USE OF CRYOPRESERVATION FOR PLANT GERMPLASM LONG-TERM CONSERVATION – CASE HISTORY: OIL PALM SOMATIC EMBRYOS

F. ENGELMANN

Office de la Recherche Scientifique et Technique Outre Mer (ORSTOM)
BP 5045, 34032 Montpellier Cedex 1, France

SUMMARY

Cryopreservation (i.e. storage at a temperature of liquid nitrogen, -196°C) is the only technique available presently for long-term conservation of plant germplasm. For every plant species, optimal conditions have to be defined for the successive steps of the cryopreservation process : choice and procurement of starting material, pre-treatment, storage, freezing, thawing, post-treatment. Resistance to liquid nitrogen temperature has been obtained with more than 70 species. The first results concerning the use of cryopreservation as a routine technique are presented in the case of oil palm somatic embryos.

INTRODUCTION

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

- orthodox seeds, which can withstand dehydration to 5% or less (dryweight basis) without damage. When dry, the viability of these seeds can be prolonged by keeping them at the lowest temperature and moisture possible;
- recalcitrant seeds, which are high in moisture and are unable to withstand much desiccation. They are predominantly seeds from tropical or subtropical trees or shrubs. 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 species of great economic importance, like oil palm, coffee, coconut, rubber, 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. poses also 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 determined 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 to 5000 - 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 often of very large size, whereas land availability drastically decreases. Moreover, as most forest trees are genetically heterozygous, it is thus necessary to preserve a large sample to maintain as much as possible the genetic variation within a population. Besides, labour costs and trained personnel requirement 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 climax forest trees, we do not possess even the rudiments of knowledge of the biology of the species.

The use of *in vitro* tissue culture techniques can be of great interest for germplasm storage of recalcitrant and vegetatively propagated species. Tissue culture systems present the following advantages:

(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 labour costs and financial terms.

MEDIUM TERM STORAGE/SLOW GROWTH

For medium term storage, growth reduction can be achieved using separately or simultaneously classical procedures, such as temperature and light intensity reduction, alterations to the basic medium, addition of growth retardants or compounds with osmotic effects like mannitol, etc. (Engelmann 1990a). These techniques are now routinely used in many laboratories for the storage of many plant species *in vitro*. Alternative methods are being developed, like partial desiccation (Nitzche 1980), encapsulation (Bapat *et al.* 1987; Bapat and Rao 1988), hypoxia using mineral oil overlays (Caplin 1950; Augereau *et al.* 1986) or controlled atmosphere (Bridgen and Staby 1981). This last technique may be of great interest for the storage of tropical species, which are often highly cold sensitive, as illustrated by the recent work of Engelmann (1990b), for the medium term storage of oil palm somatic embryos. Indeed, the standard culture temperature is not modified with technique.

LONG-TERM STORAGE/CRYOPRESERVATION

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:

- storage at a very low temperature (liquid nitrogen, -196°C)
- all biological and metabolic processes stopped
- preservation possible for a theoretically unlimited period of time
- subcultures suppressed, contaminations avoided
- space requirement limited
- maintenance and labour costs drastically reduced.

Methodology for Cryospreservation

A cryopreservation process comprises successive steps which have to be defined for every species:

- choice and procurement of material
- pre-treatment
- freezing
- storage
- thawing
- post-treatment.

1. *Choice and procurement 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 miniaturised 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 (Dereuddre *et al.* 1988).

It is sometimes necessary to set up a special culture medium in order to obtain starting material in sufficient quantities. It is the case with oil palm embryoids: only a special type of embryoids, shiny white, finger-like shaped, which are often grouped into clumps, are likely to withstand freezing. These particular embryoids are very rarely observed in standard culture conditions. Their frequency is increased by a two- month culture on a medium containing 0.3M sucrose instead of 0.1M, which is used in the standard culture medium (Engelmann & Dereuddre 1988a).

2. *Pre-treatment*

The pre-treatment corresponds to the culture of the material during a certain peiod 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 mannitol, sorbitol, sucrose, DMSO, etc. 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 act also 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 pre-treatment will have to be adapted to different clones of varieties for the same material.

3. *Freezing*

Different types of freezing processes can be carried-out: ultra-rapid (several hundreds $°C.min^{-1}$), or slow freezing (0.1 to several $°C.min^{-1}$). 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 described above correspond to different mechanisms as regards water fluxes and crystallization. During slow freezing, crystallization occurs firstly in the external medium. The water of the cells flows out to the external ice. The cells will have to be at the same time sufficiently dehydrated so as the crystallization of the residual water will cause no damage and not too much 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 components of the cells.

For every material, the following criteria will be determined:

- freezing rate: it can be very precise, as in the case of pea and strawberry meristems (Kartha *et al.* 1979, 1982), or comprise a much broader range, as in the case of oil palm somatic embryos (Engelmann & Dereuddre 1988b).
- starting and prefreezing temperature: i.e. the temperatures of beginning and end of programmed freezing. These paramaters are often very important: in the case of cassava meristems, a prefreezing temperature of -20°C ensures 91% of survival: only 3.3% are observed if the controlled freezing stops at -40°C (Kartha *et al.* 1982).

4. *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. The following calculation has been made on animal cells: the level of mutations caused by natural radiations during storage will reach an irreparable level after thawing of the stored material only after a minimum of 10 000 years.

5. *Thawing*

In the majority of the cases, thawing is carried out rapidly by immersing the cryotubes containing the samples in a water-bath thermostatically controlled at around +40°C. The aim is to avoid the fusion during thawing of the ice micro-crystals formed during freezing to larger crystals of a size which would be damageable to cellular integrity.

6. *Post-treatment*

Post-treatment consists in 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.

The osmotic shock caused by an immediate transfer on a medium with low osmotic potential has to be attenuated by succesive transfers on progressively less concentrated media. In some cases, the nature of the medium must be changed (solid versus liquid, and vice versa), in order to better the regrowth.

Recovery can eventually take place in the dark, in order to avoid photo-oxidation phenomena which can be harmful for the recovery of the material.

Finally, the hormonal content of the culture medium can be transitorily modified. It is the case with oil palm embryoids, for which an auxin has to be added during a few days after thawing in order to stimulate the recovery of the proliferation (Engelmann *et al.* 1985).

7. *Viability assessment*

The only definite assessment of viability is regrowth of the material after thawing. However, it is very important to know as soon as possible whether the material is living after freezing, whereas, in many cases, the regrowth 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. These tests are:

- FDA (fluorescein diacetate): FDA is absorbed by the living cells and transformed into fluorescein, whose fluorescence is measured in UV. This test is quantitative (Widholm 1977).
- TTC (triphenyl tetrazolium chloride): TTC is reduced into formazon, coloured red, in the mitochondria of the living cells. It is quantitative for cell suspensions (measurement of % of the control), but is only qualitative for large tissues and organs (Steponkus & Lanphear 1967).

Results

1. *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 in the laboratory, but it does not necessarily mean that the technique is effectively used for germplasm storage of many species. Table 1 presents the list of the species which have been frozen as cell suspensions, calluses, protoplasts, meristems and embryos. For more complete references, see Kartha 1985; Engelmann & Baubault 1986; Dereuddre & Engelmann 1987; Engelmann 1990a.

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

a) **cell suspensions**

Acer pseudoplatanus
Acer saccharum
Atropa belladonna
Berberis dictyophilla
Brassica napus
Brassica campestris
Capsicum annuum
Cathalantus roseus
Coleus blumei
Corydallis sempervirens
Datura innoxia
Datura stramonium
Daucus carota
Digitalis lanata
Dioscorea deltoidea
Glaucium flavium
Glycine max
Hordeum vulgare
Hyosciamus muticus
Linum usitatissimum
Medicago satiya
Myrtillocactus geometrizans
Nicotiana plombaginifolia
Nicotiana sylvestris
Nicotiana tabacum
Onobrychis viciifolia
Oryza sativa
Panax ginseng
Pennisetum americanum
Populus euramericana
Pseudotsuga menziesii
Rhazia orientalis
Rhazia stricta
Rosa Paul's scarlet
Saccharum officinalis
Solanum melongena
Sorghum bicolar
Triticum monococcum
Vinca minor
Zea mays

b) **callus**

Coleus blumei
Fragaria ananassa
Gossypium arboreum
Hordeum vulgare
Lavandula vera
Medicago sativa
Phoenix dactylifera
Populus americana
Saccharum spp.
Triticum aestivum
Ulmus americana

c) **protoplasts**

Atropa belladonna
Bromus inermis
Datura innoxia
Daucus carota
Glycine max
Hordeum vulgare
Marchantia polymorpha
Nicotiana tabacum
Oryza × *Pisum*
Triticum × *Pisum*
Triticum aestivum
Zea mays

Table 1. continued

d) **meristims**

Arachis hypogeaea
Asparagus officinalis
Beta vulgaris
Brassica napus
Brassica oleacera
Cicer arietinum
Dianthus caryophyllus
Digitalis lanata
Fragaria ananassa
Haplopapus gracilis
Lilium multiflorum
Lycopersicon esculentum
Malus domestica
Malus spp.
Manihot esculenta
Mentha spp.
Morus bombycis
Pisum sativum
Pyrus communis
Pyrus serotina
Rubus spp.
Solanum etuberosum
Solanum goniocalix
Solanum tuberosum
Xanthosoma
Vanda hookeriana
Vaccinium spp.

e) **somatic embryos**

Asparagus officinalis
Citrus sinensis
Coffea arabica
Daucus carota
Elaeis guineensis
Picea abies
Picea glauca
Pinus taeda
Xanthosoma

f) **pollen embryos**

Arachis hypogea
Arachis vilosa
Atropa belladonna
Brassica campestris
Brassica napus
Citrus spp.
Gossypium arboreum
Nicotiana tabacum
Oryza sativa
Petunia hybrida
Primula abconica
Triticum aestivum

g) **zygotic embryos**

Aesculus hypocastanea
Araraucaria excelsa
Brassica napus
Capsella bursa pastoris
Carva
Castanea
Cocos nucifera
Elaeis guineensis
Fagus
Hevea brasiliensis
Hordeum vulgare
Howea fosteriana
Juglans
Phaseolus vulgaris
Picea glauca
Quercus
Triticum aestivum
Triticale
Veitchia merrillii
Zea mays

Most of the research on trees concerns cell suspensions or calluses, which have been used more as experimental models than for practical purposes, or fruit trees. Moreover, due to the difficulty of *in vitro* culture of tree species, many of these results are incomplete. The most promising results, particularly in the frame of plant genetic resources conservation, concern the cryopreservation of zygotic embryos.

2. *Storage duration, trueness to type*

The possible variations of the material due to cryopreservation have been principally checked on the production of particular compounds by cell strains (e.g. steroid from *Dioscorea deltoidea,* alcaloids from *Catharantus roseus*). Until now, no modifications, after thawing, of the properties of the stored material have been observed. Plants obtained from frozen meristems or embryoids of several species (groundnut, potato, oil palm) 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. Until now, all storage experiments produced true-to-type material. Although, theoretically, there is no difference between 1 minute and many years of storage, since the material does not evolve when it is at -196°C, it was nevertheless important to prove the feasibility of this method of storage.

Example: Oil palm somatic embryo cryopreservation

The oil palm vegetative propagation process set up by ORSTOM and IRHO (IRHO: Research Institute for Oils and Fats, 11 square Petrarque, 75116, Paris, France) uses somatic embryogenesis (Pannetier *et al.* 1981). The present development of the process is as follows:

It is now applied in 5 different laboratories in France, Côte d'Ivoire, Malaysia and Indonesia. It has been applied to 850 ortets and 456 embryoid clones have been obtained. About 500 000 ramets have been produced and 138.5 hectares have been planted for trials in Côte d'Ivoire including 79 clones. Abnormalities have been observed on less than 5% of the total of the material.

Researches for setting up a cryopreservation process have started in France in 1982, in order to face the following problems:

- on the one hand, the risks of obtaining abnormal material which increase with *in vitro* culture duration, as it was shown in the case of oil palm (Corley *et al.* 1986). Storing the embryoids as early as possible after they have been obtained should increase our chances of storing true-to type material.
- on the other hand, the continuous production of new clones induces laboratory management problems. Cryopreservation allows to store the clones which are not used for commercial produciton, thus reducing the quantity of material which has to be regularly subcultured.

The cryopreservation process which has been set up is presented in Figure 1. The folowing conditions have been defined:

- Choice and procurement of material: only young embryoids, shining white, finger-like shaped, often grouped into clumps, are likely to withstand freezing. They are obtained in sufficient numbers after a two-month culture on a medium enriched with sucrose (1-2).
- Pre-treatment: the clumps of embryoids are placed for 7 days on a medium containing 0.75 M sucrose. Their water content decreases from 80% to around 60% (3).
- Freezing: the clumps are placed in sterile cryotubes and frozen rapidly by direct immersion in liquid nitrogen (-200°C.min^{-1}) (4a). A two-step freezing can be carried out using a programmable freezing apparatus: the cryotubes are frozen from +20°C to -100°C at a rate which can vary from 5 to 40°C.min^{-1}, then plunged in liquid nitrogen (4b).

- Thawing: the cryotubes are plunged for 1 minute in a water-bath thermostatically controlled at +40°C (6).
- Post-treatment: the embryoid clumps are cultured for 3 weeks on media added with 2,4-D and containing progressively less sucrose. Afterwards, they are transferred to the standard medium devoid of growth regulators (7a-b).

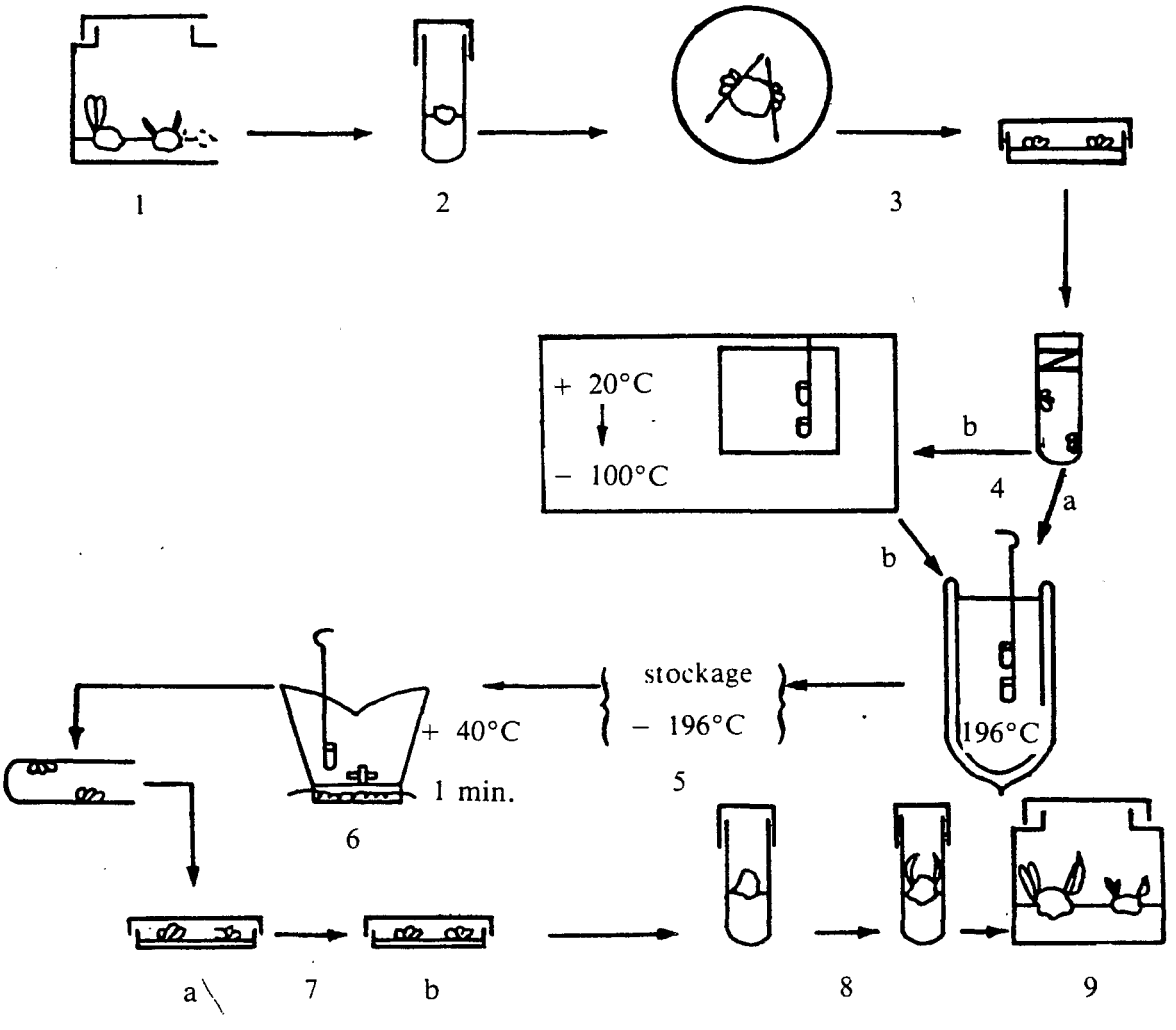

Figure 1. Schematical representation of the oil palm somatic embryo cryopreservation process (after Engelmann 1986). See in text for explanations.

The technique described above has been successfully applied to 27 different clones, with an average recovery rate of 12.5%. It has been checked with 2 clones that the extension of the storage duration to respectively 12 and 15 months in liquid nirogen did not modify the recovery rate. Finally, ramets from two cryopreserved clones have been produced and planted in the field at the IRHO La Me Research Station in Cote d'Ivoire. No difference was observed when compared with non frozen controls. The first male inflorescences, which were observed recently on cryopreserved material, are perfectly normal. New ramets coming from frozen material are now in the nursery. They will soon be planted in order to confirm these first results.

These results have been judged sufficient to decide to apply the technique in the laboratories producing oil palm embryoids through the ORSTOM-IRHO process. The experiments have started simultaneously in France, Cote d'Ivoire, Malaysia and Indonesia in 1989, on approximately 155 clones. We soon will have more information on the possibility of using cryopreservation as a routine technique for the long-term storage of oil palm embryoids.

CONCLUSION

In conclusion, tissue culture, together with cryopreservation, are of great interest for the medium and long-term conservation of plant germplasm *in vitro*. Easily usable techniques have been developed for the medium-term storage of many plant species, particularly for those coming from temperate areas. In the case of tropical species, alternative techniques, such as hypoxia, may have to be sought, due to the cold sensitivity of the material.

As concerns cryopreservation, the resistance to freezing in liquid nitrogen has been obtained for a large number of species. However, the routine use of cryopreservation still remains exceptional.

Finally, the development of these various techniques requires sophisticated equipment as well as important technical background. The development of cooperative programmes between local institutes, which have a good knowledge of the plant material, and overseas research centres, which possess the equipment and technical experience appears as the most efficient way of rapidly developing processes for the medium and long-term storage of tropical plant germplasm.

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Sitti Soetarmi Tjitrosomo
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