Annexe 8

CONSERVATION IN VITRO OF FOREST TREE GENETIC RESOURCES

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CONSERVATION IN VITRO OF FOREST TREE GENETIC RESOURCES

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1 - 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 prolongated 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 comprizes many species of great economic importance (Tabl.1).

Table 1 : Economically important plants with recalcitrant seeds (After Kartha, 1985)

Family	Scientific name	Common name
Anacardiaceae	*Mangifera indica*	Mango
Bombacaceae	*Durio zibethinus*	Durian
Dipterocarpaceae	*Dryobalanops aromatica*	Borneo camphor
Erythroxylaceae	*Erythroxylum coca*	Cocaine
Euphorbiaceae	*Hevea brasiliensis*	Rubber
Fagaceae	*Castanea* spp.	Chestnut
Fagaceae	*Quercus* spp.	Oak
Gramineae	*Zizania aquatica*	Wild rice
Guttiferae	*Garcinia mangostana*	Mangosteen
Juglandaceae	*Carya illinoensis*	Pecan
Juglandaceae	*Juglans* spp.	Walnut
Lauraceae	*Cinnamomum* sp.	Cinnamon
Lauraceae	*Persea americana*	Avocado
Meliaceae	*Swietenia* sp.	Mahogany
Moraceae	*Artocarpus heterophyllus*	Jackfruit
Palmaceae	*Cocos nucifera*	Coconut
Rubiaceae	*Coffea arabica*	Coffee
Rubiaceae	*Coffea canephora*	Coffee
Rutaceae	*Citrus* spp.	Orange, lemon, grapefruit, etc.
Sterculiaceae	*Theobroma cacao*	Cacao
Theaceae	*Thea sinensis*	Tea
Zingiberaceae	*Elettaria cardamomum*	Cardamom

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 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 to 5,000-20,000 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 oftenly of very large size, whereas land availability drastically decreases. Moreover, as most forest trees are genetically heterozygous, it is thus necessary to preserve a larger sample to maintain as much as possible of the genetic variation within a population. Moreover, 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. In this aim, 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 labour costs and financial terms

In vitro culture is commonly used for medium-term storage. For long term storage, *in vitro* culture is used jointly with cryopreservation.

2 - MEDIUM-TERM CONSERVATION

2.1 - METHODOLOGY/RESULTS

2.1.1- CLASSICAL TECHNIQUES

For medium term storage of germplasm, the various techniques listed below can be used :

(1) Alterations to the physical conditions :
- temperature
- light intensity and photoperiod

(2) Alterations to the basic medium :
± nutrients
- factor essential for normal growth

(3) Addition of:
- growth retardants (ABA,CCC)
- compounds with osmotic effects (mannitol)

The most commonly used methods concern the decrease of the culture temperature and light intensity, as illustrated in Table 2. It is important to stress the fact

that, when setting up a medium-term storage method, the aim is not to search the longest subculture period possible, as it is oftenly the case in the literature, but to determine a frequency which ensures the preservation of the material in the best possible conditions, with the minimum of losses. However, many tropical species are cold-sensitive and cannot be stored at low temperatures. For example, oil palm somatic embryo cultures show after a few weeks at 12-18°C severe injuries which drastically reduce their possibilities of proliferation recovery when the material is replaced in optimal conditions. It is thus very important to develop alternatives methods, more adaptated to tropical species.

Table 2 : *In vitro* storage strategies for short- to medium-term storage of plant genetic material (After Kartha. 1985).

Species	In vitro storage methods	Storage duration
Apple (*Malus domestica*)	Shoot-tips of apples cultured on basal medium + 5 mg/ℓ benzylaminopurine and stored at 1°C or 4°C	1 year
Cassava (*Manihot esculenta*)	Nodal cuttings from meristem-derived plantlets maintained in large vessels on nutrient medium with low osmotic concentration and activated charcoal	2 years
	Meristem-derived plants on filter paper bridges in tubes containing nutrient medium. at 20°C, 16 hr photoperiod, 100—200 lx intensity	1 year
Coffee (*Coffea arabica*)	Meristem-derived plants on 1/2 MS medium lacking in sucrose, plantlets kept in tubes at 26°C, 16 hr photoperiods at 4000 lx intensity	2½ years
Forage grasses		
Dactylis spp. *Festuca* sp. *Phleum* sp.	Plantlets maintained at 2—4°C	ca. 1 year
Lolium multiflorum	Shoot-tip plantlets stored at 2—4°C, 300 lx, 3 hr photoperiod	1 year
Forage legumes		
Medicago sp.	Plantlets in vitro stored at 2—6°C	15—18 months
Trifolium sp.	Plantlets in vitro stored at 2—6°C	15—18 months
Grape (*Vitis rupestris*)	Maintenance of shoot-tip cultures at 9°C	1 year
Potato (*Solanum tuberosum*)	Storage of meristem-derived plants at reduced temperature in the range of 6—10°C with a mean survival of 61% at 6°C; survival further increased to 83% when stored at 12°C day/6°C night	1 year
Strawberry (*Fragaria* spp.)	Meristem-derived plantlets stored at 4°C in dark with addition of 1 or 2 drops of culture media every 3 months	6 years
Sugarbeet (*Beta vulgaris*)	Cultures stored at 12°C	18 weeks
Sweet potato (*Ipomoea batatas*)	Meristem-derived plantlets on MS medium with 1.0 μ*M* each of BA and NAA + 5% each of sucrose and mannitol, at 26°C, 16 hr photoperiods and 4000 lx intensity	Drastic growth reduction within 3 months

2.1.2 - ALTERNATIVE METHODS

In this case,the aim is to modify other parameters than temperature. Thus, two methods will be tested :

- modification of the gaseous environment
- desiccation
- encapsulation

2.1.2.1 - Modification ot the gaseous environment

Several methods exist in order to decrease the quantity of oxygen available to the tissues :

- Use of a mineral oil overlay covering the tissues.
This technique was developed first by Caplin (1959) and tested recently by Augereau *et al.* (1986) and Moriguchi *et al.* (1988) for the storage of callus lines of various species. The results are presented in Table 3.

Table 3 : Summary of results concerning the medium term storage of various plant species, using mineral overlay.

Caplin, S.M., 1959
Carrot calluses : 157 days in the dark under a 45 mm mineral oil overlay.
Results : 1/4 of the control

Augereau *et al.*, 1986
-Low temperature storage
C. roseus, V. thouarsii, C. arabica : 4 months in the dark at 15°C
Results : regrowth

-Mineral oil preservation
Amsonia tabunaemontana, Atropa belladona, Catharantus roseus, Coffea, Glycine max, Papaver somniferum, Rosmarinus officinalis, Vinca minor, etc... : 6 months, 26°C, 16h photoperiod, under a30 mm mineral oil overlay.
Results : regrowth 34/37 strains

Moriguchi *et al.*, 1988
Grape callus : 360 days in the dark at 15°C under a 90 mm silicone oil overlay.
Results : 95% regrowth

- Modification of O2 partial pressure (Bridgen and Staby,1981)
The cultures are placed in an air-tight box. The oxygen pressure is lowered by using an air-ejector connected to the box, thus decreasing the quantity of oxygen available to the tissues, or by making up a mixture of oxygen and nitrogen, which is injected in the culture box. This method was successfully applied to chrysanthemum and tobacco plants using O2 concentrations of 1.3 and 8%. No effect could be observed on the growth of the plants after 6 weeks of storage.
This technique was reutilized by Engelmann (unpublished results) for the storage of oil palm somatic embryos. After 4 months of storage in an atmosphere containing 1% O2, reproliferation could be obtained very rapidly from the whole culture, whereas control cultivated in standard conditions was almost completely necrosed.

2.1.2.2 - Desiccation

This technique was firstly used by Nitzche (1980) on carrot calluses. They could be stored for one year and revived after having undergone drying for one night in the air stream of a laminar flow cabinet.

2.1.2.3 - Encapsulation

This technique is now commonly used for the production of "synthetic seeds" by coating somatic embryos in alginate beads. This technique seems very promising in a near future for preservation purposes. The protection of the material provided by encapsulation could increase its resistance to dehydration and low temperature, thus opening new possibilities for medium-term storage.

Some preliminary experiments were carried out by Bapat *et al.*(1987), Bapat and Rao (1988). Encapsulated mulberry buds could be stored for 45 days at +4°C and germinate easily. In a second experiment, sandalwood embryoids could withstand the same storage conditions and duration and give rise afterwards to secondary embryoids.

2.2 - EXAMPLES OF APPLICATION OF MEDIUM-TERM STORAGE

2.2.1 - FOREST TREE SPECIES

Until now, medium-term storage has been applied mainly to species originating from temperate climates, as listed in Table 4.

Table 4 : List of woody species conserved using low temperature storage (After Aitken-Christie and Singh, 1987).

Shoot/plantlet cultures

Cinchona ledgeriana
Eucalyptus dalrympleane
Eucalyptus gunii
Eucalyptus spp.
Malus domestica
Malus spp.
Pinus radiata
Prunus amygdalus
Prunus spp.
Punica granatum
Pyrus communis
Pyrus pashia
Salix babylonica
Sequoia sempervirens
Vitis rupestris
Vitis spp. and hybrids

Cell/callus cultures

Citrus spp.
Coffea spp.
Mallotus japonicus
Pinus sylvestris

For the storage of a given species, the following parameters have to be defined :

2.2.1.1 - Materials

- Type of culture : shoot cultures, embryos, etc.., depending on the propagation process.

- Physiological state : shoot size, juvenility, development of the root system...Very oftenly, already developed plants survive better.

- Substrate : the most suitable culture medium has to be defined. It is advised to chose a medium which is as close as possible to the standard culture medium.

- Container : test-tube, Erlenmeyer flasks, jars...The choice will depend on the size of the explant, quantity of medium, number of replicates required.

2.2.1.2 - Conditions for cold storage

- Equipment and design : the choice will depend on the mode of conservation. As a standard rule, it is better for safety reasons to have 2 replicates of the collection in 2 separate rooms, so as to further reduce the risks of accidental loss.

- Temperature : the choice will depend on the species and the type of conservation. It is recalled that most of tropical species are temperature-sensitive, thus the temperature cannot be drastically lowered without taking important risks.

- Light : it is oftenly necessary, but low intensity are sufficient, so as to reduce growth and avoid cultures to turn white and become etiolated.

- Humidity and free water : it is important to avoid excess of water which could lead to vitrification.

- Space : *in vitro* storage will lead to a drastic space reduction, when compared to the land surface which would be required for the storage *in vivo* of the same quantity of material (Tabl.5).

Table 5 : Comparison between the surfaces required for the storage *in vivo* or *in vitro* of various species

Malus domestica : 2,000 cultures = 0.28 m3 (5.7 ha)

Pinus radiata : 2,000 test tubes = 3,42 m2 (1ha)

Vitis vinifera : 800 cultivars x 6 test tubes = 2 m2 (1ha)

Manihot esculenta : 6,000 accessions (5vessels/accession) = 5 x 6 x 3 room (16 ha)

2.2.1.3 - Factors affecting cold storage

- Requirement for subculture, length of time in cold storage :
these parameters will be defined so as to ensure the maximal survival. The size of the sample to be preserved could thus be reduced.

- Stability : it can be assessed only by comparison with the control. The fact that the material regrowths after storage does not necessarily mean that stored material is free of genetic instability. However, the results in the literature are very encouraging.

2.2.1.4 - Uses for cold storage

- Short-term (less than 1 year) : in commercial laboratories, in order to smooth out production peaks.
- Long-term (more than 1 year) : for preservation of germplasm collections and commercial laboratories, use for long-term evaluation trials (Aitken-Christie and Singh, 1987)

2.2.2 - COCONUT ZYGOTIC EMBRYOS

Coconut seeds are typically heavy and large ; they are not dormant and germinate quickly. Husk complicates phytosanitary treatments. Storage and seed

exchanges are very complicated, particularly when prospecting. *In vitro* culture of zygotic embryos represents an apt and economic solution to these problems. IRHO has developed techniques for embryo field collection and *in vitro* cultivation. This method, which has been tested on more than 20 varieties, is very simple and efficient (Assy Bah, 1986; Assy Bah *et al.*, 1987, 1989). Its performances are summarized in Table 6.

Table 6 : Schematical representation and performances of the coconut mature embryo *in vitro* culture process (After Assy Bah *et al.*, 1989).

100 mature embryos cultured on medium containing 60 g/l sucrose

↓ 1 month

5-20% contamination

↓ 2 months

70-90% germination

↓ 3 months

suppression of the haustorium

↓ 5 months

transfer to the nursery on sand :
73-90% weanable plantlets

↓ 7 months

prenursery and nursery :
92-98% plantlets

Medium-term storage techniques have been set up for mature and immature embryos, which are very efficient, since embryos can be stored for 6 months without decrease in the germination rate.

2.3 - CONCLUSION

Medium-term storage techniques are now routinely used in many laboratories and international centers, e.g. Cassava at the CIAT (Cali, Colombia), potato at the CIP (Lima, Perou).

Most of the work has been carried out on species from temperate climates. Researches have to be focused now on tropical plants, in order to develop storage techniques adaptated to the biology of these species.

Medium-term storage is very important for the conservation of plant germplasm. However, if long-term preservation is required, even extended subculture intervals cannot be considered sufficient. Moreover, as the risks of genetic variation increase with *in vitro* culture duration, as illustrated in the case of oil palm (Corley *et al.*, 1986) and can lead to the loss of trueness-to-type, which is one of the aims of preservation, alternative methods have to be sought.

3 - CRYOPRESERVATION

3.1 - METHODOLOGY

Today, cryopreservation, i.e.storage at a very low temperature, usually that of liquid nitrogen, -196°C, is the only technique which is appliable 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.

3.1.1 - CRYOPRESERVATION PROCESS

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

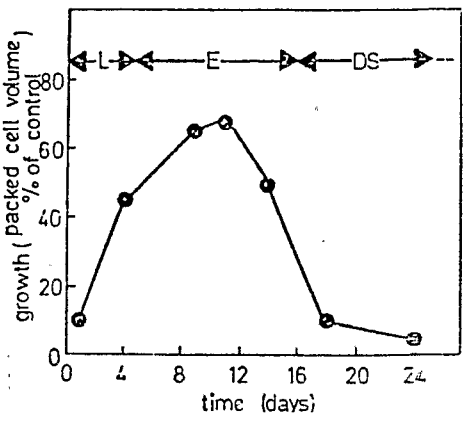
- choice and obtainment of material
- pretreatment
- freezing
- storage
- thawing
- post-treatment

3.1.1.1 - 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 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 successfuly whistand freezing (Fig.1). With carnation meristems, survival depends on their rank on the shoot axis (Fig.2).



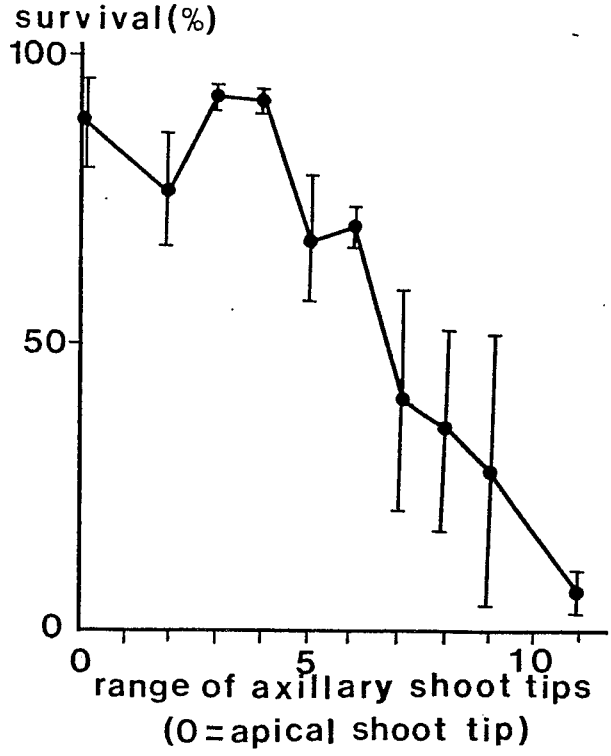


Figure 1

Figure 2

Figure 1 : Recovery growth, (estimated by packed cell volume) of suspension cultured cells of *Daucus carota* sampled during a complete growth cycle (L = lag phase ; E = exponential phase ; DS = decline of growth followed by stationary phase), frozen and thawed and returned to culture at a uniform cell density (After Kartha, 1985).

Figure 2 : Changes in the resistance of axillary shoot tips of carnation as a function of their range along the stem (range 0 corresponded to apical shoot tip). DMSO and sucrose concentrations were respectively 5% and 0.75M (After 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 oftenly 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 *et al.*, 1985).

3.1.1.2 - 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 cryoprotective substances. Some of these compounds are listed in Table 7. They 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 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.

Table 7 : Main cryoprotective substances used for cryopreservation of plant cells, tissues and organs (After Gazeau and Dereuddre, 1986)

NATURE	MOLECULAR WEIGHT	CHEMICAL FORMULA
- ALCOOL		
ETHYLENE GLYCOL	62	CH2OH-CH2OH
GLYCEROL	92	CH2OH-CHOH-CH2OH
PROPANE DIOL	75	CH3-CHOH-CH2OH
SORBITOL	182	CH2OH-(CHOH)4-CH2OH
MANNITOL	182	CH2OH-(CHOH)4-CH2OH
- SULFURED COMPOUND		
DIMETHYLSULFOXIDE (DMSO)	78	CH3-SO-CH3
- AMINO ACID		
PROLINE	115	NH2-CH-COOH-(CH2)3
- GLUCIDE		
GLUCOSE	180	CHO-(CHOH)4-CHOH
TREHALOSE	342	C12 H22 O11
SACCHAROSE	342	C12 H22 O11
- POLYMERE		
POLYETHYLENE GLYCOL (PEG)		CH2OH-(CH2OCH2)n-CH2OH
POLYLYNILPYRROLIDONE (PVP)	HIGH	CH2=CH-N-CO-(CH2)3n
HYDROXYETHYLE STARCH		

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 adaptated to different clones or varieties for the same material (Tabl.8).

Table 8 : The influence of the prefreeze incubation period on the survival of shoot tips from several potato genotypes,
after ultrarapid freezing in liquid nitrogen (After Kartha, 1985)

Incubation period (hr)	% Survival of genotypes (total frozen)			
			S. tuberosum ssp. *tuberosum*	
	S. goniocalyx[a]	*S. tuberosum* ssp. *andigena*[b]	cv. Desirée[c]	cv. Majestic[c]
0	0(15)	0(27)	0(20)	6.7(15)
18.5		24.0(25)		
24.0	50.0(18)		33.3(20)	0(18)
42.0		15.0(20)		
48.0	50.0(16)		7.1(20)	20.0(20)
65.5		17.2(29)		
72.5	35.0(17)		0(18)	6.2(19)
96.0	23.0(22)	29.7(27)	0(20)	0(19)
117.0		28.6(21)		
118.0	5.0(20)			
192.0		15.4(26)		

[a] Cryoprotectant 10% DMSO; culture medium MS + 3% sucrose + 1 mg ([illegible] BA (scored after 6 weeks).
[b] Cryoprotectant 10% DMSO; culture medium MS + 3% sucrose + 1 mg ([illegible] BA (scored after 22 days).
[c] Cryoprotectant 5% DMSO; culture medium MS + 3% sucrose + 0.25 mg ([illegible] NAA + 2.5 mg ([illegible] BA (scored after 3 weeks).

3.1.1.3 - Freezing

Different types of freezing processes can be carried-out : ultra-rapid, rapid, or slow freezing (Fig.3). In the later case, a prçogrammable freezing apparatus will be needed in order to obtain precise and reproducible freezing conditions.

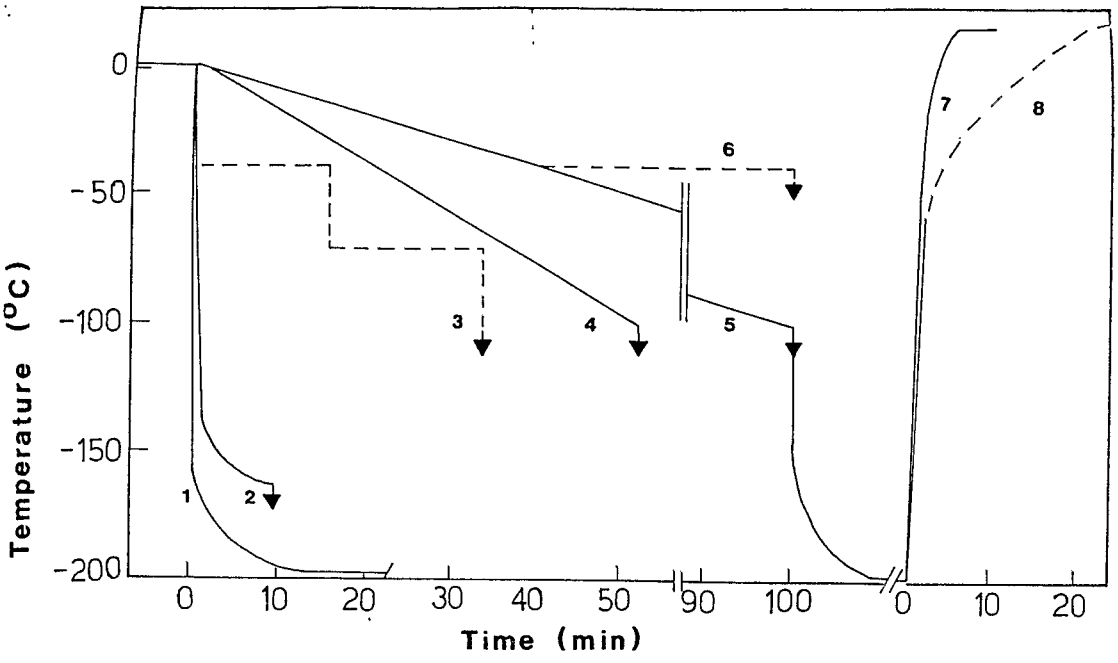


Figure 3 : Typical freeze-thaw profiles (After Withers, 1980)

1. Rapid freezing obtained by immersing specimen/ampoule in liquid nitrogen
2. Relatively rapid freezing produced by suspending specimen over liquid nitrogen ; slope of curve depends upon distance between specimen and liquid nitrogen.
3. Step-wise or prefreezing ; transition from one step to next may steep as shown here or more gentle as in the case of larger specimen.
4. Slow freezing at 2°C /min.
5. Slow freezing at 1°C/min.
6. Slow freezing interrupted by a period at a holding temperature.
7. Rapid thawing as in warm water at 35-40°C.
8. Slow thawing as in air at room temperature.

The arrows indicate the termination of the cooling by transfer to liquid nitrogen.

At the cellular level, the different freezing processes described above correspond to different mechanisms as regards water fluxes and cristallisation. During slow freezing, cristallisation 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 cristallisation 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 cristallizes in microcristals of a size which is unharmful 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 (Fig.4), or comprize a much broader range, as in the case of oil palm somatic embryos (Fig.5).
- starting and prefreezing temperature : i.e. the temperatures of beginning and end of programmed freezing. These parameters are oftenly 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 (Fig.6).

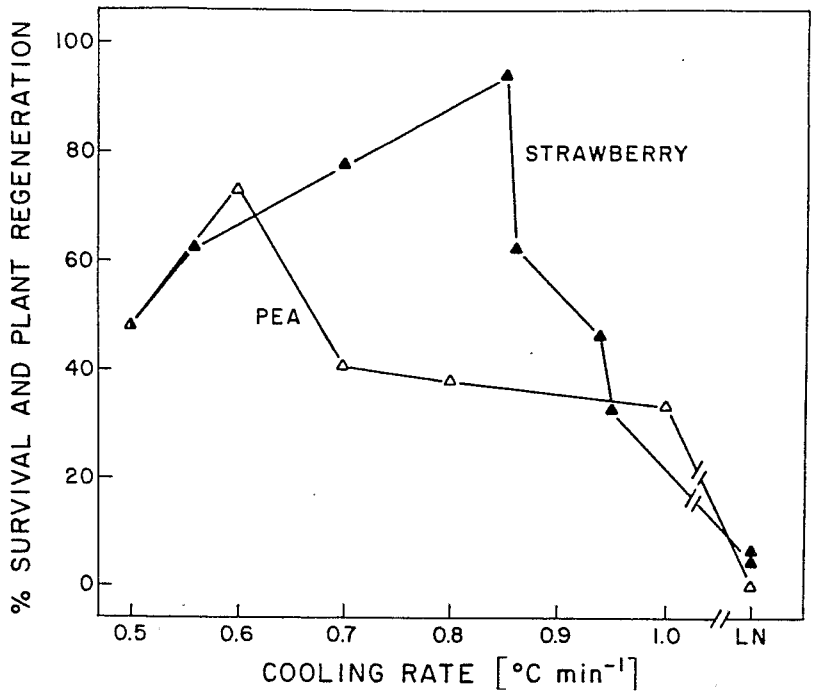


Figure 4 : Effect of cooling rate on the survival and plant regeneration from cryopreserved meristems of pea and strawberry. The meristems were precultured for 2 days on nutrient medium supplemented with 5 % DMSO and frozen using 5 % DMSO to -40°C followed by storage in liquid nitrogen (From Kartha, 1985).

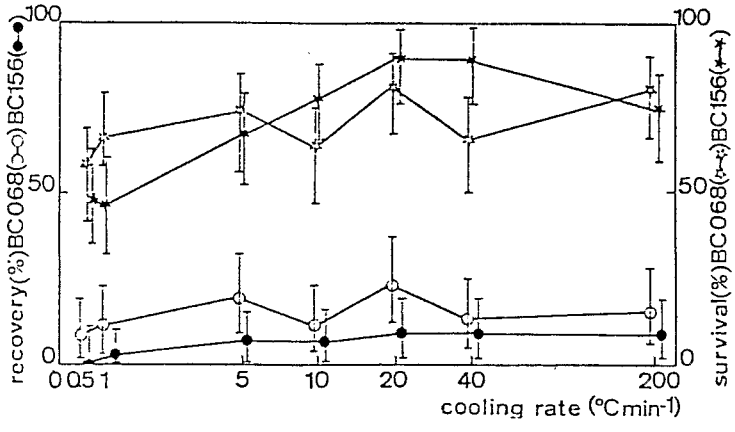


Figure 5 : Effect of cooling rate on survival and recovery of oil palm embryoid clumps from clone BC 068 and BC 156 (From Engelmann *et al.*, 1988).

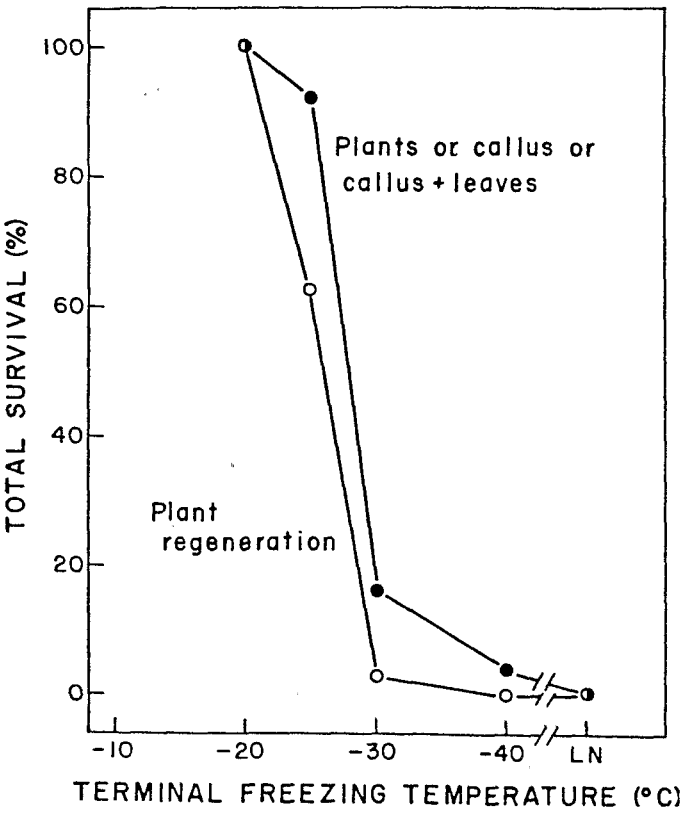


Figure 6 : Effect of terminal freezing temperature and liquid nitrogen storage on the survival and morphogenetic responses of cassava meristems. The meristems were frozen using 15% DMSO + 3% sucrose by droplet-freezing method at a cooling rate of 0.5°C/min to the indicated temperatures and stored in liquid nitrogen for 1 hr (After Kartha, 1985).

3.1.1.4 - 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.

3.1.1.5 - 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 microcristals formed during freezing to larger cristals of a size which would be domageable to cellular integrity.

3.1.1.6 - 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 successive transfers on progressively less concentrated media. In some cases, the nature of the medium must be changed (solid versus liquid, and vice et versa), in order to better the regrowth.

Recovery can eventualy take place in the dark, in order to avoid photooxidation 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.

3.1.2 - VIABILITY ASSESSMENT

The only definitive assessment of viability is regrowth 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, 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 formazan, colored in red, in the mitochondria of the living cells. It is quantitative for cell suspensions (measurment of % of the control), but is only qualitative for large tissues and organs (Steponkus and Lanphear, 1967).

3.2 - RESULTS

3.2.1 - 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 proved in the laboratory, but it does not necessarly mean that the technique is effectively used for germplasm storage of many species. The tables 9, 10 and 11 present the list of the species which have been frozen as cell suspensions, calluses or meristems.

Table 9 : List of species cryopreserved in the form of cell suspensions

Acer pseudoplatanus	*Medicago sativa*
Acer saccharum	*Nicotiana plombafinifolia*
Berberis dictyophylla	*Nicotiana sylvestris*
Brassica napus	*Nicotiana tabacum*
Catharantus roseus	*Oryza sativa*
Corydallis sempervirens	*Panax ginseng*
Datura innoxia	*Pennisetum americanum*
Datura stramonium	*Pseudotsuga menziesii*
Daucus carota	*Rosa Paul's scarlet*
Dianthus caryophyllus	*Sorghum bicolor*
Digitalis lanata	*Rhazia orientalis*
Dioscorea deltoidea	*Rhazia stricta*
Glaucium flavium	*Solanum melongena*
Glycine max	*Triticum aestivum*
Hordeum vulgare	*Triticum monococcum*
Hyosciamus muticus	*Zea mays*

Table 10 : List of species cryopreserved in the form of calluses

Lavandula vera	*Saccharum spp.*
Medicago sativa	*Triticum aestivum*
Phoenix dactylifera	*Ulmus campestris*
Populus americana	*Ulmus americana*

Table 11 : List of species cryopreserved in the form of meristems

Arachis hypogeaea
Asparagus officinalis
Brassica napus
Cicer arietinum
Dianthus caryophyllus
Fragaria ananassa
Lycopersicum esculentum
Malus domestica
Malus spp.
Manihot esculenta
Morus bombycis
Pisum sativum
Pyrus serotina
Pyrus spp.
Rubus spp.
Solanum tuberosum
Solanum goniocalix

Table 12 : List of species successfully cryopreserved as somatic,pollinic and zygotic embryos

Somatic embryos

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

Pollen embryos

Arachis hypogea
Arachis villosa
Atropa belladonna
Brassica campestris
Brassica napus
Gossypium arboreum
Nicotiana tabacum
Oryza sativa
Petunia hybrida
Primula obconica
Triticum aestivum

Zygotic embryos

Aesculus hypocastanea
Araucaria excelsa
Brassica napus
Capsella bursa-pastoris
Carva
Castanea
Cocos nucifera
Elaeis guineensis
Fagus
Gossypium arboreum
Hevea brasiliensis
Hordeum vulgare
Howea fosteriana
Juglans
Oryza sativa
Phaseolus vulgaris
Phenix dactylifera
Picea glauca
Quercus
Triticum aestivum
Triticale
Veitchia merrillii
Zea mays

Table 12 presents the results concerning the cryopreservation of embryos. It is interesting to note that many tree species are included in this list, particularly in the form of zygotic embryos.

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 uncomplete. The most promising results, particularly in the frame of plant genetic resources conservation, concern the cryopreservation of zygotic embryos.

3.2.2 - 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. steroids 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.

As concerns 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 led to the obtainment of 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.

3.2.3 - EXAMPLES

3.2.3.1 - Carnation meristem cryopreservation

Carnations are routinely propagated *in vitro* using microcuttings (Fig.7). It is very interesting to multiply on a large scale material which has been made virus-free using thermotherapy. It is thus necessary to store for the long term all these collections of virus-free material.

For cryopreservation, the following conditions have been set up for the different steps of the cryopreservation process which is presented on Fig.7) :

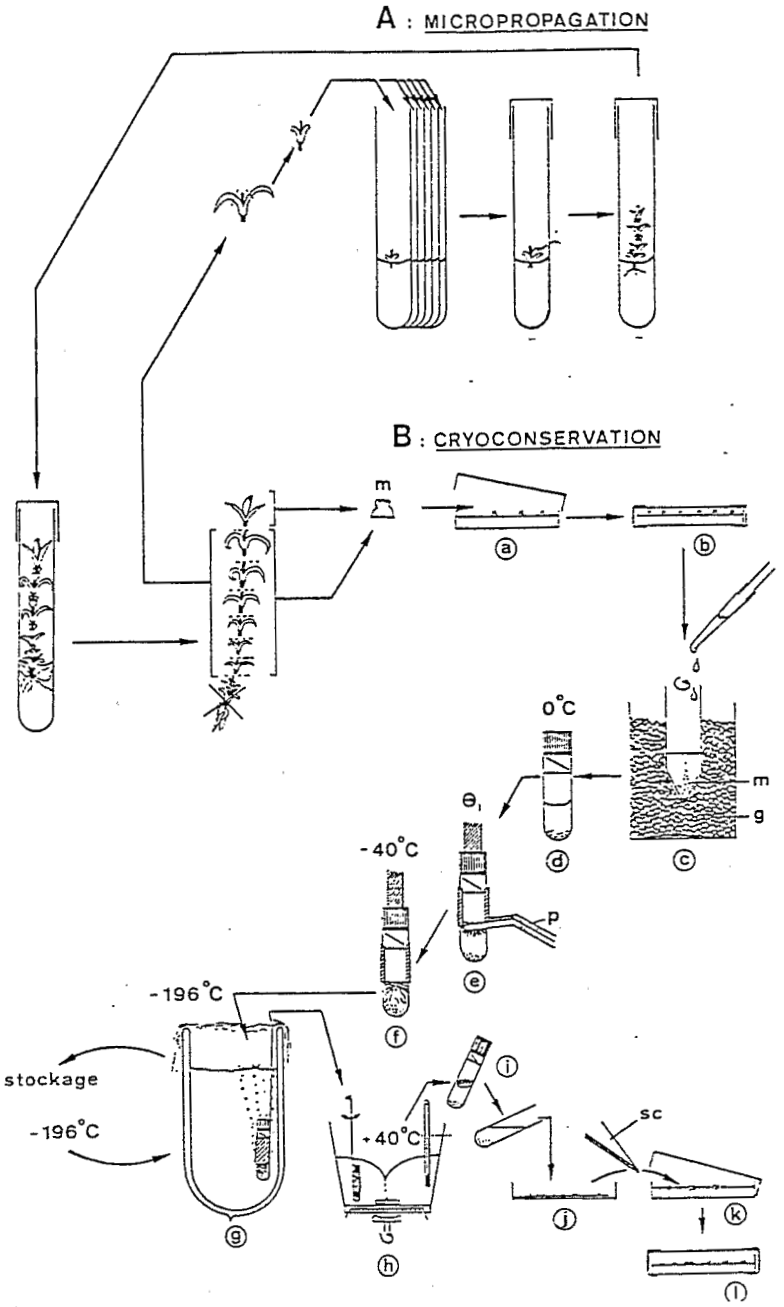


Figure 7 : Schematical representation of the carnation meristem cryopreservation process (After Fabre, 1986).

- starting material : axillary or terminal buds
- pretreatment : 24 hours at 25°C on a medium containing 0.75M sucrose (a-b), followed by 1 hour at 0°C in liquid medium containing 0.75M sucrose + 5% DMSO (c)
- freezing : 0 to -40°C at 0.5°C min-1, followed by immersion in LN (d-g)
- storage : 4.5 months in liquid nitrogen successfully tested (g)
- thawing : rapid, by immersion of the samples in a water-bath thermostated at +40°C (h)
- post-treatment : successive transfers on media with progressively decreased sucrose concentrations (j-l).

The following results have been obtained :

- Terminal buds : recovery ranging from 29.7 % to 98.9 %, depending on the variety.

- Axillary buds : 29.7% recovery.

Entire plants were obtained, which set normal flowers, compared to the non-frozen controls. These results allow cryopreservation to be used for the storage of carnation germplasm.

3.2.3.2 - Oil palm somatic embryo cryopreservation

The oil palm vegetative propagation process set up by ORSTOM and IRHO uses somatic embryogenesis. 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 production, thus reducing the quantity of material which has to be regularly subcultured.

The cryopreservation process which has been set up is presented in Figure 8. The following conditions have been defined :

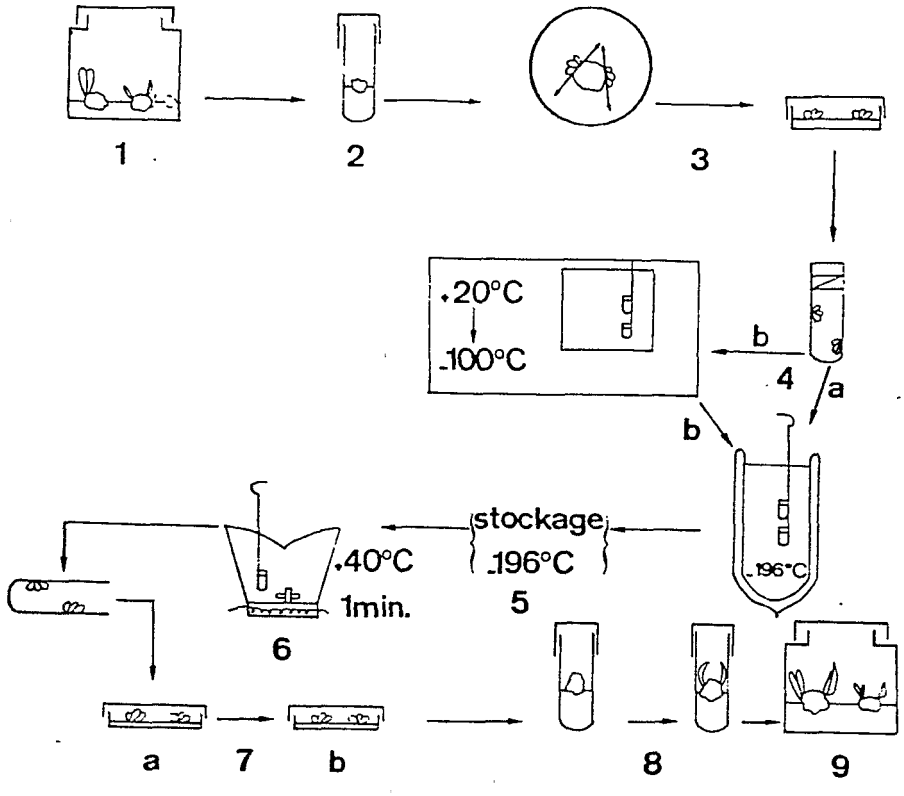


Figure 8 : Schematical representation of the oil palm somatic embryo cryopreservation process (After Engelmann, 1986).

- Choice and obtainment of material : only young embryoids , shining white , finger-like shaped, oftenly 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).

- Pretreatment : the clumps of embryoids are placed for 7 days on a medium containing 0.75M 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 in a water-bath thermostated at +40°C for 1 minute (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 on the standard medium devoid of growth regulators (7a-b).

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 nitrogen did not modify the recovery rate. Finally, ramets from two cryopreserved clones have been produced and planted in the field at the IRHO La Mé research station in Côte 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, Côte d'Ivoire, Malaysia and Indonesia in 1989, on approximatively 155 clones. The results will be available in 1990. We will then have more informations on the possibility of using cryopreservation as a routine technique for the long-term storage of oil palm embryoids.

4 - CONCLUSION

In conclusion, tissue culture techniques, together with cryopreservation techniques, are of great interest for the medium and long-term conservation of many plant species, particularly for that of tropical woody species. For that purpose, the use of zygotic embryos may present many advantages, as it is shown by the growing interest for this type of propagules in cryopreservation.

However, before routine procedures can be foreseen using *in vitro* techniques for germplasm preservation, various problems have to be faced. The germplasm has to be evaluated in order to store a representative sample of the variability of the species. Moreover, a minimal knowledge of the biology and physiology of the species is needed. The *in vitro* culture conditions have to be determined for the species which has to be conserved. Finally, trials must be carried out in order to precise the conditions for slow growth storage as well as cryopreservation.

Practical problems exist as well, particularly in the less developed countries, which have been previously mentioned : existence of minimal tissue culture facilities, lack of funds, of trained personnel, of regular and reliable liquid nitrogen supply. In this context, the development of "alternative" techniques, which must be as simple as possible, is of great interest.

The development of such processes nevertheless require sophisticated equipments as well as an important technical background in the research areas concerned. These conditions are found only in important centers of multidisciplinary research institutes like ORSTOM, for example. Success can be obtained only through a very close cooperation between the local and the overseas research institutes. This way of cooperation fits perfectly with the aims of ORSTOM. Indeed, ORSTOM has already a very large experience of the various aspects of plant germplasm preservation and is developing this research area.

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