TROPICAL PLANT GERMPLASM CONSERVATION

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SUMMARY

In vitro medium term conservation of tropical plant germplasm is used routinely in many laboratories. Growth reduction is achieved by modifying various parameters, such as temperature, culture medium, culture vessel, gaseous environment. For long term conservation, cryopreservation (i.e. storage in liquid nitrogen, -196° C) is the only current method available. For each material, optimal conditions have to be defined for each successive step of the process. Cryopreservation protocols have been set up for more than 40 different tropical species. However, routine use of cryopreservation still remains exceptional.

INTRODUCTION

As regards preservation possibilities, plant species have been divided into 2 categories (Roberts,1973):

1. Orthodox seeds which can withstand dehydration to 5% or less (dry weight basis) without damage. When dry, the viability of these seeds can be prolonged by keeping them at the lowest temperature and moisture possible.

2. Recalcitrant seeds which are high in moisture and are unable to withstand much desiccation. They are predominantly seeds from tropical or subtropical species. They can be stored only in wet medium to avoid dehydration injury and in relatively warm conditions because chilling injury is very common among these species. They remain viable only for a short time (weeks or months), even if kept in required moist conditions. This group comprises many crop species of great economic importance such as oil palm, coconut, cocoa, coffee, etc.

Moreover, long-term seed storage cannot be applied to most long-lived forest trees, including gymnosperms and angiosperms, since their juvenile period is very long and they do not produce seeds for several years. The conservation of plants which are vegetatively propagated, such as cassava, potato, yam, etc. also poses considerable problems.

In situ conservation has been made almost impossible due to the disappearance of large wild areas. Conservation ex situ is very difficult to carry out due to the following problems: an adequate sample has to be determinated for the conservation of genetic diversity. It varies from 20 to 30 plants for a single population, to several hundreds for gene pool conservation and from 5,000 to 20,000 plants, depending on the species, for the maintenance of heterozygosity. Thus, land space requirement is very important, particularly in the case of forest trees, which are usually very large, whereas land availability drastically decreases. Moreover, in the case

of genetically heterozygous species, it is necessary to preserve a larger sample to maintain as much as possible of the genetic variation within a population. Labor costs and trained personnel requirements are very important. Moreover, material in natural conditions remains exposed to natural disasters, pests and pathogens and is submitted to threats from changing government policies and urban development. Finally, for many species, we do not possess even the rudiments of knowledge of the biology of the species.

During the last years, in vitro culture techniques have been extensively developed and applied to more than 1,000 species, including many tropical species. The use of these techniques can be of great interest for germplasm collection, storage and multiplication of recalcitrant and vegetatively propagated species. Tissue culture systems present advantages which are listed below:

1. very high multiplication rates
2. aseptic system: - free from fungi, bacteria, viruses and insect pests
   - production of pathogen-free stocks
3. reduction of space requirement
4. genetic erosion reduced to zero
5. possibility of producing haploid plants
6. rescue and culture of zygotic embryos which normally abort
7. reduction of the expenses in labor costs and financial terms

However, the in vitro storage of large quantities of material induces various problems: laboratory management of plant material which needs to be regularly subcultured and risks of genetic variation which increase with in vitro storage duration and can lead to the loss of trueness to type.

The methods employed are different, according to the storage duration requested. For short and medium term storage, the aim is to reduce the growth and to increase the intervals between subcultures. This is achieved by modifying the culture conditions, mainly by lowering the culture temperature.

For long term storage, cryopreservation, i.e. storage at very low temperatures, usually that of liquid nitrogen (-196° C) is the only current method. At this temperature, all cellular divisions and metabolic events are stopped. The plant material can be stored without alterations or modifications for a theoretically unlimited period of time. Moreover, the cultures are stored in a small volume, sheltered from contaminations, with a very limited maintenance.

SHORT AND MEDIUM TERM STORAGE

Principal factors

Temperature. Growth reduction is generally achieved by lowering the culture temperature. In several cases, the cultures are maintained at standard temperature. However, satisfactory storage durations are obtained only with slow growing species. For example, Coffea arabica

plantlets conserved at 27° C can be subcultured every one year but Coffea racemosa plantlets have to be transferred every 6 months (Bertrand-Desbrunais and Charrier, 1990). The storage temperature depends on the cold sensitivity of the species. Cassava plantlets have to be stored at temperatures higher than 20° C (Roca et al., 1984). Oil palm ramets and somatic embryos do not resist to a relatively short exposure to temperatures lower than 18° C (Engelmann, unpublished results). Staritsky et al. (1985) increased the storage duration of Colocasia shoots at +3° C by exposing them for 48 hours at 18-22° C every 15 days. This sequential treatment allows for a partial reversion of the physiological disorders induced by cold.

A reduction in light intensity or a complete suppression is often used concomitantly with temperature reduction. The need for light is not systematic and varies from one species to the other.

Culture medium. Various alterations to the culture medium can be made:

1. Lowering the content in mineral elements and/or sugar. Kartha et al. (1981) could preserve coffee plantlets for 2 years on a medium devoid of sugar and with only half of the mineral solution of the standard medium.

2. Addition of cryoprotective substances or with osmotical properties. The addition of mannitol reduces significantly the growth of Colocasia and Xanthosoma shoots (Staritsky et al., 1985). However, cassava shoots deteriorated in the presence of mannitol, even at 0.1% and with a storage temperature lower than 20° C (Roca et al., 1982).

3. Growth retardants can be added: Westcott (1981) and Roca et al. (1982) used abscisic acid in order to reduce the growth of shoots of potato. However, these authors indicate that ABA is detrimental to some varieties.

4. Finally, other substances are sometimes added. Roca et al. (1984) observed that the adjunction of activated charcoal had positive effects on the storage of cassava shoots: it reduces defoliation, decreases and nearly halves shoot growth for one genotype, limits chlorophyll degradation and browning of roots.

Physiological stage of the explants. The type of explants, as well as their physiological stage, are very important. There is a minimal size for the explants. The presence of a root system increases the survival capacities, as observed by Kartha et al. (1981) with coffee plantlets and by Brizard and Engelmann (unpublished observations) with cassava plantlets. The duration between the last transfer and the moment when the cultures are placed in storage conditions can be of great importance. It is sometimes better to store the cultures immediately after the transfer, thus avoiding the appearance of necroses and production of phenolic compounds.

Culture vessel. Finally, the type of culture vessel can play a very important role. Test tubes or plastic boxes containing 10 to 20 ml

of medium are routinely used, which allows for increasing the number of replicates of each genotype and limiting the incidence of contaminations. Roca et al. (1984) indicate that, when storing cassava plantlets in 50 x 140 mm bottles instead of 25 x 150 mm test tubes, the rate of shoot elongation in larger vessels almost doubled; however, leaf fall diminished and culture viability greatly increased. In addition, leaves and roots remained healthier in the large vessels.

Modifications of gaseous environment. Growth reduction can be achieved by lowering the oxygen level. Several methods exist in order to decrease the quantity of oxygen available for the tissues. The simplest is to cover the tissues with mineral oil. This technique was first developed by Caplin (1959) with carrot calluses.

Several attempts have been made for storing organized structures using this technique (Chatti-Dridi, 1988; Engelmann et al., unpublished results; Jouve and Engelmann, submitted). Indeed, growth reduction is obtained but vitrification is often observed during storage. Moreover, when returning to standard conditions, re-growth is very slow and partial or complete necrosis of the explants is commonly observed.

Another method consists in lowering the oxygen partial pressure using controlled atmospheres or decreasing the atmospheric pressure of the culture chamber. Tobacco and chrysanthemum plantlets could be stored for 6 weeks under 1.3% oxygen, without impairing their further development (Bridgen and Staby, 1981). This technique was re-employed recently (Engelmann, 1990) for the storage of oil palm somatic embryos. After 4 months in an atmosphere containing 1% O_2, re-proliferation could be obtained very rapidly from the whole culture, whereas control embryos cultivated in standard conditions were severely damaged. This method seems particularly attractive for the storage of tropical species, due to their cold sensitivity. Indeed, growth reduction can be achieved without reducing the culture temperature.

Encapsulation. This technique is now commonly used in the "synthetic seeds" technology by coating somatic embryos in alginate beads. Some preliminary conservation experiments have been carried out recently using this technique. Mulberry buds and sandalwood somatic embryos encapsulated in alginate could be stored for 45 days at +4° C and resume growth after the storage period (Bapat et al., 1987; Bapat and Rao, 1988). The storage duration was extended recently to 4 months with Podophyllum hexandrum somatic embryos (Arumugam and Bhojwani, 1990). This technique could be very promising in the near future for conservation purposes. Indeed, the protection provided to the plant material by encapsulation could increase its resistance to dehydration and low temperature, thus opening new possibilities for medium term storage.

Desiccation. Several attempts have been made using partial desiccation of the plant material. Nitzche (1980) could store desiccated carrot calluses for one year and revive them. McKersie et al. (1990) indicate that pretreatment with ABA could increase the dehydration tolerance, thus improving the conservation possibilities.

Stability of stored plant material. If medium term storage of organized structures appears to be safer when considering trueness to type, it is not the case for the storage of cell lines or calluses. Indeed, several papers mention the loss of growth rate or/and biosynthesis capacities (Seitz, 1987). Moreover, even with organized material, such as shoot cultures, the prolonged storage in more or less detrimental conditions can lead to the selection of particular genotypes, thus leading to the loss of a great part of the genetic variability stored.

Conclusion. Conventional medium term storage techniques are now routinely employed in many laboratories and International Germplasm Conservation Centers (e.g. CIAT, CIP, CATIE). However, the management of large collections, even if the intervals between transfers are greatly extended poses considerable problems (Roca et al., 1989). Thus, complementary techniques, which suppress almost completely the needs for material maintenance, have to be sought.

CRYOPRESERVATION

Methodology

Today, cryopreservation, i.e. storage at a very low temperature, usually that of liquid nitrogen, -196° C, is the only technique which is applicable for long-term storage. The main advantages of cryopreservation, compared with other techniques are listed below:

1. All biological and metabolic processes are stopped.
2. Preservation is possible for a theoretically unlimited period of time.
3. Subcultures are suppressed, and contaminations are avoided.
4. Space requirement is limited.
5. Maintenance and labor costs are drastically reduced.

A cryopreservation process comprises successive steps which have to be defined for every species: choice and obtainment of material, pretreatment, freezing, storage, thawing, and posttreatment.

Choice and obtainment of material. As a general rule, the material will be chosen as young and as meristematic as possible. Indeed, the cells of this type of material are the most likely to withstand freezing: they are small, contain only a few vacuoles, i.e. only a small amount of water, their cytoplasm is dense, their nucleo-cytoplasmic balance is high. The material can be sampled on in vivo or in vitro plants. In vitro material is generally preferable, since the explants are already miniaturized and free of contaminations.

The physiological stage of the material is very important. In the case of cell suspensions, only material at the exponential stage of growth can successfully withstand freezing. With carnation meristems, survival depends on their rank on the shoot axis.

It is sometimes necessary to set up a special culture medium in order to obtain starting material in sufficient quantities. Such is

the case with oil palm embryoids (Engelmann and Dereuddre, 1988a): only a special type of embryoids, which are rarely observed on the standard medium, are likely to withstand freezing. Their frequency is increased by a two month culture on a medium with increased sugar content.

Pretreatment. The pretreatment corresponds to the culture of the material during a certain period of time (several minutes to a few days) in conditions which prepare it to the freezing process. It is carried out using various cryoprotective substances like sucrose, sorbitol, mannitol, dimethylsulfoxide, polyethylene glycol, etc., which differ greatly one from the other by their molecular weight and their structure. The exact mode of action of these substances is unclear: they have an osmotic role and act thus by dehydrating the cells but they may also act by protecting membranes, enzymatic binding sites from freezing injury. They are sometimes classified in penetrating and non-penetrating compounds, the first ones having both above cited effects, the second ones acting only as osmoticums.

For every species, one will have to determine the nature of cryoprotectants, their concentration as well as the duration of the pretreatment. In some cases, the pretreatment will have to be adapted to different clones or varieties for the same material.

Freezing. Different types of freezing processes can be carried out: ultra-rapid, rapid, or slow freezing. In the later case, a programmable freezing apparatus will be needed in order to obtain precise and reproducible freezing conditions.

At the cellular level, the different freezing processes correspond to different mechanisms as regards water fluxes and crystallization: during slow freezing, crystallization occurs first in the external medium. The water flows out of the cells to the external ice. The cells will have to be both sufficiently dehydrated so as crystallization of the residual water will cause no damage in order to avoid toxicity due to the concentration of the internal solutes, which increases with dehydration. During rapid freezing, intracellular ice crystallizes in microcrystals of a size which is not harmful to the integrity of the cell components.

For every material, the following criteria will be determined:

1. Freezing rate. It can be very precise, as in the case of pea and strawberry meristems, or comprise a much broader range, as in the case of oil palm somatic embryos.

2. Starting and pre-freezing temperature (i.e. the temperatures of beginning and end of programmed freezing). These parameters are often very important. In the case of cassava meristems, a pre-freezing temperature of -20° C ensures 91% survival; only 3.3% is observed if the controlled freezing stops at -40° C (Kartha et al., 1982).

Storage. The maximal storage duration is theoretically unlimited, provided that the samples are permanently kept at the

temperature of liquid nitrogen. The material remains exposed to natural radiations. However, the level of mutations caused by natural radiations during storage will reach an irreparable level after thawing of the stored material only after several thousand years.

Thawing. In the majority of the cases, thawing is carried out rapidly by immersing the cryotubes containing the samples in a water-bath thermostated at around +40° C. The aim is to avoid the fusion during thawing of the ice microcrystals formed during freezing to larger crystals of a size which would damage the cellular integrity. However, slow thawing is sometimes necessary (Withers, 1979; Marin et al., 1990).

Posttreatment. Posttreatment consists of culturing the material in conditions ensuring its recovery in the best conditions possible. Cryoprotective substances are progressively eliminated by rinsing, dilution, diffusion, for they are toxic if kept too long in contact with the material.

It is sometimes necessary to attenuate the osmotic shock caused by an immediate transfer on a medium with low osmotic potential by successive transfers of the material on progressively less concentrated media (Engelmann et al., 1985). In some cases, the nature of the medium must be changed (solid versus liquid, and vice versa), in order to better the re-growth. With cell suspensions, a transitory culture phase on solid medium is commonly used before returning to liquid conditions. Recovery can eventually take place in the dark, in order to avoid photooxidation phenomena which can be harmful for the recovery of the material (Benson et al., 1989). Finally, the composition of the culture medium can be transitorily modified by changing the hormonal content or the mineral composition.

Viability assessment

The only definitive assessment of viability is re-growth of the material after thawing. However, it is very important to know as soon as possible if the material is living after freezing, whereas, in many cases, re-growth is very slow. Two main tests exist in order to measure the viability of the material, which can be applied very rapidly after thawing. However, their major disadvantage is that they are destructive. Non-destructive methods for estimating the viability of the material are being sought (Benson and Withers, 1987). These tests are:

1. FDA (fluorescein diacetate). FDA is absorbed by the living cells and transformed into fluorescein, whose fluorescence is measured in UV. This test is qualitative (Widholm, 1977).

2. TTC (triphenyl tetrazolium chloride). TTC is reduced into formazan, colored in red, in the mitochondria of the living cells. This test is quantitative for cell suspensions but is only qualitative for large tissues and organs (Steponkus and Lanphear, 1967).

RESULTS

Various types of cultures

Today, cryopreservation has been applied to more than 70 different species. However, in many cases, resistance to freezing in LN has been proven at the laboratory level, but it does not necessarily imply that the technique is effectively used for germplasm storage of many species. Table 1 presents the list of the species of tropical origin which have been frozen as cell suspensions, calluses, meristems and embryos and for which plants were regenerated *in vitro* or *in vivo*.

Cell suspensions. For cell suspensions, routine techniques adapted to a large number of species have been proposed for several years (Withers, 1985). Concerning the setting up of particular conditions for the successive phases of a cryopreservation process, the following remarks can be made. The cells must be used during their exponential growth period. Before the application of the cryoprotective compounds, a pre-growth period of several hours or days in the presence of compounds with osmotical properties is sometimes necessary. For cryoprotection, various substances are employed, individually or in binary or ternary mixtures which are often more efficient than only one component at the same total osmolarity. Concerning the freezing procedure, slow freezing (0.1 to 1° C min^{-1}) is routinely used. Increasing the cooling rate generally leads to a decrease in viability. The pre-freezing temperature is usually between -30 and -40° C.

Rapid thawing is usually employed using a water bath thermostated at +30/40° C. Increasing the temperature of the water bath to +60 or +80° C can lead to an improvement of the results (Reuff *et al.*, 1988). The same authors mention the utilization of a microwave oven for a more homogeneous thawing, which gave very good results.

Regarding post-thaw treatments, the cells are eventually washed in order to remove the cryoprotectants (Ulrich *et al.*, 1984). However, in the majority of the cases, this treatment is deleterious to the survival of the cells due to the osmotic shock created. The cryoprotective substances are removed slowly by means of diffusion. A transitory culture on a semi-solid medium is required for recovery which lasts generally for one or two weeks, before the cells are transferred again to normal culture conditions. The re-growth medium can be transitorily modified by incorporating compounds with osmotical properties, so as to reduce the osmotic shock, altering the mineral composition, or adding activated charcoal.

Protoplasts. For protoplast cryopreservation, the conditions of the successive steps are comparable to that developed for cell suspensions, but for the posttreatments the protoplasts are re-suspended immediately in liquid medium, and the cryoprotective medium is progressively diluted (Takeuchi *et al.*, 1982).

Calluses. For callus cryopreservation, actively growing calluses are needed. They are submitted to a pretreatment with cryoprotective mixtures such as Polyethylene glycol, glucose and DMSO for rice and

date palm calluses (Finkle et al., 1982), or DMSO and glucose (Ling et al., 1987) with sugarcane calluses. Freezing is usually carried out slowly (freezing rate of 1^{o} C min^{-1}) to -23^{o} C (date palm) or -40^{o} C (sugarcane). In the case of sugarcane, survival of cryopreserved material is obtained only if the samples are held for two hours at the terminal pre-freezing temperature. Thawing is carried out rapidly and the calluses are rinsed with a simplified liquid medium containing 3% sucrose before being transferred onto standard semi-solid medium (Finkle et al., 1982). These authors underline the importance of the temperature at which the cryoprotective substances are added and removed. Survival is obtained only when these operations are carried out at 0^{o} C. Re-proliferation of sugarcane calluses is enhanced when it is performed in the dark (Ling et al., 1987).

Meristems. In the case of meristems, the aim is to preserve the whole structure, which is of macroscopic size, and to obtain its direct re-growth without adventive organogenesis. With potato, survival is improved if the meristems are placed on standard medium for 1 to 3 days before any contact with cryoprotective substances, in order to re-initiate growth (Benson et al., 1989). Pre-growth in presence of cryoprotective substances is frequently necessary (Kartha et al., 1982). Concerning the freezing procedure, there is no general rule. Ultra rapid, rapid as well as slow freezing can be employed, depending on the species. Cassava and potato meristems survive to direct immersion in liquid nitrogen (Bajaj, 1977a; Grout and Henshaw, 1978). However, Towill (1983), using potato meristems coming from in vitro cultivated plantlets, obtains re-growth using slow freezing (0.2 to 0.3^{o} C min^{-1} to -35^{o} C). The type of development after thawing depends on the freezing method. Potato meristems show callusing after rapid freezing (Benson et al., 1989). On the contrary, direct re-growth is obtained after slow freezing. Thawing is usually rapid, by immersion of the material in a water bath or in sterile medium thermostated at $35-40^{o}$ C. Recovery occurs generally directly on the standard medium.

Embryos. The main characteristics of this type of material is its size, which is generally large, according to cryopreservation standards. The embryos often comprise differentiated structures and tissues. Thus, embryos as young and as immature as possible (e.g. globular stage) will be preferentially used. Concerning embryo cryopreservation, two different categories of material can be considered: zygotic embryos, which are harvested on in vivo material and placed in vitro only after cryopreservation, and embryos which are already cultivated in vitro. The challenge is different for these two categories. For zygotic embryos, the whole structure has to be preserved in order to give rise to a whole plant, whereas with somatic embryos, only the proliferation capacities of the material must be preserved and not necessarily their structural integrity. For this latter group, standard cryopreservation techniques are used. An additional stage, prerequisite to cryopreservation, may be necessary, in order to produce a particular type of material, i.e. embryos at the right developmental stage (Engelmann and Dereuddre, 1988a). After an eventual culture for several days in the presence of cryoprotective substances, the embryos are pretreated with cryoprotective compounds. The embryos are usually frozen in liquid medium. However, dry freezing is employed with Citrus (Marin and Duran-Vila, 1988), oil palm

(Engelmann et al., 1985). The freezing rates must be precisely determined, 0.5° C min^{-1} to -42° C for Citrus somatic embryos, rapid freezing for rice pollen embryos (Bajaj, 1980). However, in the case of oil palm, a wide range of cooling rates (0.1 to 200° C min^{-1}) can be employed (Engelmann and Dereuddre, 1988b). Thawing is usually rapid, with an exception for Citrus embryos which are slowly re-warmed at room temperature (Marin and Duran-Vila, 1988). There are different possibilities offered for re-growth: the embryos may be transferred directly onto standard medium, or media modified by transitory addition of growth regulators (Engelmann et al., 1985), or compounds with osmotical properties (Bertrand-Desbrunais et al., 1988) may be used.

In the case of zygotic embryos, which are excised from the seed and frozen immediately, the cryopreservation process is generally different. The cotyledons are removed and only embryonic axes are used. The partial dehydration usually provided by the contact with the cryoprotective solution, is obtained in placing the explants under the laminar flow and letting them dehydrate in the air current. The intensity of this dehydration is adapted to the desiccation tolerance/sensitivity of the species. Rapid dry freezing is usually employed, but controlled slow cooling (2° C min^{-1}) proves to be successful with cassava embryonic axes (Marin et al., 1990). Slow thawing is usually employed. Re-growth generally takes place on the standard medium.

New Cryopreservation Techniques

The aim of these new freezing techniques is to look for eventual simplifications to the standard cryopreservation protocols.

Encapsulation. This technique is adapted from the medium term storage experiments carried out by Bapat et al. (1987). It was developed by a french research team, using pear meristems (Dereuddre et al., 1990). It is based on the fact that encapsulation protects the structure embedded and makes it resistant to treatments which otherwise would be lethal. The alginate beads containing the explants (meristems or somatic embryos) are cultivated for several hours/days in a liquid medium with high sucrose level, then partially desiccated under the laminar flow and frozen either slowing or rapidly. After slow thawing, the beads are transferred on standard medium. Re-growth of the explants excised from the beads is satisfactory.

Vitrification. This technique was developed recently by various authors (Uragami et al., 1989; Langis et al., 1989; Langis and Steponkus, 1990; Towill, 1990), using cell suspensions, protoplasts, somatic embryos and meristems of various species. In a vitrification process, the material is frozen ultra-rapidly, in order that the water vitrifies, i.e. forms an amorphous glassy structure, thus avoiding the problems caused by ice formation inside the cells. In order to achieve vitrification, a rapid and very precisely timed pretreatment in the presence of very high cryoprotectant concentrations is needed. Dilution of the cryoprotective medium, after thawing, is also very precise. It seems that, at least for cell suspensions, the complexity of the freezing phase in the standard procedure is moved to the

pretreatment phase.

Use of a Domestic Freezer. With this technique, freezing is achieved using a domestic freezer (-20° C to 40° C) (Maddox et al., 1982-83; Petiard et al., 1989). If the experimental conditions are well-defined, precise and reproducible, cooling rates, which are a prerequisite to the potential routine use of this technique, can be obtained.

Trueness to type, storage duration. The possible variations of the material due to cryopreservation have been principally checked on the production of particular compounds by cell strains (Seitz, 1987). Until now, no modifications, after thawing, of the properties of the stored material have been observed. Concerning organized structures, plants obtained from frozen meristems (Bajaj, 1983, 1985) or embryos (Engelmann, 1989) of several species appeared to be normal.

Concerning storage duration, the experience is very limited with plant material. Indeed, the maximal storage duration experimented is 4 years, in the case of cassava and potato meristems (Bajaj, 1985). Until now, all storage experiments led to the obtainment of true-to-type material.

CONCLUSION

In conclusion, tissue culture techniques, together with cryopreservation techniques, are of great interest for the medium and long-term conservation of plant germplasm, particularly that of tropical species. The following remarks can be made concerning their present and future use:

1. The development of medium term conservation techniques is easy and satisfactory storage conditions can generally be obtained without extensive research or sophisticated equipment.

2. These techniques are now routinely employed in many laboratories and International Germplasm Conservation Centers (e.g. CIAT, CIP, CATIE, INIBAP). However, the management of large collections, even if the intervals between transfers are greatly extended, poses considerable problems (Roca et al.,1989).

3. The stored material must be checked regularly, as regards its stability.

4. On the contrary, cryopreservation ensures a very good stability.

5. However, the development of a cryopreservation techniques requires extensive research and the use of very sophisticated equipment.

6. Thus, cryopreservation is presently used only at the laboratory level for the storage of small collections and its utilization on a large scale is currently exceptional.

7. The research presently focuses on the setting up of less sophisticated freezing techniques, which could facilitate the routine use of cryopreservation.

8. The safe conservation of the germplasm of a particular plant species requires the use of both storage techniques, which are complementary: medium term storage for an active collection which is used for germplasm exchange, experiments, etc., cryopreservation for a base collection which is stored for the long term.

Over the last years, national and international bodies, public research institutes and private firms have shown increasing interest in germplasm storage and cryopreservation. This encourages us to feel optimistic about the development of routine techniques for the safe storage of tropical germplasm.

Table 1. List of tropical plant species cryopreserved as cell suspensions (a), calluses (b), protoplasts (c), meristems (d), somatic (e), pollinic (f) and zygotic (g) embryos.

(a) Cell Suspensions	
Berberis dictyophilla	Withers, 1985
Berberis wilsoniae	Reuff, 1987
Brunifelsia dentifolia	Pence, 1990
Capsicum annuum	Withers and Street, 1977
Catharantus roseus	Kartha et al., 1982
	Chen et al., 1984
	Withers, 1985
Corydallis sempervirens	Withers, 1985
Dioscorea deltoidea	Butenko et al., 1984
Glycine max	Bajaj, 1976
	Weber et al., 1983
Hyosciamus muticus	Withers, 1985
Musa	Panis et al., 1990
Myrtillocactus geometrizans	Haffner, 1985
Nicotiana plombaginifolia	Maddox et al., 1983
Nicotiana sylvestris	Maddox et al., 1983
Nicotiana tabacum	Withers, 1985
	Bajaj, 1976
	Hauptman and Widholm, 1982
Oryza sativa	Sala et al., 1979
	Finkle and Ulrich, 1982
	Ulrich et al., 1984
Panax ginseng	Butenko et al., 1984
	Chen et al., 1984
	Seitz and Reinhardt, 1987
Rhazia orientalis	Withers, 1985
Rhazia stricta	Withers, 1985
Saccharum officinalis	Finkle and Ulrich, 1979, 1982
Solanum melongena	Withers, 1985
Sorghum bicolor	Withers and King, 1980
Tabernaemontana divaricata	Schrijnermakers et al., 1990
Vinca minor	Caruso et al., 1987
Zea mays	Withers and King, 1980
	Shillito et al., 1989
(b) Callus	
Glycine max	Engelmann, unpublished results
Gossypium arboreum	Bajaj, 1982
Oryza sativa	Finkle et al., 1982
	Ulrich et al., 1984
Phoenix dactylifera	Tisserat and Ulrich, 1979
	Tisserat et al., 1981
	Finkle et al., 1984
Saccharum spp.	Ulrich et al., 1979
	Ling et al., 1987

(c) Protoplasts

Glycine max	Takeuchi et al., 1982
	Weber et al., 1983
Nicotiana tabacum	Bajaj, 1988
Oryza x Pisum	Bajaj, 1983a
Zea mays	Withers, 1980

(d) Meristems

Arachis hypogeaea	Bajaj, 1979
Cicer arietinum	Bajaj, 1979
Lycopersicon esculentum	Grout et al., 1978
Manihot esculenta	Bajaj, 1977a, 1983b, 1985
	Kartha et al., 1982
Phoenix dactylifera	Bagniol et al., 1990
Solanum etuberosum	Towill, 1981
Solanum goniocalix	Grout and Henshaw, 1978
Solanum tuberosum	Standke, 1978
	Bajaj, 1985
	Benson et al., 1984, 1989
Xanthosoma	Zandvoort, 1987
Vanda hookeriana	Kadzimin, 1988

(e) Somatic Embryos

Citrus sinensis	Marin and Duran-Vila, 1988
Coffea arabica	Bertrand-Desbrunais et al., 1988
Elaeis guineensis	Engelmann et al., 1985
	Engelmann and Duval, 1986
	Engelmann and Dereuddreb, 1988
Manihot esculenta	Sudarmonowati and Henshaw, 1990
Xanthosoma	Zandvoort, 1987

(f) Pollen Embryos

Arachis hypogea	Bajaj, 1983c
Arachis villosa	Bajaj, 1983c
Citrus spp.	Bajaj, 1984
Gossypium arboreum	Bajaj, 1982
Nicotiana tabacum	Bajaj, 1977b, 1978
	Coulibaly and Demarly, 1979
Oryza sativa	Bajaj, 1981

(g) Zygotic Embryos

Carva	Pence and Dresser, 1988
Cocos nucifera	Bajaj, 1984
	Chin et al., 1989
Elaeis guineensis	Grout et al., 1983
Hevea brasiliensis	Normah et al., 1986
Howea fosteriana	Chin et al., 1988
Manihot esculenta	Marin et al., 1990
Veitchia merrillii	Chin et al., 1988
Zea mays	Delvallée, 1987; de Boucault, 1988

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