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In Vitro Conservation of Plant Genetic Resources

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I. INTRODUCTION

The conservation of biodiversity is widely recognized as a high priority area for attention in the ongoing debate linking environment and development [1,2]. In particular, the conservation of plant genetic resources for food and agriculture, one sector of biodiversity, is considered to be a major element of any strategy to achieve sustainable agricultural development, along with the conservation of other natural resources. Diverse conservation methods are pursued according to the situation at hand. These methods can be divided broadly into ex situ and in situ. The latter cover conservation in wilderness areas, reserves, protected areas, and within traditional farming systems (so-called, on-farm conservation). Ex situ conservation involves removing the plant genetic resources from their natural habitat and placing them under artificial storage conditions. The following sections examine the different ex situ options available.

A. Classic Approaches to Ex Situ Conservation

The most familiar approach to ex situ conservation is seed storage. A large proportion of agricultural crops produce seeds that can be dried to a sufficiently low moisture content that they can be stored at low temperatures. There is an interaction between moisture content, temperature, and survival in storage and longevity, so that drying to lower moisture contents permits storage at relatively higher temperatures [3]. This principle underlies current research into ultradry seed storage that should greatly reduce the constraints imposed by difficulties in maintaining sufficiently cold seed stores [4,5]. This can be an especially serious problem in developing countries and, worldwide, is a factor in the cost of operating seed stores. Nevertheless, for crops that produce seeds amenable to drying and cold storage (i.e., "orthodox" seeds), this approach to conservation is convenient, is easily adopted, and is secure. Its drawbacks relate to biological, rather than practical, features that prevent its wider application beyond orthodox seeds.

Three categories of crop present problems for seed storage. First, there are those that do not produce seeds at all, and are propagated vegetatively, for example, banana and plantain (*Musa* spp.). Second, there are crops, including potato (*Solanum tuberosum*); other root and tuber crops, such as yams (*Dioscorea* spp.), cassava (*Manihot esculenta*), and sweet potato (*Ipomoea batatas*); and sugarcane (*Saccharum* spp.), that have some sterile genotypes and some that produce orthodox seed. However, similar to temperate fruits, including apple (*Malus* spp.), these seeds are highly heterozygous and, therefore, of limited usefulness for the conservation of gene combinations. These crops are usually propagated vegetatively to maintain clonal genotypes [7]. Then, third, there are those crops that produce what are known as "recalcitrant" seeds. Several tropical fruits and timber species fall into this category, including coconut (*Cocos nucifera*), avocado (*Persea americana*), mango (*Mangifera indica*), cacao (*Theobroma cacao*), and members of the Dipterocarpaceae family [8–10]. Recalcitrant seeds cannot tolerate desiccation to moisture contents that would permit exposure to low temperatures. They are often large, with considerable quantities of fleshy endosperm. Although there are clear groups of species that can be classified categorically as orthodox or recalcitrant, there are also intermediate types for which seed storage is problematic [5,6].

Traditionally, the field genebank has been the ex situ storage method of choice for these problem materials. In some ways, it offers a satisfactory approach to conservation. The genetic resources under conservation are readily accessed and observed, permitting detailed evaluation. However, there are certain drawbacks that limit its efficiency and threaten its security [11,12]. The genetic resources are exposed to pests, diseases, and other natural hazards, such as drought, weather damage, human error, and vandalism. Nor are they in a condition that is readily conducive to germplasm exchange. Field gene banks are costly to maintain and, as a consequence, are prone to economic decisions that may limit the level of replication of accessions, the quality of maintenance, and even their very survival in times of economic stringency. Even under the best circumstances, field genebanks require considerable input in the form of land (often needing multiple sites to permit rotation), labor, management, and materials.

In the light of the problems presented by the three categories of crops outlined in the foregoing, it is not surprising that efforts have been made to improve the quality and security of conservation offered by field gene banks, and to understand and overcome seed recalcitrance to make seed storage more widely available. However, it is clear that alternative approaches to genetic conservation are needed for these problem materials and, since the early 1970s, attention has turned to the possibilities offered by biotechnology, specifically in vitro or tissue culture [13–19].

B. New Approaches to Conservation

In vitro techniques feature in the conservation strategies of animals, microbes, and plants [19–21], but it is probably fair to say that the potential for exploiting these techniques and integrating them into wider practices, including genetic improvement, is greatest for higher plants. This relates largely to the ease with which plant material can be manipulated in vitro, in particular the phenomenon of totipotency (capacity to regenerate whole plants from single cells). Early efforts in the development of new approaches to conservation focused on storage *per se*, but applications of biotechnology have been demonstrated in all aspects of conservation and use, from germ plasm collecting and exchange, to multiplication, disease indexing and eradication, characterization and evaluation, storage, stability monitoring, distribution, and utilization [19]. The main body of this chapter will deal with collecting and storage, the latter involving slow-growth storage for short- to medium-term conservation, and cryopreservation

for the long-term. However, before so doing, it is instructive to explore the broader context of conservation and use of plant genetic resources.

The interdependence of nations and regions of the world in terms of access to genetic resources [22,23] reveals a crucial role for any means that can be used to facilitate the collecting and exchange of germ plasm. However, there is an important rider to place on that; the facilitation of germ plasm exchange should not lead to any increased risk of exchanging pests and pathogens. Fortunately, biotechnology in the form of in vitro and biochemical or molecular techniques can offer ways of both eradicating and indexing for diseases. Typical illustrative examples can be found in the root and tuber crops, such as potato, sweet potato, and cassava [24,25]. Because of the predominant use of vegetative propagation techniques in these crops, there is a tendency to accumulate pathogens through successive clonal generations without the "filter" provided by seed production. Meristem-tip culture used alone or in combination with thermotherapy can effectively eliminate viral pathogens, and a combination of symptomatology, the use of grafting or inoculation onto indicator plants, enzyme-linked immunosorbent assay (ELISA), and molecular techniques, such as double-stranded (ds)RNA detection can confirm the success or otherwise of eradication [e.g., 26,27]. Precise details should be sought on a species-by-species basis from the wider literature. Overall, an important caution to apply in this area is that transfer to in vitro culture does not confer disease-free status. Indexing is the only sure way of making this judgment.

One of the most important aspects of in vitro culture, particularly for the problem crops identified earlier, is mass propagation. In vitro propagation not only facilitates the agricultural production of the crop [e.g., 28–30], but also underpins the use of all other biotechnologies in conservation and use. The advantages of being able to multiply a given genotype with relative ease, with a low risk of introducing or reintroducing pathogens, and with a low risk of genetic instability, need not be emphasized. However, the latter point of genetic instability bears further examination. Genetic stability in culture is not a given. There are clear links between the culture system in use and risk of instability through somaclonal variation [31,32]. As a broad generalization in the context of genetic conservation, the more instability-prone culture systems, such as protoplast and cell cultures, should be avoided in favor of more highly organized systems, such as shoot cultures. Somatic embryos present some attractive options here, being both relatively amenable to storage by cryopreservation and manipulable as synthetic seeds [33]. Genetic stability under conditions of in vitro conservation is dealt with later, as is the amenability of different culture systems to in vitro storage.

The application of biotechnological approaches to the genetic improvement of plants is the subject of many other sections of this book and need not be expanded on here. Suffice it to say that the generation of an awareness of the scope for applying new techniques for conservation as well as those for the use of plant genetic resources among practitioners of the respective techniques can only be beneficial. Molecular technologies based on DNA extraction and storage offer new ways of conserving as well as of using genetic information [34–36]. Although routine DNA storage for genetic conservation may be some way off in the future, it is possible to envisage applications for the storage of specific gene sequences within a broad complementary conservation strategy. Similarly, in vitro conservation techniques can facilitate the application of genetic manipulation procedures by, for example, providing a simple way of storing experimental material in the form of in vitro cultures. More importantly, perhaps, new storage techniques can relieve the burden placed on all in vitro-based procedures imposed by the need to maintain stock cultures. Applications can be envisaged in aspects ranging from basic physiological studies through to secondary product synthesis on an industrial scale, with potential savings on costs, and reduced risks of loss through human error and genetic instability.

II. COLLECTING AND EXCHANGE OF GERM PLASM

A. Problems with Conventional Methods

The problems outlined in the previous sections for the conservation of genetic resources particular crops are mirrored, and sometimes amplified, in their collecting and exchang Looking first at clonally propagated crops, the material of choice for collecting is often vegetative propagule, such as a stake, piece of bud-wood, a tuber, corm, or sucker. Only some cases are these materials adapted to survival once excised from the parent plant an almost invariably, they present a plant health risk owing to their vegetative nature a contamination with soilborne organisms [see Refs. 37,38]. The collector can compensate f these problems, to some extent, by good planning, careful selection of material, and observ tion of basic plant health precautions. Nevertheless, fundamental and unavoidable risks remai

For recalcitrant seeds, there is a dual problem. Not only are they prone to microbial attac or deterioration if exposed to unsuitable environmental conditions, or if held too long in trans to the gene bank [see Ref. 39], there are also very limited options in how to handle them on they do arrive at their destination. Seed storage under conventional conditions is not availabl and successful germination to produce a seedling for the field gene bank will require hig quality seed. A further practical problem encountered with many recalcitrant seeds is the sheer weight and bulk. If a satisfactory population sample is to be gathered, this can represent dauntingly large mass of material to transport, with attendant high costs.

Collecting the germ plasm of orthodox, seed-producing species can also be problemati Sometimes the window of opportunity for a collecting mission does not coincide with the ide stage of development of the plant. The material available for collecting may be sparse, imm ture, past its optimal state of maturity, shed from the plant, or even eaten by grazing anima [38,40,41]. Collecting expeditions in general are a logistic challenge, but when they invol exploration in remote or politically sensitive areas, or when there are factors, such as climat aberrations, to contend with, the challenge is increased and the collector needs to keep open many options as possible. In this context, the adaptation of in vitro techniques to the collecti environment, as described in the next section, illustrates one of the simplest, but most effectiv applications of biotechnology to plant genetic resources work.

With the exception of timing, where the curator has more control over the deciding when exchange material, all of the comments made earlier in relation to problems in collecting ger plasm apply to its exchange. Therefore, there is scope for using techniques that help mainta germ plasm in as healthy a general condition as possible and reduce the risk of introduci pathogens.

B. In Vitro Field Collecting

Some sporadic work had been carried out in the past on developing in vitro collecting metho but the first coherent and comprehensive examination of its potential was made by the Intern tional Board for Plant Genetic Resources (IBPGR). This took the form of a meeting of scie tists with expertise in specific crops and in vitro techniques in general [41], followed by spo sorship and encouragement of focused research [42–45]. Four crop models serve to illustra the general potential and flexibility of in vitro collecting. These are coconut, cacao, fora grasses, and *Musa* spp.

The problems inherent in collecting coconut germ plasm are obvious. The species produc large, recalcitrant seeds. However, the key to finding a solution lies in recognizing that on the small embryo is needed to propagate a coconut palm, given adequate handling technique

Research over the last 8 years has demonstrated not only the feasibility of collecting isolated embryos, but also the great flexibility that can be exercised within the basic concept.

Research by Assy-Bah and colleagues [42,43] tackled the field-collecting technique itself and the follow-up procedures needed to nurture the embryo and generate an independently growing plant. The principle used in the field collecting was the idea that, with minimal, but dexterous aseptic precautions, embryos could be isolated from nuts in the field, surface sterilized, and then either dissected at the field location or transported in endosperm plugs held in sterile salt solution for subsequent dissection in the laboratory. The basic principle demonstrated by this research is taken in the direction of greater complexity by Sossou et al. [46], who closely simulated laboratory conditions in the field, and in the direction of greater simplicity by Rillo and colleagues [47,48]. Table 1 provides details of the different approaches that have been taken with coconut and the other model crops examined herein.

The success that has been demonstrated with coconut can be repeated with other recalcitrant seeds that are physiologically similar but structurally very different, such as cacao, avocado, and *Citrus* spp. Embryos of these species have fleshy cotyledons that can be dissected away to reveal the embryo axis. In these cases, it is easier to keep the embryo axis sterile, and it can suffice to flame sterilize the fruit and, by using frequently sterilized instruments, maintain the inherent sterility of the interior of the seed [49].

A very different challenge is presented by collecting vegetative tissues. Within this broad category are a wide variety of types of material, woody and herbaceous, more or less succulent, and with great structural variations. One example presenting serious collecting problems is cacao. The seeds of cacao are recalcitrant. Accordingly, bud wood is often the target for collecting. Yet this is vulnerable owing to the potentially long transit periods involved in collecting from, say, the Amazon basin, which is the center of origin of cacao and a valuable source of genetic resources (39).

Experimentation to develop an in vitro collecting method for cacao bud wood sought to minimize the materials and equipment to be carried to the collecting site. It was based on the premise that absolute sterility would be difficult to achieve in the field and would not necessarily be essential for robust, woody material. The technique eventually developed for cacao is detailed in Table 1 [50–55]. It involves the use of drinking water-sterilizing tablets and culture medium supplemented with fungicides. Antibiotics can also be used, but this must be weighed against the inherent disadvantages in their use from the point of view of hazards to the user and the maintenance of low levels of persistent infection [52,53]. The general approach used for cacao has been used successfully for other similar materials, such as woody shoots of coffee (*Coffea* spp.), *Prunus* spp., and grape *Vitis* spp. [49,54].

Forage grasses present problems in their collecting because often the only material available is the herbaceous shoot or tiller. Ruredzo [44] developed a successful procedure for collecting such material as detailed in Table 1. This was used to acquire material for the forage germ plasm collection at the International Livestock Centre for Africa (ILCA).

The final example of stem tissue to be considered is exemplified by *Musa* spp. that is collected in the form of stem suckers, which are large, fleshy, and likely to be covered in soil. The strategy that has proved successful with this material involves surface sterilization, combined with extensive dissection to reach naturally aseptic inner tissues. A sucker of some 30×8 cm is reduced to a shoot tip less than 1×1 cm [49,55].

These examples illustrate the flexibility of in vitro collecting. There is no one formula to be followed, nor need there be. The approach to be taken should be based on prior knowledge of the requirements of the species and explant in question, combined with the collective experience gained with diverse species in different collecting environments. As in any germ plasm transfer operation, particular attention should be given to phytosanitary considerations. In vitro

Table 1 Summary of Conditions Used in the In Vitro Collecting of Diverse Specimens

Species	Explant	Surface sterilization	Initial handling	Laboratory treatment	Re
Coconut (*Cocos nucifera*)	Embryo in endosperm plug, extracted with cork borer	Calcium hypochlorite at 45 gL^{-1}	Endosperm plug inoculated into sterile solution of KCl at 16.2 gL^{-1}	Repeat sterilization if necessary. Embryo dissected, inoculated onto semisolid medium, cultured under standard conditions, transferred to the nursery.	42
Coconut (*Cocos nucifera*)	Embryo in endosperm plug, extracted with cork borer	Commercial bleach (8%Cl)	Embryo dissected at field work bench and inoculated onto semisolid medium	Embryo cultured under standard conditions, transferred to the nursery.	50
Coconut (*Cocos nucifera*)	Embryo in endosperm plug, extracted with cork borer	None	Endosperm plugs placed in a bag of freshly gathered coconut milk, held in a cool box	Endosperm plug surface sterilized, embryo dissected and inoculated onto standard culture medium	47
Coconut (*Cocos nucifera*)	Embryo in endosperm plug, extracted with cork borer	Inoculation is carried out in an inflatable glove box sterilized with alcohol. Sterilization with 5% calcium hypochlorite for 15–20 min. Embryo is then excised and sterilized with 2% calcium hypochlorite for 2–5 min, followed by washing in sterile water.	Embryos are placed on standard culture medium in screw-top flasks	Standard culture procedure	46
Cacao (*Theobroma cacao*)	Stem nodal cutting	Drinking water sterilizing tablets containing "Halozone" (*p*-carboxybenzenesulfondichloroamide),4 mg/tablet: 10 tablets dissolved in 100 mL of boiled water, plus 0.05% FBC protectant fungicide	Inoculation onto semisolid medium containing fungicide Tilt MBC at 0.1% with or without antibiotics rifamycin at 30 mgL^{-1} and trimethoprim at 30 mgL^{-1}	Continued culture or resterilization using standard treatments, or grafting	45

Table 1 Continued

Species	Explant	Surface sterilization	Initial handling	Laboratory treatment	Ref.
Digitaria decumbens (forage grass)	Stem cutting	Drinking water-sterilizing tablets containing "Halozonc" (*p*-carboxybenzene-sulfondichloroamide) at 1 g tablets/L of boiled water	Inoculation onto culture medium containing 1.5 gL^{-1} benlate and 0.1 mgL^{-1} rifamycin	Standard culture conditions; transfer to soil after 14 weeks	44
Cotton (*Gossypium* sp.)	Stem nodal cutting	20% commercial bleach in 30% ethanol for 45 s; no washing	Inoculation onto solid culture medium containing half-strength salts, 1% glucose, antibiotics rifamycin at 15 mgL^{-1} and trimethoprim at 15 mgL^{-1}, fungicide Tilt MBC at 1 gL^{-1}, NAA at 1 mgL^{-1} and casein hydrolysate at 0.5 gL^{-1}	Resterilize with 4% bleach, treat with rooting hormone and plant in soil/sand/vermiculite mix with lime and slow-release nutrients	51

collected explants should be treated with the same care and observance of regulations as any other type of collected material.

C. In Vitro Germ Plasm Exchange

In vitro techniques have been used widely for several years for the international distribution of plant genetic resources. Notable examples that have been tried and tested, yielding routine procedures include potato, cassava, yam, and *Musa* spp. The techniques used are based on standard mass propagation procedures, with minor but important modifications of detail to increase structural stability in transit. For example, the concentration of agar or other gelling medium used for preparing the culture medium may be raised to increase its firmness. Also, plantlets may be transferred in sterile heat-sealable polyethylene bags, rather than the more fragile glass or plastic containers [56,57]. For species that produce storage propagules, such as stem tubers, capable of regenerating plants, a further option is available. This approach has been used successfully in potato and yam, the tubers being more resilient with the result of producing a more robust system for germ plasm exchange [58].

III. SLOW-GROWTH STORAGE

A. Classic Techniques

Standard culture conditions can be used only for medium-term storage of naturally slowly growing species. For example, plantlets of *Coffea arabica* can be conserved on standard

medium at 27°C for 1 year without subculturing [59]. However, such examples are in the minority. Accordingly, techniques have been developed for reducing the growth rate of cultures. Classic slow-growth storage techniques involve modification of the physical environmental conditions or culture medium, or both. The most successful and widely applied technique is temperature reduction. A decrease in light intensity or culture in the dark is often used in combination with temperature reduction. Strawberry (*Fragaria* × *ananassa*) plantlets have been conserved in the dark at 4°C [59]. Regular addition of a few drops of liquid medium to the cultures maintained the plantlets viable for up to 6 years.

Apple (*Malus domestica*) and *Prunus* shoots survived 52 weeks at 2°C [61]. Temperatures in the range of 0°–5°C can be employed with cold-tolerant species, but higher temperatures have to be used with tropical species that are often cold-sensitive. Roca et al. [62] indicated that cassava shoot cultures have to be stored at temperatures higher than 20°C. Oil palm (*Elaeis guineensis* Jacq.) somatic embryos and plantlets cannot withstand even short-term exposure to temperatures lower than 18°C [63]. In contrast, banana in vitro plantlets can be stored at 15°C without transfer for up to 15 months [64,65].

It is also possible to limit growth by modifying the culture medium. Reduction in the concentration of mineral elements and elimination of sugar allowed the conservation of *C. arabica* plantlets for 2 years [66]. Addition of osmotic growth inhibitors (e.g., mannitol) or hormonal growth inhibitors (e.g., abscisic acid) is also an efficient way to achieve growth reduction [67–72].

The type of culture vessel, its volume and the volume of medium, and the closure of the culture vessel influence the survival of stored cultures [18,73]. Roca et al. [62] indicated that storing cassava shoot cultures in larger vessels improved their condition and maintenance of viability during storage. Replacing cotton plugs by polypropylene caps, thereby reducing the evaporation rate of the culture medium, increased the survival of *Rauvolfia serpentina* during storage [74]. As well as standard glass and plastic vessels, the use of heat-sealable polypropylene bags has been reported [57].

At the end of a storage period, cultures are usually transferred onto fresh medium and placed in optimal culture conditions for a short period to stimulate regrowth before entering the next storage cycle. (But note treatment of strawberry plantlets discussed earlier [60]).

B. Alternative Techniques

Alternative slow-growth techniques include modification of the gaseous environment and desiccation or encapsulation of explants. Growth reduction can be achieved by lowering the quantity of oxygen available to the cultures. The simplest method consists of covering the tissues with paraffin oil, mineral oil, or liquid medium. This technique was first developed by Caplin [75], who stored carrot (*Daucus carota*) callus under paraffin oil for 5 months. It was employed more recently by Augereau et al. [76] with *Catharanthus* calluses and by Moriguchi et al. [77] with grape calluses. Florin [78] showed that 86 and 50% of a collection of 313 different callus lines could be stored with the same technique for 6 and 12 months, respectively. Similarly, 13 of 20 cell suspensions from eight different species survived after 6 months of storage under liquid medium without shaking (78).

Attempts to store microcuttings under mineral oil have been performed with pear (*Pyrus communis*) [79], coffee [80], and several ginger (*Zingiber officinale*) genotypes [81]. Growth reduction was achieved in all cases, but hyperhydration of explants was often observed during storage. After return to standard conditions following 4 months in storage, regrowth of surviv-

ing cultures was very slow for coffee, and partial or complete necrosis of explants was noted with pear. However, this storage technique was very efficient with some ginger genotypes, which could be conserved under mineral oil with high viability for up to 2 years [81].

Reduction in the level of available oxygen can also be achieved by decreasing the atmospheric pressure of the culture chamber or by using a controlled atmosphere. Tobacco (*Nicotiana tabacum*) and chrysanthemum (*Chrysanthemum morifolium*) plantlets were stored under low atmospheric pressure (with 1.3% O_2) for 6 weeks [82]. Oil palm somatic embryos could be conserved for 4 months at room temperature in a controlled atmosphere with 1% O_2, and proliferated rapidly after subsequent transfer to standard conditions [83].

Desiccation as a means of storage of embryogenic cultures was first described by Jones [84]. Embryogenic cultures of carrot were left on semisolid medium for up to 2 years at 25°C. Supply of a sucrose solution to the cultures resulted in "germination" of the embryos that, on planting out, produced healthy individual plants. More recently, Senaratna et al. [85] have shown that alfalfa (*Medicago sativa*) somatic embryos, dehydrated progressively using saturated salt solutions, could be conserved with 10–15% moisture content for 1 year at room temperature. They displayed only a 5% decrease in their conversion rate after storage. Similarly, Lecouteux et al. [86] stored carrot somatic embryos for 8 months at 4°C without viability loss.

Large-scale propagation by means of somatic embryogenesis is being developed for elite genotypes of numerous crop species, leading to the production of large numbers of synchronously developing embryos. These embryos can be encapsulated in a bead of gel (e.g., calcium alginate), containing nutrients and fungicides, thereby forming synthetic seeds which, theoretically, can be stored and sown directly in vivo similar to true seeds. The production of synthetic seeds has been developed for many plant species [33,87,88]. The application of synthetic seed technology to somatic embryos or shoot tips also appears of interest in a germ plasm conservation context. However, only a limited number of short- to medium-term storage experiments have been performed with encapsulated material (but see Section IV for long-term storage by cryopreservation).

Encapsulated axillary buds of mulberry (*Morus indica* L.) and somatic embryos of sandalwood (*Santalum album*) can be stored for 45 days at 4°C [89,90] and somatic embryos of interior spruce (*Picea glauca*) for only 1 month [91]. Storage for longer periods was achieved if beads were placed in liquid medium at low temperature. Under these conditions, Machii [92] conserved mulberry apices for 80 days and Shigeta et al. [93] carrot somatic embryos for 3 months. Mathur et al. [94] reported that encapsulated shoot tips of *Valeriana wallichii* could be conserved over 6 months at 4°–6°C without affecting viability, but no detailed results were provided. Redenbaugh et al. [33] mention that the rapid survival loss of encapsulated material that is generally observed is mainly due to the encapsulating matrix, which dehydrates rapidly and limits the respiration of the embedded embryos.

IV. CRYOPRESERVATION

A. History

Cryopreservation (i.e., storage at ultralow temperatures in a cryogenic medium, such as liquid nitrogen) has the potential to achieve the goal of suspending metabolism and, to all intents and purposes, suspending time. Cryopreservation has a relatively long history in microbiology for the storage of stock cultures, and in livestock husbandry for the storage of semen of elite male cattle [20]. Research into the response of higher plant systems to cooling to ultralow temperatures has been carried out over the past 40 years, following two main themes: (1) to gain an

understanding of the physiological and biochemical processes involved in the transitions to an from the frozen state, including cold acclimation; and (2) to preserve plant material in a viabl state [15,95–98]. Although the two themes have rather different motivations, the respectiv lines of research have much of mutual interest, and some of the most successful cryopreserva tion work has involved attention to the underlying processes of cryoinjury and cryoprotectior rather than an empirical approach alone.

Some sporadic reports of successful cryopreservation of in vitro systems appeared in th late 1960s, but the first report of exposure of cultured plant material to the temperature o liquid nitrogen was made by Quatrano in 1968 [99], using cultured cells of flax (*Linun usitatissimum*). This research was explicitly conducted with a genetic conservation motive highlighting the early awareness and convergence of interests of scientists in the genetic conservation and in vitro culture communities. The methods adopted closely followed the clas sic procedures found to be successful with other living systems [20,95,100]; namely, chemica cryoprotection, slow, dehydrative cooling, storage in liquid nitrogen, rapid thawing, washing and recovery. As will be described in the following section, subsequent studies did much t optimize and elucidate the flexibility of this approach to plant cryopreservation, to extend it t other culture systems, and importantly, to illustrate its limitations.

Recent years have seen a diversification of cryopreservation techniques, providing th interested scientist with a portfolio of general and specific options from which to choose These options seek to match both varying biological requirements and varying infrastructura situations, from the highly sophisticated to the minimally equipped laboratory. They thereby extend cryopreservation to a wide range of users.

B. Classic Techniques

Most of the early work on the cryopreservation of in vitro plant cultures focused on a method based on chemical cryoprotection and dehydrative cooling. This was particularly successful with cell suspension cultures, which is not surprising when the underlying biophysical events are explored. The vast majority of higher plant somatic cells, be they in vivo or in vitro, are not inherently freeze-tolerant. The transition of extra- and intracellular water into ice causes damage of a physical or biochemical nature [101–105].

The dynamics of the freezing process are particularly important. Extracellular freezing commonly occurs first, causing a flow of water from the cytoplasm and vacuole to the extracellular space where it freezes [106–108]. Depending on the rate of cooling, different amounts of water will leave the cell before the intracellular contents solidify [109]. Rapid cooling will result in more water remaining within the cell and causing potentially damaging ice than in slow cooling. Ice causes damage when formed in the freezing process *per se*. It can also cause damage during rewarming owing to the phenomenon of recrystallization, in which ice melts and reforms at a thermodynamically favorable, larger, and more damaging crystal size. This can be mitigated by rapid thawing [110,111]. Slow cooling reduces this risk, but can incur different damaging events owing to the concentration of intracellular salts and changes in the cell membrane [112–114]. Shrinkage of the protoplast and loss of surface area in the plasmalemma can render the protoplast incapable of resuming its original volume and surface area after thawing, resulting in rupture [115,116].

Light and electronmicroscopic studies of cell suspension cultures and isolated protoplast systems have helped clarify the nature of damage under different cooling regimens [e.g., 105,117–120]. They have also revealed the mitigating effects of cryoprotectants [105,118]. Cryoprotectants facilitate the flow of water across the cell membrane, and protect both molec-

ular and gross structures through a range of modes of action, including colligative effects and free radical scavenging [121–123].

Studies of the effect of cooling rates on survival in cryopreserved cell suspension culture systems clearly illustrate the existence of an optimum cooling rate, commonly in the region of $-1°C$ or $-2°C\ min^{-1}$, providing the central strategy of the classic approach to cryopreservation [see Refs. 15,16]. A strong body of research followed the initial elucidation of this approach, to explore other attendant factors, including the effect of culture conditions before cryoprotection, the age of the cells at the time of harvest for cryopreservation, immediate postthaw treatment, and recovery growth conditions, as well as looking more closely at the temperature excursions [e.g. 124–128]. Linear cooling and warming are not the only or necessarily the most successful options and may, in fact, prove more difficult to achieve, other than in complex, costly equipment [129]. Withers and King [128–130] describe improvised and simpler apparatus that can offer reproducible, but nonlinear slow cooling.

Some key findings are as follows: The age of cells at the time of harvesting for storage can affect their survival. This is linked to cell size and water content. Rapidly growing cells are small and have a relatively low water content. Modification of the pregrowth medium used for the passage before cryopreservation by, for example, the addition of osmotically active compounds, such as mannitol and sorbitol, can lead to reduction in cell size and an increase in freeze tolerance. For cell suspension cultures, in particular, mixtures of cryoprotectants are much more effective than single cryoprotectants, and preparation in culture medium is usually beneficial. Removal of cryoprotectants after thawing has not been demonstrated to be essential, and there is clear evidence for a detrimental effect of washing. Similarly, recovery growth on solid medium is generally much more effective than dilution in liquid medium [128,130,131]. If toxicity is suspected, precautions can be taken, such as moving cells on a supporting filter paper through a series of dishes of solid culture medium [132].

Table 2a provide examples of the application of the classic approach to cryopreservation for a range of in vitro plant cultures systems. Its flexibility is evident, but it is also clear from the studies carried out to date that the approach is most successful with culture systems that consist of small units of uniform morphology, such as would be found in a protoplast culture, an exponentially growing cell suspension culture, or fragmented callus culture. The approach is less successful with culture systems that consist of larger units comprising a mixture of cell sizes and types, such as shoot tips or relatively mature somatic embryo cultures.

A clue to an alternative approach that might be taken with organized cultures can be found in some data in which wide ranges of cooling rates are explored and an upturn in survival is seen at the fastest rates. This is interpreted as being due to the formation of microscopically small ice crystals that develop without damaging cell structures. As long as these can be thawed again without recrystallization, by applying an adequately rapid warming rate, the specimen can survive. By this approach, problems in achieving uniform dehydration in a large, dense mass of tissue, such as a shoot tip, and the differential requirements of small, highly cytoplasmic meristem cells and large, more vacuolated cells elsewhere in the shoot tip that limit slow cooling's success are bypassed. Details of several successful reports of ultrarapid cooling are given in Table 2b. In general, although this approach is practically uncomplicated and inexpensive to carry out, reservations must be expressed over its practicality in the context of genetic conservation. It often entails the use of naked specimens, specimens in open containers, and specimens in droplets of medium on a sheet of aluminium (e.g., 136–139). In all of these cases, there is a risk of microbial contamination either during the cooling and warming stages or during storage, where the specimens do not easily lend themselves to organized management.

Table 2 Examples of (a) the Classic, Slow, Cooling-Based Approach to Cryopreservation of In Vitro Plant Cultures, and (b) Rapid Cooling

Species	Culture system	Pregrowth	Cryoprotection	Cooling, storage, warming	Recovery	Ref.
(a)						
Sycamore (*Acer pseudopatanus*)	Cell suspension	Culture for 3–4 days in medium containing 6% mannitol	0.5 M DMSO + 0.5 M glycerol + 1 M sucrose	$-1°C\ min^{-1}$ to −35°C, hold for 30 mins; transfer to liquid nitrogen; thaw in water bath at +40°C	Layer cells in suspending liquid over semisolid medium	131
Soyabean (*Glycine max*)	Protoplasts	Protoplasts isolated from exponentially growing cells	5% DMSO + 10% glucose	$-10°C\ min^{-1}$ to −35°C; transfer to liquid nitrogen; thaw in water bath at +40°C	Wash in liquid medium; transfer to standard medium	133
Potato (*Solanum tuberosum*)	Shoot-tip	Shoot-tip dissected from glasshouse or in vitro plants; incubate overnight in standard liquid medium	10% DMSO	$-0.20°C\ min^{-1}$ to −35°C; transfer to liquid nitrogen; thaw in water bath at +37°C	Wash twice with liquid medium; transfer to semisolid medium	134
Pear (*Pyrus* spp.)	Shoot-tip	Culture in vitro plantlets at 22°C/16 h day, −1°C/8 h night for 7 days; dissect shoot-tips and pregrow for 48 h in medium containing 5% DMSO	10% polyethylene glycol + 10% glucose + 10% DMSO	$-1°C\ min^{-1}$ to −40°C; transfer to liquid nitrogen; thaw in water bath at +40°C for 1 min; transfer to +23°C	Wash in liquid medium; drain; transfer to semisolid medium	135

(b)

Carnation (*Dianthus caryophyllus*)	Shoot-tip	Dissect shoot-tip from cold-hardened plant	5% DMSO	Place in ampule and plunge into liquid nitrogen.	Culture under standard conditions	136
Potato (*Solanum tuberosum*)	Shoot-tip	Dissect shoot-tip from plantlet and culture on filter paper floating on liquid medium for 2 days.	10% DMSO	Collect shoot-tip on hypodermic needle and plunge into liquid nitrogen; thaw by plunging into liquid medium at 34°–40°C	Culture on filter paper bridge over liquid medium	137
Oilseed rape (*Brassica napus*)	Shoot-tip	Dissect shoot-tip from in vitro plantlet; incubate for 24 h in medium containing 5% DMSO	15% DMSO	Collect shoot-tip on hypodermic needle and plunge into liquid nitrogen; thaw by plunging into liquid medium at room temperature (+40°C)	Transfer without washing to semisolid shoot-induction medium	138

C. New Techniques

1. Principle

In classic cryopreservation techniques, the removal of cellular water and the behavior of remaining cellular water during the freezing and thawing processes are critical to success. In contrast with these freezing-based techniques, new cryopreservation techniques are based on the phenomenon of vitrification. *Vitrification* can be defined as the transition of water directly from the liquid phase into an amorphous phase or glass, while avoiding the formation of crystalline ice [140]. (*Note*: Vitrification in the present context should not be confused with the phenomenon of "hyperhydration," which sometimes goes by the same name.)

In vitrification-based procedures, cell dehydration is performed before freezing by exposure of samples to concentrated cryoprotective media or air desiccation. This is followed by rapid cooling. As a result, all factors that affect intracellular ice formation are avoided. Glass transitions (changes in the structural conformation of the glass) during cooling and rewarming have been recorded with various materials using thermal analysis [141–144]. Dumet et al. [145] showed that increased survival rates for cryopreserved oil palm somatic embryos were correlated with the progressive disappearance of ice crystallization peaks and their replacement by glass transitions.

Vitrification-based procedures offer practical advantages in comparison with classic freezing techniques [146]. Similar to ultrarapid freezing, they are more appropriate for complex organs (shoot tips or embryos) which contain a variety of cell types, each with unique requirements under conditions of freeze-induced dehydration. By precluding ice formation in the system, vitrification-based procedures are operationally less complex than classic ones (e.g., they do not require the use of controlled freezers) and have greater potential for broad applicability, requiring only minor modifications for different cell types.

Luyet [147] was the first to envisage the use of vitrification for cryopreserving biological specimens, but it is only somewhat recently that numerous reports on cryopreservation of plant material using vitrification-based procedures have appeared in the literature [146–149]. Four different procedures based on the phenomenon of vitrification can be identified: encapsulation–dehydration, desiccation, pregrowth–desiccation, and a procedure that actually goes by the name of vitrification.

2. Encapsulation–Dehydration

The encapsulation–dehydration technique is based on the technology developed for the production of synthetic seeds, by which embryos are encapsulated in a bead of calcium alginate gel [33]. Cryopreservation using encapsulation–dehydration has been applied mainly to shoot apices of various species, and also to carrot, walnut (*Juglans regia*), and coffee somatic embryos, and to oil seed rape (*Brassica napus* L.) microspore embryos (Table 3).

The encapsulation–dehydration technique permits freezing explants of large dimensions; pear shoot tips up to 5 mm in length [153], and heart or torpedo stage embryos (2–3 mm in length) have been successfully cryopreserved [162,164].

Before the cryopreservation procedure itself, plant material is often submitted to various treatments that increase survival potential. For cold-tolerant species, such as pear, apple, or mulberry, mother plants [152,165] or apices [151] can be placed at a low temperature (0–5°C) for several weeks. Scottez [153] showed that this cold treatment resulted in an increased quantity of unsaturated fatty acids in the pear apices. Before encapsulation, apices of mulberry are transferred daily onto solid media with progressively increased sucrose concentrations to initiate dehydration [151].

Table 3 List of Plant Species and Specimens (Apices, Somatic and Microspore Embryos) That Have Been Successfully Cryopreserved Using the Encapsulation–Desiccation Technique

Specimen	Species	Ref.
Shoot apex	Potato	150
	Apple	152,151
	Pear	142,153
	Mulberry	151
	Carnation	154
	Grape	155
	Chicory	156
	Eucalyptus	157
	Coffee	158
	Cassava	159
	Sugarcane	160
Somatic embryo	Carrot	161
	Coffee	162
	Walnut	163
Microspore embryos	Oilseed rape	164

The stages of the process after encapsulation are pregrowth, desiccation, cooling, warming, and recovery growth. Pregrowth is performed in liquid medium enriched with sucrose (0.3–1 M) for periods of between 16 h [151] and 7 days [150]. Partial replacement of sucrose with other sugars (raffinose, maltose, glucose, or trehalose) did not improve the survival of cryopreserved grape (*Vitis vinifera*) shoot tips [155]. For plant species that are sensitive to direct exposure to high sucrose levels, a progressive increase in sucrose concentration is used [157,158, 166].

Encapsulated samples are desiccated either in the air current of a laminar airflow cabinet or by using silica gel. The latter method is preferred because it provides more precise and reproducible desiccation rates [160]. The optimal water content of desiccated beads is about 20% (fresh weight basis), ranging from 13% with coffee somatic embryos [162] to 30–35% with apple, grape, mulberry [151], and cassava apices [158].

Cooling is usually carried out rapidly, by direct immersion of samples in liquid nitrogen. However, controlled slow cooling, down to −100°C, led to improved survival of grape apices [166]. In contrast, the survival rate of sugarcane (*Saccharum* spp.) apices was higher after rapid than after slow cooling [167,168]. These results suggest continued dependence on control of the residual water content in the specimen.

Storage is usually performed at −196°C. Scottez [153] showed that survival of pear apices was not modified after 2 years of storage in liquid nitrogen. The same author demonstrated that samples could also be conserved for 1 year at a higher temperature (−75°C) which, nevertheless, is below the temperature of ice recrystallization (−50 to −70°C). Similarly, apices of apple, pear, and mulberry have been stored for 5 months in a deep-freezer at −135°C [151]. In these cases, the use of liquid nitrogen is not necessary for storing the plant material, giving obvious advantages in situations where its regular supply is unreliable. Importantly, survival of explants after thawing is independent of the rewarming rate.

For recovery, samples are usually placed directly under standard culture conditions. However, survival of cryopreserved apices of sugarcane was improved if they were placed for 1 week in the dark, on a medium supplemented with growth regulators [160]. Extraction of apices from the alginate beads was necessary to allow regrowth in the case of grape and pear

[165,166]. Recovery growth of cryopreserved material is usually direct and rapid, without callus formation. Histological studies performed with apices of several plant species revealed that the structural integrity of most meristematic cells is preserved after cryopreservation by encapsulation–dehydration [158]. Therefore, recovery growth originates from the entire meristematic zone. This is contrary to what is generally observed after classic cryopreservation, during which many cells are destroyed, frequently leading to callus formation during recovery. With sugarcane, apices withstood freezing as a whole and cell divisions could be observed within 2 days after thawing [167].

Successful extension of encapsulation–dehydration protocols has been performed with 11 varieties of pear [153], 9 varieties of apple [151,152], and 14 varieties of sugarcane [158]. With all three species, even though genotypic variations were noted, survival rates were sufficiently high to envisage large-scale routine application of the cryopreservation protocols developed.

3. *Desiccation*

Cryopreservation using a desiccation procedure is very simple because it consists of dehydrating the plant material, then freezing it rapidly by direct immersion in liquid nitrogen. Desiccation has been applied mainly to zygotic embryos of a large number of species [see Ref. 169 for a review]. Experimentation has been carried out with only one other type of material, shoot tips of mulberry [170].

The physiological state of the starting material is an important parameter. In *Coffea arabica*, mature embryos (1 week before harvest) showed higher survival rates than immature ones (2 months before harvest) [171]. High variability in the survival rates of embryos extracted from seeds of some recalcitrant seed-producing trees (*Aesculus*, *Castanea*, and *Quercus*) harvested at different periods was noted by Pence [172].

Desiccation is usually performed by placing the embryos or embryonic axes in the air current of a laminar airflow cabinet. However, more precise and reproducible desiccation can be achieved by placing plant material in a stream of compressed air [173] or in an air-tight container with silica gel [144]. The duration of desiccation varies with the size of the embryos and their initial water content. Optimal survival rates are generally noted when embryos are dehydrated down to 10–20% moisture content (fresh weight) [169]. Dehydration must be sufficient to ensure survival after freezing, but not so intense to induce extended desiccation injury. In optimal cases, no significant difference is observed in the survival rates of desiccated control and cryopreserved embryos, as noted with tea (*Camellia sinensis*), banana, and hazelnut (*Corylus* spp.) [171,174,175].

Regrowth of plant material after warming is usually direct, but modified regrowth patterns are occasionally observed. Chin et al. [176] noted the nondevelopment of the haustorium and more rapid leaf expansion of cryopreserved embryos of *Veitchia* and *Howea*, in comparison with unfrozen controls. Abnormal regrowth of a fraction of cryopreserved embryos in the form of callusing or incomplete development occurred with *Castanea* and *Quercus* [172], *Hevea brasiliensis* [177], and oil palm [178].

Modified recovery conditions, notably of the hormonal balance of the culture medium, can significantly improve the survival rate of the cryopreserved material, as observed with coffee embryos [171,179].

4. *Pregrowth–Desiccation*

Cryopreservation using a pregrowth–desiccation procedure comprises the following steps: pregrowth treatment with cryoprotectants, desiccation, rapid cooling, storage, and rapid warming. This technique has been applied to only a limited number of specimens: stem

segments of in vitro plantlets of asparagus (*Asparagus officinalis* L.) [180], somatic embryos of melon (*Cucumis melo*) and oil palm [181,182], microspore embryos of rapeseed [164], and zygotic embryos of coconut [183].

The application of cryoprotectants is usually performed before desiccation. However, in the coconut embryos, dehydration was carried out before preculture with cryoprotectants [183]. The duration of treatment with cryoprotectants varies between 20 h for coconut [183] and 7 days with oil palm somatic embryos [182]. Sugars (sucrose or glucose) are generally employed for preculture. However, abscisic acid only was used for the pretreatment of melon somatic embryos [181].

Various methods have been employed for desiccation: coconut embryos were placed either in the air current of a laminar airflow cabinet [183]. Asparagus stem segments, rapeseed and oil palm embryos were placed in an air-tight chamber containing silica gel [164,180,182], and melon somatic embryos were placed over a salt solution, ensuring a constant relative humidity [181]. Optimal water contents (fresh weight) for storage range between 11.8% for melon somatic embryos [181] and 25–30% with oil palm somatic embryos [182].

All materials cryopreserved using pregrowth–desiccation are cooled rapidly by direct immersion in liquid nitrogen. Storage is usually performed in liquid nitrogen. Experiments with oil palm somatic embryos have shown no modification in the recovery rate after 1 or 52 months of storage at −196°C [169]. More recently, Dumet et al. [184] have been able to conserve oil palm somatic embryos for 6 months at −80°C (i.e., below the glass transition temperature) without any modification in recovery rate compared with embryos stored at −196°C.

Warming is generally carried out rapidly except for stem segments of asparagus and rapeseed microspore embryos, which were rewarmed slowly at room temperature. Specimens are usually transferred directly onto standard medium for recovery. However, oil palm somatic embryos were cultured on media with a progressively reduced sucrose concentration and transitory supplement of 2,4-dichlorophenoxyacetic acid (2,4-D), to stimulate proliferation [182].

Cryopreservation usin ' pregrowth–desiccation has ensured satisfactory survival rates with all materials tested, and recovery is usually rapid and direct. Alterations in regrowth pattern have been observed only with coconut embryos and oilseed rape microspore embryos. The haustorium of frozen coconut embryos browned rapidly and did not develop further [183]. Even though up to 93% of oilseed rape embryos withstood freezing, only 43% of them developed directly into plantlets. The remaining 50% produced calluses or secondary embryos [180].

Pregrowth–desiccation has been tested with four varieties of coconut, giving recovery rates of between 33 and 93% [183]. Large-scale application of this technique has been performed in the case of oil palm somatic embryos. Eighty clones are now routinely stored at −196°C [185].

5. *Vitrification*

Vitrification proc edures consist of the following steps: treatment ("loading") of samples with cryoprotective substances, dehydration with a highly concentrated vitrification solution, rapid cooling and warming, removal ("unloading") of the vitrification solution. Vitrification solutions are complex mixtures of cryoprotective substances that have been selected in view of their ability to vitrify (i.e., form an amorphous glassy structure) during cooling. The most commonly employed are derived from the solution proposed by Sakai's group which comprises 22% glycerol, 15% ethylene glycol, 15% propylene glycol, 7% dimethyl sulfoxide (DMSO), and 0.5 M sorbitol [141] and by Steponkus' group [186], which consists of 40% ethylene glycol, 15% sorbitol, and 6% bovine serum albumin.

Vitrification procedures have been developed for about 20 species, using protoplasts, cell suspensions, shoot apices, and somatic embryos [see Ref. 149 for a review]. The plant material is often submitted to various treatments before the cryopreservation procedure itself, to increase its survival potential. In the cold-tolerant species, the in vitro mother plants can be cultured at low temperature for several weeks [144,187]. Explants have been placed for 1 or 2 days on a medium supplemented with a high sugar concentration or cryoprotective agents. Mint (*Mentha* spp.) shoot tips have been cultured thus for 2 days on a medium containing 0.75 M sucrose and 4% DMSO [188].

Explants are then loaded (i.e., suspended) in a medium containing cryoprotective substances (ethylene glycol, glycerol, sucrose) for a short period (5–90 min, depending on the material). This reduces their sensitivity to the vitrification solutions. Survival of rye (*Secale cereale*) protoplasts after exposure to a vitrification solution increased from 4% without loading with 1.5 M ethylene glycol to 65% with loading [189].

The duration of contact between the plant material and the vitrification solutions is a critical parameter owing to their high toxicity. The period generally increases with the size of explants treated. Rye protoplasts have to be dehydrated for only 60 s [189], whereas the optimal dehydration period is 80 min for apple and pear shoot tips [187]. Encapsulated carnation (*Dianthus caryophyllus* L.) apices are treated for up to 5 h with a vitrification solution comprising 38% sucrose and 35% ethylene glycol [154].

Dehydration of samples at 0°C instead of room temperature reduces the toxicity of the vitrification solutions and increases the potential period of exposure to vitrification solution, thereby giving more flexibility for handling the plant material during this critical phase of a vitrification protocol. Survival of asparagus cell suspensions dropped rapidly after 5 min of dehydration at 25°C, whereas if it were performed at 0°C, dehydration for between 5 and 60 min ensured satisfactory survival rates, with an optimum at 20 min [190].

Once dehydrated, samples are cooled rapidly by direct immersion in liquid nitrogen to achieve vitrification of intracellular solutes. Reduction in the quantity of suspending cryoprotectant solution and the use of containers of a small volume (e.g., 500 µL plastic straws) led to increased cooling rates. Asparagus cell suspensions have been enclosed in 50 µL of medium in 500 µL plastic straws, thus giving a cooling rate of –990°C min^{-1} [191]. Mint and sweet potato shoot apices were cooled ultrarapidly (–4800°C min^{-1}) without cryoprotective medium [188,192].

Potato and carnation apices cryopreserved using a vitrification procedure have been stored at –196°C for 1 and 2 years, respectively, without any modification in their survival rate [154,193].

Rewarming of samples has to be performed as rapidly as possible to avoid devitrification processes, which would lead to the formation of ice crystals that would be detrimental to cellular integrity. Thus, samples are immersed in a water bath or liquid medium held at 20°–40°C. However, Steponkus et al. [146] have advised holding vitrified samples in air for a few seconds before plunging them in a thermostated water bath. This is to achieve slow rewarming through the glass transition region (ca. –130°C) to minimize mechanical fracturing of the glass caused by excessive thermal gradients [194].

After warming, the highly concentrated vitrification solution must be removed progressively to minimize osmotic shock. This is usually performed by diluting the vitrification solution in liquid medium supplemented with 1.2 M sucrose or sorbitol, before transferring the explants to standard medium.

Vitrification procedures generally lead to high survival rates, and direct and rapid recovery is usually observed. However, Towill [188] mentioned callus formation and abnormal development of some mint apices after vitrification.

Vitrification experiments involving a large range of genotypes are still infrequent. In the case of mulberry apices, experiments performed with 13 cultivars or species gave survival rates ranging between 40 and 80% [144]. More recently, 45–47% and 40–72.5% survivals have been noted with apices of five varieties of apple and eight cultivars of pear, respectively [187].

V. GENETIC STABILITY OF IN VITRO CONSERVED GERM PLASM

It is implicit in the genetic conservation context that genetic stability should be a very high priority. The facility of cloning in vitro does then offer, superficially, a very attractive means of perpetuating given genotypes, particularly for traditionally vegetatively propagated material. However, assumptions of clonal integrity in the in vitro situation may be unsafe. It is more pragmatic to consider supposed clones to be very tight populations with potential for deviation from the original distribution of genotypes. This then leads to a consideration of the factors that might contribute to such a deviation, namely creation and selection. Genetic variation may arise by somaclonal variation, with obvious implications for the choice of culture system used for genetic conservation. It may be intrinsic in the cultured material, possibly linked to its genetic structure, such as in sugarcane or banana, for which polyploids are more prone to instability than diploids [195]. Selection may occur under conditions that either cause differential damage of a lethal nature or that favor the growth of one genotype over others in a mixture. The issues to be taken into consideration and the information now available on genetic instability in material conserved in vitro are rather different for slow growth and cryopreservation, as described in the following sections.

A. Slow Growth

It has long been documented that in cultures comprising a mixture of genotypes, the different components of the mixture may not grow at the same rate [e.g., 196]. Under the stressed conditions implicit in slow growth, the risk of selection must be considered to be greater than under standard growth conditions. Accordingly, it is important to minimize the initial risk of instability and take measures to minimize additional risks and monitor cultures at intervals to detect variation. One of the most effective ways of minimizing risks of instability both at the outset and during slow growth storage is through control of the culture system. If the cultures are maintained in a highly organized state, as shoots, plantlets, or embryos, the risk of somaclonal variation is much lower than if they were in the form of cells or calluses. This will apply when there is an effective choice available, but sometimes it will be unavoidable to use cells or calluses, in which event, the choice of slow-growth storage must be questioned.

There have been few controlled experiments to monitor the genetic stability of cultures over time in slow-growth storage. However, such evidence as is available would suggest that organized cultures need not incur unacceptable risks. The genetic integrity of cassava cultures maintained in slow growth at CIAT over a period of 10 years, was confirmed when tested by isozyme analysis, DNA analysis, and by monitoring their morphology when returned to the field [26]. A slight suggestion of some acclimatization to slow-growth conditions [197] was not confirmed by any of the other analytic criteria.

There are differences from species to species in their susceptibility to somaclonal variation. When this is combined with the clear differences in response to storage under slow-growth conditions experienced not only between species but also between cultivars, it is clear that one cannot simply extrapolate from one fortunate example to all others. Thus, there is a pressing need for controlled experiments to test the genetic integrity of cultures stored in slow growth,

in comparison with controls maintained under normal-growth conditions, and ideally, with cryopreserved specimens, to gain an insight into the relative risks. An interesting model on which to conduct such an experiment would be *Musa*. Mass propagation in vitro is widely used for bananas and plantains, and a risk of somaclonal variation, even under optimal propagation conditions, is recognized. This is strongly linked to genotype. Studies of this phenomenon and parallel development of morphological, biochemical, and molecular methods for characterizing variants, have yielded a wealth of information on patterns of variation and potential markers to use in monitoring instability [19,198–202].

The risks of selection under slow-growth conditions should not be evaluated alone. Culture under any conditions carries risks. The risks of loss through human error are reduced by most storage measures. Risks caused by equipment failure are highly variable and, under some slow-growth conditions, might be considered to be greater than in normal growth. Thus, the equation to be drawn is not simple and must pragmatically take into account all of the pertaining circumstances, not least of all the risks of not applying the best available storage conditions.

B. Cryopreservation

Cryopreservation involves a series of stresses that may destabilize the plant material and lead to modifications in recovered cultures and regenerated plants. Therefore, it is necessary to verify the genetic stability of material recovered from cryopreserved samples before this technique is routinely used for the long-term conservation of plant germ plasm. Even though freezing protocols have been developed for many species, only a limited number of studies have considered this aspect. No modification at the phenotypic, biochemical, chromosomal, or molecular level that could be attributed with certainty to cryopreservation has yet been reported. This correlates with observations from other biological systems.

In cell suspensions, numerous examples are now available to illustrate that cryopreserved cells maintain their biosynthetic and morphogenic potential [203–205]. The only published exception concerns lavender *Lavandula vera* cell suspensions submitted to repeated freeze–thaw cycles: the number of colonies recovered from cryopreserved cells increased with the number of freeze–thaw cycles, suggesting that the selection of more freeze-tolerant cells was taking place [206]. However, no modifications were noted in the biosynthetic and regenerative capacities of cryopreserved cells, implying a change in population structure, rather than genetic change.

Plants regenerated from cryopreserved apices of strawberry and cassava were phenotypically normal [207,208]. No differences were noted in the vegetative and floral development of several hundred oil palm plants regenerated from control and cryopreserved somatic embryos [73]. Harding and Benson [209] noted that the ability of potato plants that were regenerated from cryopreserved apices was not impaired. However, following recovery of apices on certain media, plants failed to produce flowers in the first regeneration cycle. The authors suggest that this is more likely to be attributable to tissue culture than to cryopreservation as such.

Electrophoretic profiles of two enzymatic systems were comparable in plants regenerated from control and cryopreserved apices of sugarcane [210] and sweet orange (*Citrus sinensis* L., Osb.) somatic embryos [211]. With the latter material, there was no modification noted in the pattern of total soluble proteins. The ploidy level was not modified by cryopreservation in plants regenerated from oilseed rape somatic embryos [164] and sensitive dihaploids of potato [212]. Finally, restriction fragment length polymorphism (RFLP) patterns of plants regenerated from cryopreserved potato shoot-tips [213], embryogenic cell suspensions, and apices of sugarcane [210,214] were identical with those of unfrozen controls.

VI. CONCLUSIONS

A. Current Use of In Vitro Conservation Techniques

Classic in vitro slow-growth conservation techniques have been developed for a wide range of species, including temperate woody plants [215], fruit trees [18], and horticultural crops [216], as well as many tropical species [13,73,217]. A recent literature survey [71] indicated that shoot tips and node cuttings are the explants most frequently employed for slow-growth storage. For the tuber-producing species, medium-term storage of microtubers may represent an interesting solution, as shown [218] in experiments performed with potato. The slow-growth method most commonly employed is temperature reduction, the next most common is manipulation of the culture medium, then a combination of both parameters.

However, there are still only a few examples of in vitro slow-growth storage being used routinely as a complementary technique for the conservation of genetic resources of a given plant species. These notably include banana, potato, and cassava, which are conserved in regional and international germ plasm conservation centers such as INIBAP (now part of IPGRI), CIAT, CIP, and IITA.

Alternative medium-term conservation techniques are still at the experimental stage. Low-oxygen storage at room temperature may be interesting for tropical, cold-sensitive species because it allows growth reduction at the normal growth temperature. However, it still has to be tested with additional species and over longer storage periods.

Medium-term storage of desiccated (and possibly encapsulated) somatic embryos will facilitate the management of large-scale production of elite genotypes. For genetic resources conservation, encapsulated apices stored at low temperature may become the material of choice. However, further research is needed to increase the duration of storage.

Cryopreservation techniques have been developed for about 80 different plant species cultivated under various forms as cell suspensions, calluses, apices, somatic and zygotic embryos [1 ',18,19,205,219,220]. Most of this work has been performed in the framework of academic studies and has involved only one or a few genotypes. However, owing to the development in the last 3–4 years of new cryopreservation procedures for apices and embryos (encapsulation–desiccation, desiccation, pregrowth–desiccation, and vitrification), reports involving many genotypes or varieties are becoming more frequent. These new freezing procedures generally lead to satisfactory survival rates with a wide range of genotypes by using the same technique. The best example of large-scale experimentation is potato, for which a cryopreservation technique has already been successfully applied to more than 60 different varieties [221].

There is an increasing number of examples for which techniques can be considered operational. This is notably true with sugarcane, because cryopreservation procedures are now available for cell suspensions [222], embryogenic calluses [223], and apices [158,160].

Routine application of cryopreservation is still restricted almost exclusively to the conservation of cell lines in research laboratories [203]. The only example of routine application of cryopreservation to another type of material is oil palm. For this species, somatic embryos of 80 different clones are stored in liquid nitrogen, and frozen samples are thawed on request for plant production [185].

B. Future Needs and Prospects for In Vitro Conservation

When comparing the current status of in vitro conservation for plant genetic resources with the situation 20, or even 10, years ago, dramatic advances can be appreciated. Whereas in the 1970s it was a suggestion largely supported by extrapolation from the cryopreservation of

other biological systems, it is now a very realistic option for many species and culture systems. It has, through the development of slow-growth storage, revolutionized the medium-term genetic conservation of a substantial number of clonally propagated staple crops of the developing world. In vitro active gene banks of these crops can be maintained, independently of prevailing climatic conditions; thus, an important world collection of *Musa* is housed in Belgium where it is free of the risks that would be ever present if maintained in a *Musa*-growing country [65]. Similarly, collections can be safely duplicated to second sites and moved from location to location with ease.

A natural caution in the take-up of in vitro conservation has been observed over recent years. This was fully understandable at a time when the techniques were at a more experimental stage. However, there are now many instances where both slow growth and cryopreservation techniques could be more widely applied, to the benefit of both germ plasm management and research. Slow growth could be more widely used to provide safer alternatives to the field gene bank for species that are readily propagated in vitro by low-risk methods. Cryopreservation is not yet at as advanced stage of development as slow growth, particularly for organized cultures, despite the recent advances in technological development described here. Nevertheless, for cell and callus cultures, especially embryogenic systems, the effort to tailor published methods to specific materials would almost certainly pay off.

For organized shoot and embryo cultures, it must be recognized that there is still some way to go before routine methods can be applied without any such tailoring. However, it is time that the primary focus of experimentation for such materials moved from the research laboratory to the gene bank, or at least to research units within institutes that have a conservation mandate. Close liaison between in vitro conservation specialists and gene bank personnel will assure the development of method and conservation routines that meet the necessary criteria required in the gene bank context. This will also allow the know-how of the gene bank staff in relation to genetic stability and genetic characterization to be incorporated into in vitro conservation research and development.

Above all else, it is important that in vitro conservation be demystified to convey it as a realistic option that is not in the province of high biotechnology, but a very practical, convenient approach to consider alongside other conservation methods. An important step in that process will be development of an understanding for the flexibility of in vitro conservation protocols. A level of precision and exactitude that is unrealistic and, in fact, unnecessary will serve as a serious deterrent to the take-up of any technique.

The locations in which in vitro conservation could be most beneficial are developing country laboratories and gene banks in which facilities, although adequate, may not be practically adaptable to the many different conditions described in the literature. Thus, it is important to obtain a clear idea of the flexibility of storage protocols (i.e., the window within which effective conservation may be achieved). This is truly a situation in which "the best is the enemy of the good." Optimizing storage conditions to a fine degree of tuning for every genotype will be far less effective than developing more generalized procedures that have an adequate degree of flexibility that can accommodate slightly different culture conditions or a different pair of hands applying them.

Another crucial issue is the complementarity of in vitro conservation for other genetic conservation strategies [12,224,225]. In vitro conservation is not being presented as a replacement for conventional ex situ and in situ approaches. It is one more tool to place in the hands of the curator to cover the conservation requirements of the gene pool in question. In some circumstances it will be appropriate; in others it will not. Factors to take into account in making the selection will be effectiveness, security, cost, available facilities, and needs for access by users, including breeders. Sometimes the need will be to store clonal genotypes for

the short- to medium-term; sometimes, the need will be to store genes for the long term. Each situation needs to be matched by the best combination of conservation options, each option offering advantages and offsetting disadvantages of other methods. In this context, it is easy to see that in vitro conservation could be taken up much more readily than at present, its research and development, proceeding toward routine use with the safety net of other conservation methods that, at the same time, serve the purpose of experimental controls against which to evaluate its effectiveness.

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