Alternative methods for the storage of recalcitrant and intermediate seeds: slow growth and cryopreservation

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Introduction

A large proportion of plant species produce seeds that can be dried to a sufficiently low moisture content that permits them to be stored at low temperatures. These seeds are termed orthodox (Roberts 1973). There are also crops that produce what are known as recalcitrant seeds. A number of tropical fruit and timber species fall into this category, including coconut, avocado, mango, cacao and members of the Dipterocarpaceae family (Chin and Pritchard 1988). Recalcitrant seeds cannot tolerate desiccation to low moisture contents and remain viable only for a short time (weeks or months), even if stored optimally (high humidity and temperature). Recently, an intermediate category has been described (Ellis *et al.* 1990), comprising seeds which can be desiccated to fairly low moisture contents, but which do not withstand exposure to low temperature. Even though storage duration of those seeds can be extended to several months or years, their long-term conservation as seeds is still impossible.

Traditionally, the field genebank has been the *ex situ* method of choice for those species which produce recalcitrant seeds or which are vegetatively propagated. However, this method of conservation presents certain drawbacks which limit its efficiency and threaten its security. Genetic resources in field genebanks remain exposed to pests, diseases and natural hazards such as drought, weather damage, etc. Moreover, land requirements and cost of maintaining field collections can become very extensive, particularly in the case of large plants such as many forest trees. *In vitro* culture techniques offer alternative approaches for the genetic conservation of these problem species.

The aim of this paper is to present a brief overview of the present use of *in vitro* culture techniques for the conservation of genetic resources of tropical forest trees.

In vitro culture for germplasm conservation

The use of *in vitro* culture techniques is of great interest for collecting, storage and exchange. In addition, tissue culture techniques provide the opportunity for very high multiplication rates in an aseptic environment. Meristem culture and thermotherapy allow the production of disease-free material. Moreover, the space requirements and labour costs are considerably less than those of field genebanks. Additionally, tissue culture techniques greatly facilitate the international exchange of plant germplasm, reducing the volume to be shipped and satisfying phytosanitary regulations by providing sterile conditions.

In vitro propagation techniques have been described for more than 1000 plant species, among them approximately 100 forest tree species, mainly from temperate origin (Thorpe *et al.* 1991). Tissue culture of forest trees is faced with specific difficulties at various stages of the propagation process, including notably high levels of contamination in the initial explants, high secretion of polyphenols and tannins which inhibit the development of the explants and often cause necrosis, hyperhydration and low rooting ability (Harry and Thorpe 1994). A major problem

is the difficulty of using explants from mature trees since they are often recalcitrant in culture or display growth patterns characteristic of mature material (e.g. plagiotropic growth habit) which are very difficult to modify. Therefore, most of the propagation processes have been established using juvenile tissues (excised embryos or seedling parts), i.e. unproven material.

In the case of tropical forest trees, the search for the development of tissue culture techniques is fairly recent and it is mainly restricted to a limited number of species (Rao 1988; Umaly *et al.* 1988, 1992; Khuspe *et al.* 1994; Aziah and Darus 1995).

In vitro field collecting

The use of *in vitro* methods when collecting germplasm has many advantages and applications. For example, this technology is ideal when only vegetative explants are available, when clonal genotypes are required, or when collecting missions fail to coincide with seed production. They are even more useful for collecting short-lived recalcitrant seeds. The methods include collecting and inoculating *in vitro* embryos or vegetative tissues into culture vessels in the field.

In the case of coconut, embryos are extracted from the nut in the field, surface-sterilized, then either dissected and inoculated at the field location or transported in endosperm plugs held in a sterile salt solution for subsequent dissection in the laboratory (Assy-Bah *et al.* 1987). Similar methods have been established with embryos of cacao, avocado and *Citrus* spp. Introduction *in vitro* of woody shoots has been successfully performed with cacao, coffee, *Prunus* spp. and *Vitis* spp. (Withers 1995).

Slow growth

Standard culture conditions can be employed for medium-term storage of naturally slow-growing species. However, in the majority of cases, reduction of the growth rate is achieved by modifying the physical environment conditions and/or the culture medium, temperature reduction being the most widely employed (Aitken-Christie and Singh 1987; Withers 1992). Temperatures in the range of 0-5°C can be employed in the case of cold-tolerant species, but higher temperatures have to be used with tropical species which are generally cold-sensitive.

Alternative slow-growth techniques include modification of the gaseous environment and desiccation and/or encapsulation of explants (Engelmann 1991). Growth reduction can be achieved by modifying the quantity of oxygen available to the cultures. This is performed either by covering the explants with paraffin oil, mineral oil or liquid medium, or by using controlled atmospheres. If immersion appears adequate for the storage of unorganized cultures such as cell suspensions or calluses, its application to shoot cultures often leads to rapid necrosis and/or to hyperhydration of tissues. Oil palm somatic embryos could be stored for 4 months under controlled atmosphere with 1% O_2 and proliferated rapidly after subsequent transfer to standard conditions (Engelmann 1990). However, this technique should be experimented with other materials and for longer storage durations.

Desiccation as a means of storage of embryogenic cultures has been applied to various species, including alfalfa and carrot somatic embryos (Redenbaugh 1993). Storage durations of up to 1 year were recorded with both materials.

Encapsulation of somatic embryos and shoot-tips in alginate beads has been employed for medium-term storage of various materials (Bapat *et al.* 1987; Bapat and Rao 1988). However, the maximum storage duration reported did not exceed 6 months and no detailed results were provided. Redenbaugh (1993) mentions that the rapid survival loss of encapsulated material which is generally observed is mainly

due to the encapsulating matrix, which dehydrates rapidly and limits the respiration of the embedded embryos.

Standard *in vitro* slow-growth procedures have been developed for numerous forest tree species, mainly from temperate climates (for reviews see Aitken-Christie and Singh 1987; Mascarenhas and Agrawal 1991).

Cryopreservation

Classical cryopreservation procedures comprise a pretreatment with cryoprotective substances followed by slow controlled freezing. Such procedures have been successful with culture systems consisting of small units of uniform morphology such as would be found in a protoplast culture, an exponentially growing cell suspension culture or a fragmented callus culture (Withers and Engelmann 1995; Kartha and Engelmann 1994). However, this approach is less successful with culture systems that consist of large units comprising a mixture of cell sizes and types, such as shoot-tips, zygotic embryos or relatively mature somatic embryo cultures. Recently, reproducible and efficient methods have been proposed for these materials with the emergence of new techniques such as encapsulation/dehydration, vitrification, desiccation and pregrowth-desiccation (Withers and Engelmann 1995).

Encapsulation-dehydration

The encapsulation-dehydration technique is based on the technology developed for the production of synthetic seeds (Redenbaugh 1993). For cryopreservation, apices or somatic embryos are encapsulated in a bead of alginate and pregrown for various durations in liquid medium with high sucrose concentrations. Beads are then partially dehydrated under the air current of a laminar flow cabinet or using silica gel, down to a water content of ca. 20%. Freezing is usually rapid, by direct immersion of the samples in liquid nitrogen. For recovery, samples are usually placed directly under standard culture conditions. Growth recovery of cryopreserved material is generally rapid and direct, without callus formation. This technique has been applied to somatic embryos of carrot, apices of several temperate as well as tropical species (Dereuddre 1992; Withers and Engelmann 1995), and recently to zygotic embryos of two tropical forestry species, *Swietenia macrophylla* and *Shorea leprosula* (Marzalina *et al.* 1994; Krishnapillay, unpubl.). Successful extension of encapsulation-dehydration protocols has been performed with 11 varieties of pear, 9 varieties of apple and 14 varieties of sugarcane. With all three species, even though genotypic variations were noted, survival rates were sufficiently high to envisage large-scale routine application of the cryopreservation protocols developed.

Vitrification

Vitrification consists of placing samples for pretreatment in extremely concentrated cryoprotective solutions and freezing them ultrarapidly. In these conditions, the intracellular solutes vitrify, i.e. form an amorphous glassy structure, thus avoiding the formation of intracellular ice crystals, detrimental for cell survival. Vitrification procedures have been developed for cell suspensions, somatic embryos and apices of various species (Sakai 1993). However, the cryoprotective mixtures employed are often toxic due to their very high concentration, thus making this technique difficult to apply to species which proved to be sensitive to cryoprotectants.

Desiccation

Cryopreservation using a desiccation procedure is very simple since it consists of dehydrating the plant material, then freezing it rapidly by direct immersion in liquid

nitrogen. Desiccation has been applied mainly to zygotic embryos of a large number of species (Engelmann 1992; Engelmann *et al.* 1995), including tropical forest trees such as *Dipterocarpus alatus*, *D. intricatus* and *Pterocarpus indicus* (Krishnapillay *et al.* 1992, 1994).

The physiological state of the starting material is an important parameter. In the case of *Coffea arabica*, mature embryos showed higher survival rates than immature ones. High variability in the survival rates of embryos extracted from seeds of some recalcitrant seed-producing trees (*Aesculus*, *Castanea* and *Quercus*) harvested at different periods was noted by Pence (1992).

Pretreatment of the embryos with cryoprotective substances before desiccation led to positive results with *Artocarpus heterophyllus* and *Dryobalanops aromatica* (Chin *et al.* 1988).

Desiccation is usually performed by placing the embryos or embryonic axes in the air current of a laminar flow cabinet. However, more precise and reproducible desiccation can be achieved by placing plant material in a stream of compressed air or in an airtight container with silica gel. The duration of desiccation varies with the size of the embryos and their initial water content. Optimal survival rates are generally noted when embryos are dehydrated down to 10-20% water content (fresh weight basis). Dehydration must be sufficient to ensure survival after freezing but not too intense to induce extended desiccation injury. In optimal cases, no significant difference is observed in the survival rates of desiccated control and cryopreserved embryos (Krishnapillay *et al.* 1995).

Regrowth of plant material after rewarming is usually direct but modified regrowth patterns such as callusing or incomplete development are observed in some cases. Modified recovery conditions, notably the hormonal balance of the culture medium, can significantly improve the survival rate of the cryopreserved material, as observed with coffee embryos.

Pregrowth-desiccation

Cryopreservation using a pregrowth-desiccation technique comprises the following steps: pregrowth treatment with cryoprotectants, desiccation, rapid cooling, storage and rapid warming. This technique has been applied to only a limited number of specimens: stem segments of *in vitro* plantlets of asparagus, somatic embryos of melon and oilpalm, microspore embryos of rapeseed and zygotic embryos of coconut (Withers and Engelmann 1995). Cryopreservation using pregrowth-desiccation has ensured satisfactory survival rates with all materials tested and recovery is usually rapid and direct. In the case of coconut zygotic embryos, experiments performed with four different varieties led to recovery rates between 33 and 93%. Large-scale application of this technique has been performed in the case of oilpalm somatic embryos. Eighty clones are now routinely stored at -196°C (Dumet 1994).

Conclusion

In vitro conservation techniques for the safe storage of plant germplasm have become technically more feasible, particularly as a result of recent advances in cryopreservation. However, for many tropical tree species, there are several prerequisites which have to be fulfilled before *in vitro* conservation becomes possible. A minimal knowledge of the biology and physiology of the species to be conserved is needed in order to identify which type of explants can be used as a storage propagule and to define the necessary *in vitro* culture techniques. In many cases, simple *in vitro* culture procedures (embryo culture, shoot micropropagation) have to be established and/or optimized. Additional research aiming at the development of *in vitro*

conservation techniques, including slow growth storage and cryopreservation, and at the understanding of the biological mechanisms determining recalcitrance is also required.

Significant progress should be achieved by establishing and/or reinforcing collaborative links between research institutes from tropical and temperate countries working in the area of forest tree genetic resources conservation.

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