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The Implications of Biotechnology in Germplasm Conservation and Utilization

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The current preferred method of germplasm preservation is seed storage at low temperature. However, for vegetatively propagated plants and plants producing recalcitrant seeds, long-term seed storage is inappropriate or impossible. A new method under consideration in biotechnology for germplasm banks is *in vitro* culture. About 350 species can be cultivated by *in vitro* culture, in the form of excised shoot-tips or meristems, adventitious budding from callus or cell-suspension culture and somatic embryogenesis from an original explant. It is possible to initiate cultures from immature anthers, ovules, protoplasts and inflorescences. Germplasm storage may be short term (with frequent periodical subcultures), medium term (with reduced growth rate) or long term in liquid nitrogen. *In vitro* storage appears to be more suitable for species that produce recalcitrant seeds (Withers and Williams, 1982) and for vegetatively propagated plants.

CONSERVATION OF GENETIC RESOURCES

Plants producing recalcitrant seeds

Usually, seeds are used for storage. Seeds may be either orthodox or recalcitrant, according to their ability to tolerate desiccation. Most temperate species produce orthodox seeds. Recalcitrant seeds, which are produced by many perennial species, are unable to withstand desiccation and freezing (Roberts, 1973), and such seeds pose considerable problems with respect to long-term preservation (*see* Table overleaf).

Vegetatively propagated plants

Genetic erosion of many economically important crop plants, including potato, cassava, yam and sweet potato, has been taking place in several ways: replacement of native cultivars by

TABLE 1 Dessication-sensitive seeds

Desiccation-sensitive seeds are produced by:
1 Species from aquatic habitats (such as wild rice)
2 Large-seeded woody plants (such as rubber, cacao, coconut, mango, avocado, jackfruit and mangosteen)
3 Herbaceous tropical perennials (such as plantain, cassava, sweet potato, yam, banana and sugarcane)
4 Trees of temperate latitudes (such as oak, chestnut, maple and walnut)

high-yielding varieties; destruction of natural habitats; and loss of collected material. In these cases germplasm preservation has been applied to wild species and primitive cultivated forms. While genebanks could be set up for the storage of seeds from wild species and fertile cultivars, conservation of sterile cultivars and clones would require a meristem-cryopreservation procedure.

Several international centers, such as the Centro Internacional de la Papa (CIP) in Peru, the Centro Internacional de Agricultura Tropical (CIAT) in Colombia and the International Institute for Tropical Agriculture (IITA) in Nigeria, maintain collections of these vegetatively propagated plants.

Two of the most problematical crops are coconut, *Cocos nucifera*, and oil palm, *Elaeis guineensis*. These species are heterozygous and the juvenile stage exceeds 6-7 years. Coconut seeds are recalcitrant and methods for vegetative propagation do not yet exist (Pannetier and Buffard-Morel, 1982). For oil palm, a method has been established which ensures continuous production of plantlets by means of theoretically indefinite adventitious embryogenesis (Pannetier et al., 1981).

IN VITRO CULTURE FOR GERMPLASM PRESERVATION

Whereas asexual preservation is needed for selected clones, *in vitro* culture is an appropriate tool only for short- or medium-term storage (Dereuddre, 1985). There are several types of *in vitro* storage.

Maintenance at normal growth

This method is useful for shoot-tips and indefinite somatic embryogenesis propagation; it is inappropriate for callus or cell suspension cultures, which often require the presence of 2.4-D. Indeed, repeated subcultures in the presence of auxins can lead to abnormal progeny and loss of totipotency. Calluses and cell cultures subcultured for an extended period have a tendency to undergo chromosomal variations and changes at the ploidy level (D'Amato, 1978). Haploid cultures, in particular, are known to be highly unstable and can revert in a few weeks or months to a diploid state (D'Amato, 1977). Maintenance of these cultures as haploids is highly desirable.

Medium-term storage

Growth-limiting conditions offer the possibility of reducing the frequency of subcultures; culturing at reduced temperatures (4-10°C) and addition of mannitol or growth inhibitors, such as abscissic acid, to the growth medium (Westcott 1981a, 1981b) may be appropriate for many species originating from temperate regions. Low temperature and abscissic acid seem to be unsuitable for the majority of tropical species. Other techniques have been developed for tropical species, such as the use of low concentrations of sucrose in the culture medium (Kartha et al., 1981) or the addition of polyols.

The Office de la Recherche Scientifique et Technique d'Outre-Mer (ORSTOM) is developing the preservation of coffee germplasm by *in vitro* culture of immature zygotic embryos of various coffee species (Bertrand-Desbrunais, unpubl.).

For medium-term storage, hypoxia has been applied to several types of callus (Augereau et al., 1986). However, periodic subcultures (every 1 or 2 years) remain necessary, with regular controls of the culture's ability to initiate regrowth.

IN VITRO CULTURE FOR THE MOVEMENT OF PLANT GERMPLASM

In vitro methods can be used to move plant germplasm in collecting missions and for exchange with other collections (Withers, 1986).

In vitro collecting

The possibility of using collecting techniques based on *in vitro* methods has been explored in recent years for both vegetative material and recalcitrant seeds. A range of techniques have been developed from the use of a fully equipped local laboratory to the use of an outdoor working area. The most successful attempt has been the use of an outdoor working area to collect zygotic embryos of coconut in the field to solve the problems encountered in transporting and storing coconut seeds. This work, supported by the International Board for Plant Genetic Resources (IBPGR), will interface with *in vitro* methods for clonally propagating coconut carried out by the Office de la Recherche Scientifique et Technique d'Outre-Mer (ORSTOM)/Institut de Recherche sur les Huiles et Oléagineux (IRHO). Direct inoculation into culture on media has been successfully carried out under field collecting conditions. An alternative method holds embryos in endosperm cores in a salt solution for brief periods before inoculation in a laboratory (Assy-Bah, 1986; Assy-Bah et al. 1987)

In vitro exchange

This operation has been carried out successfully for several years by some of the IARCs. The IBPGR *in vitro* conservation databases show a steady increase in reports of the distribution

of culture. In addition, *in vitro* culture can help to prevent phytosanitary, quarantine and related problems. According to Withers (1988), *in vitro* movement of plant germplasm has enormous advantages but the apparent cleanliness of cultures could promote a false sense of security.

CRYOPRESERVATION AS A TOOL FOR LONG-TERM STORAGE

Cryopreservation in liquid nitrogen is the most convenient technique for long-term storage and is now applied to a wide range of species. This technology can be applied to protoplasts, cell suspensions, calluses, meristems and shoot-tips, as well as to somatic, pollinic and zygotic embryos. Seeds may also be stored in liquid nitrogen.

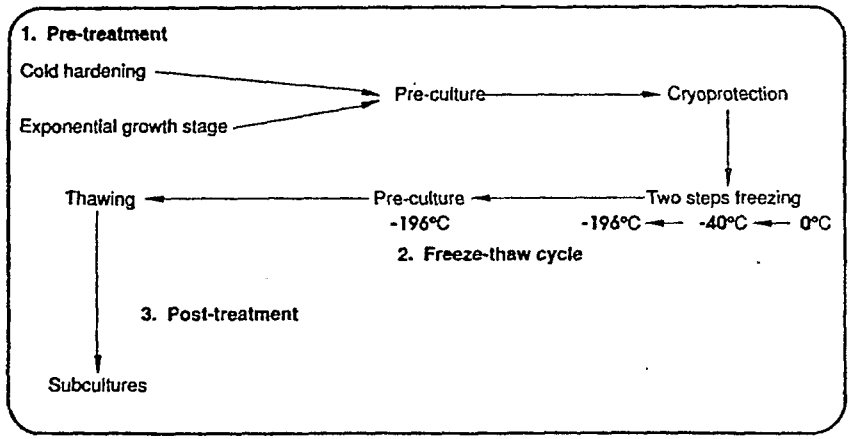
Cryopreservation offers several advantages as a method for storage. Stability for a hypothetically infinite period without deterioration is ensured; indeed, at the temperature of liquid nitrogen, cells no longer divide and all metabolic processes come to a halt. The material investment is relatively low if *in vitro* techniques are routinely used in the laboratory. The liquid nitrogen storage containers are compact and require only periodic filling.

Principles of cryopreservation

The cryopreservation process includes three main steps: pre-treatment, which prepares the cells and organs for resistance to the second step, the freeze-thaw cycle, and post-treatment, which may increase the growth recovery rate (*see* Figure 1).

The example of oil palm will serve to illustrate the process (Engelmann et al., 1986). The pre-treatment includes two or three successive steps. Cells or organs may be in a fast-growing

FIGURE 1 Cryopreservation process



stage (exponential growing phase). Pre-culture is carried out at room temperature or under the same conditions as in the routinely cultured cells, callus or donor plants. The cryoprotective mixture is generally added progressively at 0°C. Cooling is achieved in two steps: after a first slow cooling to -40°C, the samples are directly immersed in liquid nitrogen. Freezing is generally induced artificially a few degrees below the freezing point of the cryoprotective medium. For thawing, the cryobiological ampoules are transferred directly from liquid nitrogen into a water bath at 40°C. Post-treatment involves several subcultures in gradually reduced concentrations of cryoprotectant, and finally on standard medium. We have registered survival rates up to 30%. We have recently obtained plantlet regeneration after the freeze-thaw cycle for *Coffea arabica* (Bertrand-Desbrunais et al., 1988).

Cryopreservation of meristems

With cell suspensions, growth recovery occurs only if a certain proportion of cells survives to the freeze-thaw cycle. In the case of organized structures, such as meristems and embryos, entire organs must be preserved.

Cryopreservation of meristems and shoot tips is recommended for long-term storage of plants micropropagated by *in vitro* culture. It appears to be the main technique for preservation of genetic stability and generation of true-to-type progenies. However, it is necessary to avoid adventitious organogenesis and the possible genetic changes that might occur if morphogenetic calluses are allowed to proliferate from the explant after storage. The explants used for cryopreservation are apical or lateral shoot-tips. They include the apical dome and one to three leaf primordia; they measure about 0.5 mm in length, and are thus larger than those utilized to obtain virus-free plants.

Cryopreservation of meristems should permit the storage of five important types of material (Kartha, 1982):

- disease-free micropropagated plants;
- germplasm from recalcitrant seeds;
- sterile hybrids;
- vegetatively propagated plants;
- plants producing seeds contaminated by pathogens.

Thirteen species have been cryopreserved in liquid nitrogen in the form of meristems. They include: *Arachis hypogaea, Brassica napus, B. oleracea, Cicer arietinum, Lycopersicum esculentum, Manihot esculenta, Pisum sativum, Solanum etuberosum, S. goniocalyx* and *S. tuberosum*. The results depend very much on the variety (Bajaj, 1981; Towill, 1984).

Cryopreservation of embryos

Embryo storage is another important tool for the conservation of genetic resources. It can be used for:

- storage of pollinic embryos, which could allow regular plantlet production, until now impossible because of the seasonal production of such structures;
- conservation of genetic resources of plants producing recalcitrant seeds, when micropropagation by means of somatic embryogenesis or meristem culture does not exist;
- storage of embryogenic lines;
- storage of hybrid embryos which abort at early developmental stages.

Cryopreservation of embryos appears more critical than cryopreservation of meristems. Since it implies survival of the two apical meristems and of hypocotyl tissues, cryopreservation of embryos requires the use of material at early stages of development (globular or early heart-shaped embryos).

Survival rates differ according to the type of embryo. Direct regrowth (that is, without intermediary callus formation) has been observed in only a few cases: zygotic embryos of *Brassica napus* and oil palm (Grout et al., 1983); somatic embryos of oil palm, via somatic embryogenesis (Engelmann et al., 1985); and pollinic embryos of *Brassica napus* (unpubl.). In other cases, intermediary callus formation occurred (Bajaj, 1984). Embryos successfully cryopreserved in liquid nitrogen are listed in Table 2.

Table 2 Species successfully cryopreserved as somatic, pollinic and zygotic embryos

Somatic embryos	Pollinic embryos	Zygotic embryos
Elaesis guineensis	*Arachis hypogea*	*Brassica napus*
	Brassica campestris	*Cocos nucifera*
	Gossypium arboreum	*Elaeis guineensis*
	Nicotiana tabacum	*Oryza sativa*
	Oryza sativa	*Zea mays*
		Gossypium arboreum

Generally, younger (globular) embryos give rise directly to plantlets, whereas older embryos (late-heart or torpedo stage) survive only partially, because of their size, and form calluses. In most cases, auxins such as 2.4-D are still added to the post-treatment medium in order to ensure regrowth of the frozen-thawed material (Bajaj, 1984).

STABILITY

Organogenesis is preserved after long-term storage in liquid nitrogen, as noted for somatic embryogenesis in carrot cell suspensions. Oil palm somatic embryos reproliferate normally after 7-18 months of storage in liquid nitrogen without any loss of the resumption rate (Engelmann, 1986), giving rise to plantlets, the development of which in the nursery has so far been comparable to that of non-frozen controls.

Regeneration of plantlets after storage is also maintained in date palm (Tisserat et al., 1981) and alfalfa (Finkle et al., 1985). No differences in isoenzyme patterns were found between plantlets produced by unfrozen calluses and those produced by cryopreserved calluses (Ulrich et al., 1982). Microtuberization in potato plants regenerated from freeze-preserved meristems is maintained even after 2-4 years of storage (Bajaj, 1985).

CONCLUSION

The increasing number of species propagated *in vitro*, which include many tropical crop species with recalcitrant seeds, emphasizes the problem of storage. Much experience has been gained in several international centers, including CIP, CIAT and IITA, concerning the handling of *in vitro* collections and germplasm exchange. However, in the case of slow growth, used for medium-term storage, genetic stability must be assured, and a renewal process must be devised which permits medium-term storage (Withers, 1986).

The development of cryopreservation has been considerable during the past decade. Nevertheless, although cryopreservation of cell suspension cultures can be used routinely without major problems, difficulties still remain when freezing organized and/or macroscopic structures. These difficulties will be resolved both by improving cryopreservation technical processes and by developing more fundamental research.

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