflow cabinet. However, more precise and reproducible desiccation conditions are achieved by placing the embryos in a stream of compressed air (Pammenter *et al.*, 1991) or in an airtight container with silica gel. Optimal survival rates are obtained when the water content of the embryos is around 10–20% (fresh weight basis) (Engelmann, 1992).

Freezing is usually rapid but positive results have been obtained using slow cooling with several tropical forest tree species (Chai *et al.*, 1994; Normah and Marzalina, 1995).

Regrowth of the frozen material usually takes place in standard conditions even though modification of the hormonal balance of the recovery medium proved beneficial with coffee zygotic embryos (Abdelnour-Esquivel *et al.*, 1992; Normah and Vengadasalam, 1992). Direct development of the embryos into plantlets is common but abnormal development patterns have been noted in some cases, such as the nondevelopment of the haustorium in the case of *Howea* and *Veitchia* (Chin *et al.*, 1988), or callusing and/or incomplete development with *Hevea* (Normah *et al.*, 1986), *Castanea* and *Quercus* (Pence, 1992), and oil palm (Engelmann *et al.*, 1995a).

Pregrowth

This technique consists of cultivating the plant material for different durations (hours to weeks, depending on the material) in the presence of cryoprotectants, then freezing rapidly by direct immersion in liquid nitrogen. It was first developed by Monnier and Leddet (1978) with zygotic embryos of *Capsella bursa-pastoris*, then applied to embryos of bean, wheat and maize (Zavala and Sussex, 1986; Delvallée *et al.*, 1989), and more recently to meristematic clumps of banana (Panis, 1995).

Culture for 3 days up to 3 weeks on solid medium with high sucrose concentration (0.3 to 0.9 M) was employed with *Capsella*, maize and banana whereas a short treatment with liquid medium containing polyethylene glycol, glucose and DMSO was sufficient to ensure survival of bean and wheat zygotic embryos.

The pregrowth technique was successfully applied to five banana cultivars, resulting in survival rates ranging between 6 and 42.5% (Panis, 1995). Modifications in the sucrose concentration and the duration of the pregrowth treatment should lead to improvements in the survival rate.

Pregrowth-desiccation

Pregrowth-desiccation is a combination of the two techniques described immediately above. In this technique, samples are treated with cryoprotectants, partially desiccated, cooled and rewarmed rapidly. This technique has been applied to a limited number of specimens only (Table 6.1): somatic embryos of oil palm, date palm, coffee, pea and melon; stem segments of *in vitro* plantlets of asparagus; microspore embryos of rapeseed; and zygotic embryos of coconut.

Culture of samples on media containing cryoprotectants usually takes place before desiccation. However, coconut zygotic embryos are dehydrated before preculture with cryoprotectants (Assy-Bah and Engelmann, 1992a). Sugars (sucrose, glucose) are generally employed for preculture. The duration of the preculture varies between 20 h for coconut (Assy-Bah

and Engelmann, 1992a) and 7 days for oil palm (Dumet *et al.*, 1993).

Various methods are employed for desiccation: coconut embryos are placed in the air current of a laminar airflow cabinet (Assy-Bah and Engelmann, 1992a); asparagus stem segments and oil-palm somatic embryos are dehydrated using silica gel (Uragami *et al.*, 1990; Dumet *et al.*, 1993); melon somatic embryos are placed over a saturated salt solution (Shimonishi *et al.*, 1991); and date palm, coffee and pea somatic embryos are dehydrated using a stream of compressed air (Mycock *et al.*, 1995). The optimal water content (fresh weight basis) varies between 11.8% for melon and 25–30% for oil-palm somatic embryos.

Desiccated samples are frozen rapidly by direct immersion in liquid nitrogen. Specimens are usually stored in liquid nitrogen. However, Dumet *et al.* (1994) have demonstrated that oil-palm somatic embryos could be conserved for 6 months in a deep freezer at −80°C, i.e. below the glass transition temperature, without any modification in their recovery rate.

After rewarming, samples are usually placed directly under standard culture conditions. However, oil-palm somatic embryos are cultured on media with progressively reduced sucrose concentration, with transitory addition of 2,4-dichlorophenoxyacetic acid (2,4-D) to stimulate reproliferation (Dumet *et al.*, 1993).

Recovery of samples is usually rapid and direct. Alterations in the regrowth pattern of cryopreserved specimens were observed with coconut and oilseed rape microspore embryos. The haustorium of cryopreserved coconut embryos browned rapidly and did not develop further (Assy-Bah and Engelmann, 1992a). Only half of frozen rapeseed microspore embryos developed directly into plantlets, whereas the other half produced callus and/or secondary embryos (Uragami *et al.*, 1993).

Pregrowth-desiccation has been tested with four varieties of coconut, giving recovery rates ranging between 33 and 93% (Assy-Bah and Engelmann, 1992a). This technique is routinely employed for the long-term storage of 80 clones of oil-palm somatic embryos (Dumet, 1994).

Droplet freezing

The droplet freezing technique has presently been applied to potato apices only (Schäfer-Menuhr, 1994). Apices are pretreated for 2–3 h in liquid medium supplemented with DMSO, placed on an aluminium foil in 5 µl droplets of cryoprotective medium and frozen rapidly by direct immersion in liquid nitrogen. This procedure is adapted from the classical procedure developed by Kartha *et al.* (1982) with cassava where apices placed in droplets of liquid medium were frozen slowly in a programmable freezer.

The droplet freezing method has been successfully applied to 100 varieties of potato with recovery rates ranging between 5 and 100% (Schäfer-Menuhr, 1995). Around 25 000 apices are stored for the long term in liquid nitrogen at DSM, Braunschweig, Germany.

Genetic stability of in vitro *conserved material*

Genetic conservation is based on the assumption that the material is stored under conditions ensuring genetic stability. However, there are various factors linked with *in vitro* culture/conservation procedures which can be a source of variation. These factors, as well as the various approaches for assessing the genetic stability of plants recovered from *in vitro* culture, have been reviewed recently by Harding (1995). Genetic variation can pre-exist in the collected material, possibly linked to its genetic structure, such as in sugar-cane or banana where polyploids are more prone to instability than diploids. It can also arise from somaclonal variation, particularly if the propagation system includes a dedifferentiated phase or from stresses imposed by the conservation procedures. The information available on the genetic stability of plant material conserved *in vitro* is presented below.

Slow growth

Different genotypes do not grow at the same rate when placed in culture. This induces a risk of selection when plants are placed under stress conditions, such as slow growth. It is thus important to minimize the risk of selection. One of the most efficient ways is to use, whenever possible, differentiated, organized structures such as shoot tips or embryos for which the risks of variation are minimal (D'Amato, 1985).

A limited number of experiments only have been performed to assess the genetic stability of plants during slow-growth storage. The most exhaustive study has been performed in the framework of a joint project between CIAT and IPGRI which aimed at monitoring the genetic stability of cassava shoot cultures stored *in vitro* (CIAT-IPGRI, 1994). Isozyme and DNA analysis as well as examination of the morphology of plants regrown in the field did not reveal any modification after 10 years of storage under slow growth. In contrast, growth of potato shoot tips on mannitol-supplemented medium, which caused morphological changes in the material, was correlated with DNA hypermethylation, which may be an adaptive response to conditions of high osmotic stress (Harding, 1994). This clearly illustrates the importance of defining storage conditions which are likely to cause as little stress as possible to the plant material.

Cryopreservation

Cryopreservation involves a series of stresses which might lead to modifications in the recovered cultures and regenerated plants. It is therefore necessary to verify that the genetic stability of the cryopreserved material is not altered before using this technique routinely for the long-term storage of germplasm. There is now increasing evidence that, provided the cryopreservation technique applied ensures the greatest possible maintenance of the integrity of the frozen specimen, there will be no modification at the

phenotypic, biochemical, chromosomal or molecular level attributable to cryopreservation; none has been reported to date.

It has been shown that cell suspensions of numerous species maintain their biosynthetic and morphogenic potential after cryopreservation (Withers, 1985; Kartha, 1987; Seitz, 1987). The only exception concerns lavender cell suspensions exposed to successive freeze-thaw cycles, for which the number of colonies recovered from cryopreserved cells increased with the number of freeze-thaw cycles (Watanabe *et al.*, 1985). However, no modifications were noted in the biosynthetic capacities of cryopreserved cells, suggesting a change in population structure rather than genetic change. A similar observation was made by Bercetche *et al.* (1990), who noted that cryopreserved *Picea abies* embryogenic calluses recovered faster than nonfrozen controls; nonembryogenic tissues were killed by freezing, thus leading to the production of a more homogeneous population, with a higher embryogenic potential. This suggests that cryopreservation could be used as a tool to 'rejuvenate' cultures when their proliferation capacities are decreasing.

Plants regenerated from cryopreserved apices of strawberry and cassava were phenotypically normal (Kartha *et al.*, 1980; Bajaj, 1983). No differences were noted in the vegetative and floral development of several hundreds of oil palms regenerated from control and cryopreserved somatic embryos (Engelmann, 1991a). In contrast, Fukai *et al.* (1994b) showed that a high percentage of plants regenerated from apices of a periclinally chimeric chrysanthemum cultivar frozen using a classical protocol had an altered flower colour. These apices had been severely harmed during freezing, leading to callusing during recovery. Regenerated plants had thus an adventitious origin which explained the disturbance in the initial chimeric structure. The same group compared the effect of freezing carnation apices using a classical protocol and the encapsulation-dehydration technique (Fukai *et al.*, 1994a). Encapsulation-dehydration ensured 100% recovery after freezing, and regrowth was rapid and direct. In contrast, after slow, controlled freezing, the recovery rate was 50% only, and callusing was observed during regrowth. The two above examples underline the importance of selecting the most appropriate freezing technique for any given material, not only as regards recovery rates but also as regards recovery pattern and genetic stability.

Electrophoretic profiles of two enzymatic systems were comparable in plants regenerated from control and cryopreserved apices of sugar-cane (Paulet *et al.*, 1994) and sweet orange somatic embryos (Marin *et al.*, 1993). The ploidy level of plants regenerated from oilseed rape somatic embryos and sensitive dihaploids of potato was not modified by cryopreservation (Uragami *et al.*, 1993; Ward *et al.*, 1993). Restriction fragment length polymorphism (RFLP) patterns of plants regenerated from sugar-cane embryogenic cell suspensions were identical to those of unfrozen controls

(Chowdhury and Vasil, 1993). Several molecular types were uncovered in plants recovered from both control and cryopreserved sugar-cane apices, thus indicating that the variation was not due to freezing but was pre-existing among the *in vitro* mother plants (Glaszmann *et al.*, 1996).

Finally, DNA analysis showed that a gene integrated in the genome of a navel orange cell suspension was maintained after freezing and one-year storage in liquid nitrogen (Kobayashi *et al.*, 1994).

Comparative studies

A limited amount of work has been published on the comparative effects of slow growth and cryogenic storage on the stability of plant material. Mannonen *et al.* (1990) showed that the production of secondary metabolites by cell cultures of *Panax ginseng* and *Catharanthus roseus* decreased drastically after 6 months of culture either in standard conditions (i.e. with weekly subcultures) or in slow growth under mineral oil, whereas the productivity of cell suspensions cryopreserved and stored in liquid nitrogen during the same period was identical to that of the original cultures.

Modifications in the RFLP pattern were observed in potato plants stored for 6 months under slow growth on a medium supplemented with mannitol, whereas no such modifications were noted in plants regenerated from cryopreserved apices (Harding, 1991).

6.5. Present use of *in vitro* conservation techniques

Classical *in vitro* conservation techniques have been developed for a wide range of species, including temperate woody plants (Aitken-Christie and Singh, 1987), fruit trees (Withers, 1992), horticultural species (Engelmann, 1991b), as well as numerous tropical species (Dodds, 1991; Engelmann, 1991a; Zakri *et al.*, 1991).

However, a recent FAO survey (Anonymous, 1994) indicated that only 37 600 accessions are conserved *in vitro* worldwide. Slow-growth conservation is used routinely for the conservation of only a few species, including banana, potato and cassava, in regional and international germplasm conservation centres such as INIBAP, CIP, CIAT and IITA.

Alternative medium-term conservation techniques are still at the experimental stage. Low-oxygen storage might be interesting for storing cold-sensitive tropical species since it allows growth reduction without decreasing the temperature. However, complementary experiments involving additional species and longer storage periods are still required.

Medium-term storage of desiccated somatic embryos will be mainly applicable for the management of large-scale production of elite genotypes. Encapsulated apices might be employed for medium-term genetic

resources conservation. Research is still needed to refine the protocols and extend the storage periods.

Cryopreservation procedures have been developed for around 100 different plant species cultured in various ways including cell suspensions, calluses, apices, and zygotic and somatic embryos (Withers, 1992; Kartha and Engelmann, 1994; Engelmann *et al.*, 1995b; Withers and Engelmann, 1997). Most of this work has been performed in the framework of academic studies and has involved only one or a few genotypes. However, there are recent reports of large-scale experimentation of classical freezing techniques involving cell suspensions from several hundred genotypes (Cyr *et al.*, 1994; Park *et al.*, 1994). In the case of organized structures such as apices or embryos, the development of new cryopreservation procedures has also allowed experiments involving a relatively wide range of genotypes/varieties (Fukai *et al.*, 1991a,b). In the case of potato, apices from more than 100 different varieties have been successfully frozen and around 25 000 apices are presently stored in liquid nitrogen (Schäfer-Menuhr, 1994).

There is an increasing number of cases where cryopreservation techniques can be considered operational. However, their routine application is mostly restricted to the conservation of cell lines in research laboratories (Withers, 1985). The only example of routine application of cryopreservation to another type of material is oil palm, where 80 clones of somatic embryos are stored in liquid nitrogen and samples thawed upon request for plant production (Dumet, 1994).

6.6. Conclusion

Significant advances have been made during the past decade in the development of *in vitro* techniques for the conservation of plant genetic resources. *In vitro* collecting, slow-growth and cryopreservation procedures are now available for a wide range of plant species. There is an increasing number of cases where slow growth and cryopreservation could be used to improve the conservation of genetic resources of problem species. Obviously, slow-growth techniques are in a more advanced state of development and should become more widely applied once their flexibility, simplicity and practicality are clearly demonstrated.

For most undifferentiated cultures, such as cell suspensions and calluses, cryopreservation procedures can be set up rapidly with only slight adaptations of already published procedures. For differentiated materials such as embryos or apices, even though the situation has considerably improved with the emergence of new (vitrification-based) freezing procedures in recent years, the development of a cryopreservation protocol for any new material still requires considerable inputs. However, it is time to

move the experimentation from the research laboratory to the genebank, or at least to research units which have a conservation mandate. This will allow the development of methodologies which meet the criteria required in the genebank context and the incorporation into *in vitro* conservation research and development the knowledge of the genebank staff in relation to genetic stability and genetic characterization (Withers and Engelmann, 1997).

Finally, it is important to stress that *in vitro* conservation should not be seen as a replacement for conventional *in situ* and *ex situ* approaches. *In vitro* conservation offers genebank curators a set of additional tools to allow them to improve the conservation of germplasm collections for which they are responsible.

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