7

Cryopreservation of Zygotic and Somatic Embryos of Tropical Species Producing Recalcitrant or Intermediate Seeds

Dominique Dumet[1], Patricia Berjak,[1] and Florent Engelmann[2]

INTRODUCTION

Biodiversity—genetic, taxonomic, and ecological diversity—has been produced by millions of years of mutation and natural selection (Krogstrup et al., 1992). In contrast, within the past 10,000 years, human activity has resulted in an accelerating decline in the biodiversity of the Earth. It has, in fact, been estimated that 25% of the 250,000 vascular plant species could become extinct within the next 50 years (Raven, 1987, as cited by Schemske et al., 1994). Real and potential genetic erosion has attracted wide attention, leading to the drawing up of the International Convention on Biological Diversity of 1993 which addresses a broad spectrum of issues related to the protection of biodiversity.

Biodiversity can be conserved either *in situ* or *ex situ*. In the former instance, species are conserved in their natural habitat, including nature reserves. The advantage of this form of conservation is that it facilitates the ongoing processes of natural evolution in both the physical and biological contexts (Krogstrup et al., 1992). Although *in situ*

[1]Plant Cell Biology Research Unit, Department of Biology, University of Natal, Durban 4041, South Africa.

[2]International Plant Genetic Resources Institute (IPGRI), Via delle Sette Chiese 142, 00145 Rome, Italy.

conservation is probably the best way to preserve biodiversity, it is nevertheless expensive in terms of the area required and in the cost of establishment and maintenance of natural reserves.

Ex-situ conservation involves removing plants and/or propagules from their natural habitat and storing the collections in botanic gardens, arboreta, seedbanks or in *in vitro* culture. Seedbanks are probably the most common and convenient way of storing germplasm, as maintenance of such facilities is a relatively low-cost operation that does not require very sophisticated technology (Krogstrup et al., 1992). However, conventional seedbanking, which implies maintenance of low relative humidity/low temperature conditions, is not suitable for all seed-types.

Seeds have been characterized as showing orthodox, recalcitrant or intermediate storage behavior (Roberts, 1973; Ellis et al., 1990, 1991). Mature, orthodox seeds have moisture contents of 20% or less on a wet-mass basis, and can be further dried (generally to moisture contents of around 5%) without loss of viability in most species (Chin and Roberts, 1980). According to Roberts (1973), the lower the water content of an orthodox seed, the longer its lifespan. Additionally, a quantitative relationship has been shown to exist between seed longevity and storage temperature (Dickie et al., 1990), whereby the lower the temperature, the more prolonged the lifespan of the seeds of many species. In this regard, according to recommendations of the IBPGR (1976), orthodox seeds should be dried to a moisture content range of 5–1%, and stored hermetically at temperatures of –18°C or less. More recently, however, Vertucci and Roos (1993) have suggested that the optimal seed moisture content might depend on the storage temperature, and that the ultradry condition may actually reduce longevity because of the effects of overdehydration.

In contrast to the desiccated condition of mature, orthodox seeds, those categorized as recalcitrant (Roberts, 1973) or intermediate (Ellis et al., 1990, 1991) are relatively highly hydrated at maturity. The notable difference between these two seed categories, however, is that while recalcitrant seeds remain sensitive to water loss, intermediate seeds may be dehydrated to relatively low water contents (12–5%, depending on the species; Ellis et al., 1990, 1991). Chilling sensitivity is an additional feature of many recalcitrant seed species (Chin and Roberts, 1980; Berjak et al., 1995), and of dehydrated intermediate seeds (Ellis et al., 1990, 1991). As a result of their postharvest characteristics, neither recalcitrant nor intermediate seeds are amenable to low moisture/low temperature storage conditions. Presently, the only option for the storage of *intact* recalcitrant or intermediate seeds is their maintenance in an hydrated or dehydrated condition respectively,

at the lowest temperature that is commensurate with viability retention (Chin and Roberts, 1980; Berjak et al., 1989; Ellis et al., 1990, 1991). However, such conditions facilitate only relatively short-term storage, varying from a few days in the case of highly recalcitrant seeds (Berjak et al., 1984) to several months for intermediate types (Ellis et al., 1990, 1991).

Cryopreservation (i.e., storage at temperatures between ≈ −80 and ≈ −196°C) presently appears to offer the only option for long-term conservation of the germplasm of species with recalcitrant or intermediate seeds. Once tissue is cooled down to sufficiently low temperatures, biological and most degradative processes are halted, theoretically facilitating storage for thousands of years (Ashwood-Smith and Friedman, 1979). However, it should be noted that some authors contend free radical generation may continue even at cryo-storage temperatures (Benson, 1990) and that this process could well occur under all seed storage conditions (Hendry, 1993; Smith and Berjak, 1995).

While it might be possible to cryostore suitably dehydrated, intact, intermediate seeds, this is not the case for hydrated, recalcitrant seeds, which are relatively large to very large (e.g. diameter 50 mm or more in the case of avocado, mango, and coconut). However, in the great majority of cases, the embryonic axes constitute only an insignificant proportion of the total seed mass (Berjak et al., 1989), offering, after excision, an appropriate source of gemplasm for cryopreservation. Furthermore, for species of economic importance (e.g. oil palm, date palm, coffee) there has been extensive development of systems for *in vitro* propagation to produce somatic embryos (Pannetier et al., 1981; Tisserat, 1984; Hatanaka et al., 1991). Cryopreservation of somatic embryos has great potential for the storage of selected genotypes, particularly in cases where the seeds are highly heterozygous although, in the interests of preserving biodiversity, conservation of zygotic embryonic axes should not be neglected.

The water content of both zygotic and somatic embryos of recalcitrant and intermediate species remains high throughout their development. According to Crowe et al. (1990), most tissue water is "free" (i.e., freezable), as opposed to a very small proportion that is described as "bound" (i.e., non-freezable) and is frequently constitutive of macro molecules. During cooling, free water forms ice crystals, which have the potential to cause lethal mechanical damage. Furthermore, as ice is formed, the intracellular milieu becomes increasingly desiccated, which may cause perturbation because of changes in osmotic potential and pH (Mazur, 1984; Kartha, 1985; Franks, 1990).

Several procedures may be followed in order to limit lethal ice crystal formation during freezing:

1) Reduction or elimination of the freezable water by dehydration of the tissue prior to freezing.
2) Increasing the cooling rate. According to Mazur (1984), when the cooling rate is extremely rapid, water movement cannot occur, thus facilitating the formation of only very small (micro) ice crystals. In this regard, the smaller the ice crystals, the less the consequent damage.
3) Pretreatment of the embryos with cryoprotectants, i.e., molecules known to improve the low-temperature tolerance of tissues. The suggested modes of action of cryoprotectants have been reviewed by Withers (1990) and will not be considered here.

This chapter highlights the critical facets of the cryopreservation processes that have been developed for zygotic and somatic embryos of several tropical species that produce recalcitrant or intermediate seeds.

STERILIZATION AND *IN VITRO* CULTURE CONDITIONS

Recalcitrant seeds almost invariably harbor a mixed external and internal microflora (Mycock and Berjak, 1990) which contributes adversely to their already curtailed lifespan (Berjak, 1996). This microflora can be the cause of significant problems in the *in vitro* propagation of isolated embryonic axes. As a consequence, rigorous sterilization procedures must be followed to eliminate fungi and bacteria that would otherwise proliferate in the culture. After surface sterilization of fruits and/or seeds, embryonic axes are excised aseptically in a laminar flow cabinet. Alternatively, axes may be excised from unsterilized seeds and then subjected to surface sterilization, using the least injurious treatment, which usually involves a solution of sodium hypochlorite, mercuric chloride or ethanol (Table 1), often with a few drops of a wetting agent (e.g. Tween 20®) added. However, while a particular treatment might be adequate in eliminating all associated microflora in one instance, it is not guaranteed to succeed in all cases. For example, while sterilization of axes of *Camellia sinensis* with a 1% solution of sodium hypochlorite was successful, this procedure was found to be inadequate for axes of *Syzygium guineense,* for which 0.1% mercuric chloride had to be employed (Dumet, unpublished data). Even in the case of an individual species, sterilization procedures must sometimes be amended: e.g. heavy bacterial contamination occurred in the case

Table 1. Sterilization processes, principal culture media, and *in vitro* development of zygotic embryonic axes of various species

Species	Sterilization	Growth media for embryonic axes						Embryonic Axis Development	References
		Salts	Vitamins	Sugars	Growth Regulator ($mg \cdot l^{-1}$)	Charcoal (g)	pH		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Artocarpus heterophyllus	• Seeds: 0.5% mercuric chloride, axes excised aseptically	MS*	MS	?	1 NAA 1 BAP	2	?	Shoot and root	Chandel et al., 1994
	• Axes: 1% sodium hypochlorite	MS/2	MS/2	3%	—	—	5.6–5.8	Shoot and root	Dumet and Berjak, 1995
	• Axes: 0.1% mercuric chloride 10 min	MS	MS	3% sucrose	—	—	5.8	Radicle clearly visible	Fu et al., 1993
Azadirachta indica	• Axes: 1% sodium hypochlorite 20 min	MS/2	MS/2	3%	—	—	5.6–5.8	Root and shoot elongation	Dumet and Berjak, 1995
Camellia sinensis	• Axes: 1% sodium hypochlorite 10 mn + 0.2% hibitane (Hibiclens; Stuart Pharmaceutical, Wilmington, DE)	MS	MS	3% sucrose	—	—	5.6–5.8	Greening and organ development	Wesley-Smith et al., 1992
	• Axes: 1% sodium hypochlorite 20 min	MS/2	MS/2	3% sucrose	—	—	5.6–5.8	Seedlings	Dumet and Berjak, 1995
	• Fruit: 0.5% mercuric chloride, axes excised aseptically	MS + Yeast extract		?	?	—	?	Shoot and root	Chandel et al., 1994

(Contd)

Table 1. ***(Contd)***

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
	• Fruit: 0.1% mercuric chloride, axes excised aseptically	NN**	NN	?	?	?	?	Seedlings	Chaudhury et al., 1991
Cocos nucifera	• Embryo: cylinder of albumen, 3% sodium hypochlorite 20 min, embryos excised aseptically	MS	MW***	6% sucrose	—	2	5.5	Seedlings	Assy-Bah et al., 1989
Coffea sp. *C. canephora, C. arabica* and *Arabusta*	• Fruit: 70% ethanol 15 min, axes excised aseptically	MS**	MS	3% sucrose	1 BA	—	5.7	Seedlings	Abdelnour-Esguivel et al., 1992
C. liberica	• Axes: 5% commercial Chlorox 6 min	MS	MS	3%	0.1 kinetin 0.1 2,4-D 0.1 BA 0.5 IBA	2	?	Seedlings	Normah and Vengada-salam, 1992
Elaeis guineensis	• Kernels: 0.1% mercuric chloride 20 min, axes excised aseptically	MS	MS	3%	0.01 NAA 0.1 kinetin	—	5.7	Seedlings	Engelmann et al., 1995
Euphoria longan	• Axes: 70% alcohol 5–10 s	?	?	?	?	?	5.8	Radicle and plumule elongation	Fu et al., 1993
Hevea brasiliensis	• Seeds: 20–30% Chlorox, axes excised aseptically	MS	MS	?	0.15 kinetin 0.2 NAA 0.5 GA	4	?	Seedlings	Normah et al., 1986

(Contd)

Table 1. ***(Contd)***

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Landolphia kirkii	• Axes: 2.5% sodium hypochlorite 10 min + 0.2% hibitane (Hibiclens; Stuart Pharmaceutical, Wilmington, DE)	MS	MS	3%	0.15 kinetin 0.2 NAA 0.5 GA	4	?	Greening and root development	Vertucci et al., 1991
Litchi chinensis	• Axes: 70% alcohol 5–10 s	?	?	?	?	?	5.8	Radicle and plumule elongation	Fu et al., 1993
Mangifera indica	• Seeds: 0.1% mercuric chloride 10 min, axes excised aseptically	MS/2	MS/2	5%	—	—	5.8	Leaf production from axes	Fu et al., 1990
Theobroma cacao	• Fruit: 70% ethanol 15 min, axes excised aseptically	MS	MS	3%	1.5–3 NAA	0.5	5.6	Callus and somatic embryo-genesis	Pence, 1991
Trichilia dregeana	• Axes: 1% sodium hypochlorite	MS/2	MS/2	3%	—	—	5.6–5.8	Greening and callus	Dumet and Berjak, 1995

*Murashige and Skoog (1962); **Nitsch and Nitsch (1967); ***Morel and Wetmore (1951)
BAP: benzylaminopurine; 2,4-D: 2,4-dichloro-phenoxyacetic acid; IBA: indole-3-butyric acid; NAA: naphtaleneacetic acid; BA: 6-benzylaminopurine; GA: gibberellic acid

of one particular sample of *C. sinensis* seeds, necessitating the addition of Hibitane® to the sterilant used for the excised axes (Table 1).

Depending on the species, the composition of *in vitro* culture media may be more or less complex. The medium acts as a substitute for the storage tissues of the intact seed, in which all the constituents necessary for germination and early seedling growth are contained. In the case of some species, a basic MS medium (Murashige and Skoog, 1962) with various combinations of vitamins and sugar(s) allows embryonic axes to develop into seedlings. For other species, incorporation of growth regulators into the medium is essential. Development of the appropriate medium on a species basis requires a considerable expenditure of time and effort, notwithstanding which plantlet establishment might still prove elusive. In the case of some species, despite intensive trials with various combinations of components, incomplete development is currently still being obtained (Table 1). Although it is probable that the more mature an embryonic axis, the simpler will be its *in vitro* cultivation, it appears that there are greater problems with associated microorganisms (e.g. for coffee [Abdelnour-Esquivel et al., 1992] and *S. guineense* [Dumet, unpublished data]).

DESICCATION

Desiccation Procedures

Despite the fact that a seed species may be recalcitrant (and thus, by definition, desiccation-sensitive), if water is removed rapidly, then axes withstand a considerable degree of dehydration (Berjak et al., 1989). Dehydration may be achieved by one of several means:

1) In a sterile airstream in a laminar flow cabinet. This method has been used in several cases, e.g. for zygotic axes of rubber (Normah et al., 1986), tea (Chaudhury et al., 1991), coffee (Abdelnour-Esquivel et al., 1992), and oil palm (Engelmann et al., 1995), as well as for somatic embryos of oil palm (Dumet et al., 1993a) and coffee and date palm (Mycock et al., 1995).
2) In airtight containers containing dry silica gel, which has been used for zygotic embryonic axes of *Euphoria longan* (Fu et al., 1993) and somatic embryos of oil palm (Dumet et al., 1993a, b).
3) Using a compressed airstream (flash-drying) as carried out for the dehydration of zygotic axes of *Landolphia kirkii* (Pammenter et al., 1991; Vertucci et al., 1991) and *C. sinensis* (Wesley-Smith et al., 1992; Berjak et al., 1993).

4) Under vacuum, as described for the dehydration of *Artocarpus heterophyllus* (jackfruit) zygotic axes by Fu et al. (1990).

Depending on the mode of dehydration, the rate of water loss varies, which might be reflected in the degree of desiccation tolerance achieved (see below).

Desiccation Tolerance

As with intact seeds, relative desiccation tolerance of isolated embryonic axes varies among recalcitrant and intermediate species. However, it is highly relevant that whereas intact recalcitrant seeds cannot tolerate a significant degree of desiccation, the isolated axes can, but it is important to note that such desiccated axes will not retain viability for any length of time unless they are cryostored. Embryonic axes of tea, which is a recalcitrant species, can be dried down to a moisture content of 13% (FWB; i.e. 0.14 g water per g dry mass, $g \cdot g^{-1}$) (Chaudhury et al., 1991; Dumet and Berjak, 1995). Over 80% of the embryonic axes of coffee, an intermediate species, have been reported to survive when desiccated to water contents of 15–20% (0.17–0.25 $g \cdot g^{-1}$) and 15.8% (0.18 $g \cdot g^{-1}$) for *Coffea liberica* and *C. arabica* respectively (Normah and Vengadasalam, 1992; Abdelnour-Esquivel et al., 1992). In the case of neem (a species which has been variously categorized as orthodox, intermediate or recalcitrant, depending on provenance (Gamene et al., 1994; Sacandé et al., 1995; Berjak et al., 1995)), viability is not adversely affected when axes are desiccated down to 0.23 $g \cdot g^{-1}$ (Dumet and Berjak, 1995).

Depending on the species, the degree of desiccation tolerance of somatic embryos also varies. Hatanaka et al. (1994) reported that 50–80% of *Coffea canephora* embryos withstood dehydration to a water content of 20% (0.25 $g \cdot g^{-1}$), but 80% loss of viability occurred when clumps of somatic embryos of oil palm were desiccated to water contents below 0.7 $g \cdot g^{-1}$ (41 % FWB) (Dumet et al., 1993a).

Optimization of Desiccation Tolerance

Various studies have indicated that several parameters, e.g. developmental stage, desiccation rate or pretreatment conditions, can modify the ability of both zygotic axes and somatic embryos to survive water loss.

Desiccation Rate: Pammenter et al. (1991) have shown that flash-dried axes of *Landolphia kirkii* could be dehydrated down to a water content of 0.3 $g \cdot g^{-1}$ (23% FWB) without damage, whereas viability of axes in slowly dried, intact seeds was lost in the water content range 0.9–0.7 $g \cdot g^{-1}$ (47–41% FWB). In contrast, for axes of *Artocarpus*

heterophyllus, Fu et al. (1993) reported that the relatively slower the desiccation rate, the lower the water content tolerated without loss of viability. Those authors achieved the slower drying rate using silica gel, and the faster by vacuum, and pointed out that depletion of oxygen during vacuum drying might have contributed to viability loss. The benefit of relatively less rapid dehydration has also been suggested by the work of Dumet et al. (1993a): those authors suggested that oil palm somatic embryos desiccated with silica gel might adapt better to the imposed water stress and thus to lower water contents, than those dried more rapidly in a laminar flow cabinet.

Developmental Stage: More mature embryonic axes of *Camellia sinensis* and *A. heterophyllus* have been shown by Chandel et al. (1994) to be relatively more desiccation tolerant, but, in contrast, those authors indicated that mature axes of *Theobrama cacao* were less tolerant than those excised in an immature condition. Berjak et al. (1993) have also drawn attention to the importance of developmental status of zygotic axes in relation to relative tolerance of water loss.

Conditioning Prior to Desiccation: Conditioning embryos—certainly in the case of somatic embryos—can substantially improve their desiccation tolerance. This has been shown for oil palm by Dumet et al. (1993b), wherein somatic embryos pretreated on a medium containing 25% sucrose survived desiccation down to a water content of 0.5 $g \cdot g^{-1}$ (33% FWB), while only 20% of the embryos not pretreated survived to 0.7 $g \cdot g^{-1}$ (41% FWB). Sucrose has been suggested to be involved in desiccation-tolerance mechanisms of orthodox seeds (by water substitution, e.g. Crowe et al., 1988) and/or by facilitating vitrification (Williams and Leopold, 1989; Koster, 1991). However, in the case of the oil palm somatic embryos, these phenomena are inappropriate, as neither water replacement nor vitrification would occur under ambient conditions at a water content of 0.5 $g \cdot g^{-1}$. Furthermore, sucrose pretreatment is not specific in conferring greater desiccation tolerance in oil palm somatic embryos, since fructose, galactose, and glucose are also effective (Dumet et al., 1994).

The effect of sucrose in conferring greater desiccation tolerance on zygotic embryonic axes has been tested for four recalcitrant species, with conflicting results (Dumet and Berjak, 1995). In the case of *A. heterophyllus* and *Trichilia dregeana,* axes pretreated with sucrose could be dehydrated successfully to lower water contents than those not pretreated. However, similar treatment had no effect on the desiccation tolerance of axes of *C. sinensis* (tea) and actually had a negative effect on those of *Landolphia kirkii,* which showed reduced tolerance

after sucrose pretreatment. As mentioned earlier for *L. kirkii,* more rapid water loss facilitated survival of axes of lower water contents than did slower dehydration. It is possible that pretreatment of the excised axes of *L. kirkii* on a high-sucrose medium causes dehydration to proceed at a damagingly slow rate. However, when appropriate dehydration rates are achieved by this osmotic effect, then such treatment could be beneficial in facilitating further water loss from the axes (Dumet and Berjak, 1995). Those results indicate that if osmotic dehydration is to be used to advantage, then parameters need to be determined on a species basis.

Improvement of desiccation tolerance of somatic embryos has also been achieved by encapsulation in sodium alginate. Hatanaka et al. (1994) showed that encapsulated embryos of *C. canephora* survived dehydration to 13% water content (0.14 $g \cdot g^{-1}$), while viability retention was not as good for those not encapsulated.

CRYOPROTECTION

Pretreatment with cryoprotectants prior to freezing may increase survival to very low temperatures. As previously mentioned, sucrose can improve desiccation tolerance in certain cases and this sugar can also act as a cryoprotectant. Oil palm somatic embryos have been shown to survive liquid nitrogen immersion after being pretreated on a medium containing 25% sucrose (Engelmann et al., 1985; Dumet et al., 1993a). Sucrose also showed a specific cryoprotectant effect in the case of relatively hydrated oil palm somatic embryos, whereas other sugars, although acting to promote desiccation tolerance, did not afford cryoprotection (Dumet et al., 1994). High sucrose pretreatment has also been used successfully for cryopreservation of *C. canephora* somatic embryos in liquid medium (Tessereau, 1993).

Use of a combination of cryoprotectants can also be beneficial. Mycock et al. (1995) have shown that somatic embryos of date palm and coffee survived cryopreservation after being treated with mixtures of sucrose and glycerol (5% followed by 10%), while for immature zygotic embryos of coconut, combination of 60% glucose and 10–15% glycerol were used as pretreatments for cryopreservation (Assy-Bah and Engelmann, 1992a).

However, even if cryoprotectant pretreatment permits some survival of cryopreservation, improved survival rates are obtained when such treatments are followed by partial desiccation. This has been clearly shown for somatic embryos of oil palm (Dumet et al., 1993a) and coffee and date palm (Mycock et al., 1995). While the usual procedure is that

desiccation follows cryoprotectant treatment, Assy-Bah and Engelmann (1992b) dehydrated mature embryos of coconut prior to the application of cryoprotectants, using a combination of 60% glucose and 15% glycerol. It should be noted that when certain cryoprotectants or cryoprotectant combinations are used, it might be necessary to eliminate them from the tissues by several rinses in distilled water after the embryos have been thawed (see below).

FREEZING

Freezing Processes

Various processes, affording different cooling rates, have been used.

Slow cooling can be performed by using a programmable freezing apparatus, the most common rates used being 0.5 to 1.0°C·min^{-1} (Kartha and Engelmann, 1994). Alternatively, the specimen can be slowly precooled at a temperature of –18°C in a deep freezer before being immersed in liquid nitrogen. Slow cooling rates have been successfully used for the cryopreservation of desiccated zygotic axes of *Euphoria longan* (Fu et al., 1990) as well as for hydrated somatic embryos of coffee (Tessereau 1993). In the latter case, freezing of cryoprotected embryos was carried out in a liquid medium.

Freezing by rapid cooling rates has frequently been employed. In these cases, specimens have been enclosed either in cryovials or aluminium foil, or have been held within stainless steel mesh baskets, which are then rapidly immersed in liquid nitrogen. Such procedures have been used for the cryopreservation of zygotic axes of several species, such as rubber, coconut, oil palm, and coffee, among others (Normah et al., 1986, 1992; Abdelnour-Esquivel et al., 1992; Assy-Bah and Engelmann, 1992a, b; Engelmann et al., 1995). Somatic embryos of oil palm, coffee, and date palm have been similarly successfully frozen (Engelmann et al., 1985; Dumet et al., 1993a, b; Mycock et al., 1995).

The cooling rate achieved by direct immersion of a specimen in liquid nitrogen will vary according to its volume and to the mode of its introduction into the cryogen (Wesley-Smith et al., 1992). Those authors discuss the phenomenon that enclosing containers, or forceps holding the specimen, are heat-sinks which substantially slow cooling rates, achieving perhaps a cooling rate of around 200°C·min^{-1}. Wesley-Smith et al. (1992) achieved a cooling rate in the region of 500°C·s^{-1} for axes of *C. sinensis* which were propelled by a spring-loaded device directly into subcooled liquid nitrogen (–210°C). The same technique

has been used successfully for the cryopreservation of somatic embryos of date palm and coffee (Mycock et al., 1995).

Optimization of Freezing Tolerance

Freezing Rate and Water Content: Wesley-Smith et al. (1992) have clearly shown that in order to achieve successful cryopreservation, the higher the water content of the embryonic axes, the faster must be the freezing rate. Those authors achieved 100% survival of embryonic axes of *C. sinensis* in the water content range 1.6–1.1 $g.g^{-1}$ (61–52% FWB), which they were rapidly propelled into subcooled liquid nitrogen (see above), whereas none in this water content range survived cooling at either 200 or $10^{\circ}C \cdot min^{-1}$. However, when the water content was moderately reduced below 1.1 $g \cdot g^{-1}$, then some survival was obtained at a cooling rate of $200^{\circ}C \cdot min^{-1}$, and a similar result was obtained at very reduced water content (0.6–0.44 $g \cdot g^{-1}$) after cooling at $10^{\circ}C \cdot min^{-1}$ (Wesley-Smith et al., 1992).

The benefit of such rapid freezing has also been demonstrated for somatic embryos of coffee dried down to a moisture content range of 0.26–0.21 $g \cdot g^{-1}$ (20–17% FWD): 70% survived cryopreservation when propelled into subcooled liquid nitrogen, as opposed to 8–10% when plunged into the same cryogen at –196°C (Mycock et al., 1995). Those authors reported a similar (although less marked) trend for somatic embryos of date palm. However, according to Fu et al. (1993), embryonic axes of *Euphoria longan* at a water content of 0.21 $g \cdot g^{-1}$ (17% FWB) survived cryopreservation better when subjected to pre cooling at –18°C than when directly immersed in liquid nitrogen. This suggests that, at least for excised zygotic axes, factors other than only the cooling rate need to be taken into consideration when devising a protocol for cryopreservation.

Optimal Water Content for Cryopreservation: As long as desiccation effects are not injurious, the optimal water content for cryopreservation is generally between 10 and 16% (fresh-weight basis) (Engelmann, 1992). However, optimal water content appears to vary with the species and the nature of the embryo. Use of differential scanning calorimetry (DSC) has shown that increasing survival of embryos is correlated with diminishing levels of freezable water (Vertucci et al., 1991; Wesley-Smith et al., 1992; Dumet et al., 1993b). In the case of oil palm somatic embryos, maximum survival was obtained when all freezable water had been removed (Dumet et al., 1993b). However, this is not necessarily always the case: for example, zygotic axes of *Landolphia kirkii* that retained a small amount of freezable water survived cooling to –80°C, whereas

those that had been dehydrated to the level of non-freezable water only did not (Vertucci et al., 1991). Axes of *C. sinensis* that had been dehydrated near to the level of non-freezable water only were described as having sustained desiccation injury, which in itself could have been worsened by the stress imposed by freezing (Wesley-Smith et al., 1992). It is probable, as discussed by those authors, that the formation of very small ice crystals is not injurious and thus that retaining a measure of freezable water and manipulating the freezing rate is a better procedure than excessive desiccation prior to freezing.

Developmental Stage: At a similar water content, mature embryonic axes of *L. kirkii* have been shown to be more tolerant of cryopreservation at –80°C than immature axes (Vertucci et al., 1991). Other studies correlating the response to freezing with the developmental stage were not found in the literature. However, it seems reasonable to suppose that there might be an optimal developmental stage that would facilitate successful cryopreservation, and that this is likely to be at full maturity. However, a major difficulty with recalcitrant seeds is that there are no clear-cut indications as to what constitutes full maturity and, indeed, it appears that seeds of the same species may be shed at varying stages of maturity (Finch-Savage, 1996). This underscores the importance of gaining as complete an appreciation of individual seed species as possible, in order to develop protocols which have the potential to achieve successful cryopreservation.

THAWING

When cryovials are used for liquid nitrogen storage of material, these are usually plunged into a water bath at 30–40°C in order to warm the specimen relatively rapidly, which avoids ice crystallization during the process. This procedure has been used for zygotic axes and somatic embryos of coffee and oil palm (Engelmann et al., 1985; Abdelnour-Esquivel et al., 1992; Normah et al., 1992; Dumet at al., 1993a, b). Hydrated thawing, by plunging the specimens themselves directly into a culture medium, either preheated to 37°C or maintained at ambient temperature, has been used for zygotic axes of tea (Wesley-Smith et al., 1992) and also somatic embryos of coffee and date palm (Mycock et al., 1995). Fu et al. (1990), in comparing the effects of different thawing temperatures on axes of *Euphoria longan,* concluded that temperatures in the range 10–30°C facilitated better survival than thawing carried out at 0 or 5°C. It must be noted, however, that adverse effects of such low temperatures might be caused by chilling injury, rather than by the rate of thawing.

CONDITIONING AND EMBRYO DEVELOPMENT AFTER FREEZING

After thawing, both zygotic axes and somatic embryos must be transferred onto a culture medium for further development; it is essential that the optimal medium has been developed and tested for individual species beforehand. Although in most instances the medium developed for unfrozen controls is quite suitable, sometimes the incorporation of growth regulators may be necessary to ensure normal post-thawing development. This was found to be the case for oil palm somatic embryos, which required short-term addition of 2,4-D (0.2 $mg \cdot l^{-1}$) to increase recovery after cryopreservation (Engelmann et al., 1985; Dumet et al., 1993). A similar situation has been described for zygotic axes of *Coffea liberica* (Normah and Vengadasalam, 1992), which initiated normal development that then ceased until IBA and BA (0.1 $mg \cdot l^{-1}$ and 0.5 $mg \cdot l^{-1}$ respectively) were added to the medium. Abdelnour Esquivel et al. (1992) mention that the lower survival of cryopreserved immature axes of *C. arabica,* compared with mature ones, could be almost totally overcome by placing them on a modified medium supplemented with GA_3 (100 $mg \cdot l^{-1}$) for recovery.

A further consideration is that complete (normal) development of embryos might not occur after cryopreservation. For example, mature embryos of coconut no longer produced haustoria after retrieval from liquid nitrogen, although normal plantlet development did occur (Assy-Bah and Engelmann, 1992b). In other cases, only some regenerative tissue survived cryopreservation, leading to the formation of callus: this was observed in the case of cryopreserved zygotic axes of *Theobroma cacao* (Pence, 1991) and *Trichilia dregeana* (Dumet and Berjak, 1995). In such cases, when the protocol for *in vitro* propagation of somatic embryos from callus has been developed, the situation poses no problem, as in the case of *T. cacao* (Pence, 1991).

CONCLUSIONS AND PERSPECTIVES

The requirements for successful cryopreservation differ for different species.

In some case embryos will withstand the removal of most (or all) of their free water, thus eliminating lethal ice formation and creating the potential for high survival rates after cryopreservation. However, factors additional to those involved with water, may play a part in the adverse reaction of embryonic axes or somatic embryos to freezing. For example, Vertucci et al. (1991) found that despite very low water contents, *Landolphia kirkii* axes frozen to –80°C did not survive as well as did controls that had not been frozen. Moreover, whatever their

water content, no axes of *L. kirkii* survived if the temperature were lowered to –150°C. Wesley-Smith et al. (1992) reported that embryonic axes of *Camellia sinensis* dehydrated so that no crystallizable water remained, showed lower survival rates after cryopreservation than those not frozen. Those authors suggested that phase transitions of certain intracellular macromolecules occurring at very low temperatures might underlie damage in partially dried, frozen tissues.

For some species, potentially lethal damage of embryonic axes occurs at relatively high water contents, considerably in excess of the level of non-freezable water only (Pammenter et al., 1993). The causes of desiccation sensitivity of recalcitrant seeds have not yet been elucidated, whereas several factors have been implicated in the desiccation-tolerance mechanism of orthodox seeds. Desiccation tolerance has been associated in the latter with high sugar contents, particularly of sucrose in association with oligosaccharides such as raffinose and/or stachyose, as well as with the presence of LEA (late embryogenic abundant) proteins (Bartels et al., 1988; Bocchichio et al., 1988; Caffrey et al., 1988; Koster and Leopold, 1988; Williams and Leopold, 1989; Leopold, 1990; Blackman et al., 1991, 1992). However since the presence of certain sugars or of LEAs alone is insufficient to confer desiccation tolerance, it is thought that other factors must also be involved (Berjak et al., 1989; Blackman et al., 1991; Farrant et al., 1993). The presence of highly vacuolated tissue (Farrant et al., 1992) or of latex (Dument and Berjak, 1995) has been suggested as being implicated in desiccation sensitivity.

Use of particular rates of dehydration, and/or pretreatments, and/or by choosing specific developmental stages, might improve the relative desiccation tolerance of isolated embryonic axes. As the minimum water content commensurate with viability retention increases, the freezing rate becomes a parameter of prime importance. If embryonic axes lose viability at relatively high water contents, then very fast cooling rates are essential to avoid lethal ice formation. One exception seems to be the case of embryos frozen in liquid medium, when slow freezing of cryoprotected material may be the method of choice.

The heterogeneity of embryonic axes from recalcitrant and intermediate seeds probably results from the difficulty of ascertaining the precise maturation stage. In contrast to orthodox seeds, in which maturation drying occurs, there appears to be no easily recognisable, but reliable, sign of maturity in recalcitrant or intermediate seeds. An approximation of the state of maturity is usually made on the basis of fruit or seed color. However, germinable seeds may be extracted from still-unripe fruits, as observed for *Strychnos madagascariensis* (Dumet, unpublished observations), and the axes from such seeds may be

amenable to cryopreservation. For other species, however, this is not the case, and it is possible that developmental stages that are optimal for cryopreservation may differ from species to species.

Thus it should be apparent that the achievement of successful embryo cryopreservation and the subsequent regeneration of a plant is no easy task; many parameters have to be ascertained and tested on a species basis.

References

Abdelnour-Esquivel, A., Villalobos, V. and Engelmann, F., 1992. Cryopreservation of zygotic embryos of *Coffea* spp. Cryo-Lett., 13: 297–302.

Ashwood-Smith, M.S. and Friedmann, G.B., 1979. Lethal and chromosomal effects of freezing, thawing, storage time and X-irradiation on mammalian cells preserved at −196°C in dimethylsulfoxide. Cryobiology, 16: 132–140.

Assy-Bah, B. and Engelmann, F., 1992a. Cryopreservation of immature embryos of coconut (*Cocos nucifera* L.). Cryo-Lett., 13: 67–74.

Assy-Bah, B. and Engelmann, F., 1992b. Cryopreservation of mature embryos of coconut (*Cocos nucifera* L.) and subsequent regeneration of plantlets. Cryo-Lett., 13: 117–126.

Assy-Bah, B., Durand-Gasselin, T., Engelmann, F. and Pannetier, C., 1989. Culture *in vitro* d'embryons zygotiques de cocotier (*Cocos nucifera* L.). Méthode, révisée et simplifiée, d'obtention de plants de cocotiers transférables au champ. Oléagineux, 44: 515–523.

Bartels, D., Singh, M. and Salamini, F., 1988. Onset of desiccation tolerance during development of barley embryos. Planta, 175: 485–492.

Benson, E.E., 1990. Free radical damage in stored plant germplasm. IBPGR, Rome, 128 pp.

Berjak, P., 1996. The role of micro-organisms in deterioration during storage of recalcitrant and intermediate seeds. In: A.S. Ovédraogo, K. Poulsen and F. Stubsgaard (Eds.), Intermediate/Recalcitrant Tropical Forest Tree Seeds. IPGRI, Rome, pp. 121–126.

Berjak, P., Dini, M. and Pammenter, N.W., 1984. Possible mechanisms underlying the differing dehydration responses in recalcitrant and orthodox seeds: Desiccation-associated subcellular changes in propagules of *Avicennia marina*. Seed Sci. Technol., 12: 365–384.

Berjak, P., Farrant, J.M. and Pammenter, N.W., 1989. The basis of recalcitrant seed behaviour. Cell biology of the homoiohydrous seed condition. In: R.B. Taylorson (Ed.), Recent Advances in the Development and Germination of Seeds. Plenum Press, New York, pp. 89–108.

Berjak, P., Vertucci, C.W. and Pammenter, N.W., 1993. Effects of developmental status and dehydration rate on characteristics of water and desiccation-sensitivity in recalcitrant seeds of *Camellia sinensis*. Seed Sci. Res., 3: 155–166.

Berjak, P., Campbell, G.K., Farrant, J.M., Omondi-Oloo, W. and Pammenter, N.W., 1995. Responses of seeds of *Azadirachta indica* (neem) to short-term storage under ambient or chilled conditions. Seed Sci. Technol., 23: 779–792.

Blackman, S.A., Obendorf, R.L. and Leopold, A.C., 1992. Maturation proteins and sugars in desiccation tolerance of developing soybean seeds. Plant Physiol., 100: 225–230.

Blackman, S.A., Wettlaufer, S.H., Obendorf, R.L. and Leopold, A.C., 1991. Maturation proteins associated with desiccation tolerance in soybean. Plant Physiol., 96: 868–874.

Bocchichio, A., Vazzana, C., Raschi, A., Bartels, D. and Salamini, F., 1988. Effect of desiccation on isolated embryos of maize. Onset of desiccation tolerance during development. Agronomie, 8: 29–36.

Caffrey, M., Fonseca, V. and Leopold, A.C., 1988. Lipid-sugar interaction. Relevance to anhydrous biology. Plant Physiol., 86: 754–758.

Chandel, K.P.S., Chaudhury, R. and Radhamani, J., 1994. Biological mechanisms determining the recalcitrance in seeds of tea, cocoa and jackfruit. IPGRI/NBPGR Report, IPGRI, Rome.

Chaudhury, R., Radhamani, J. and Chandel, K.P.S., 1991. Preliminary observations on the cryopreservation of desiccated embryonic axes of tea (*Camellia sinensis* [L.]. O. Kuntze) seeds for genetic conservation. Cryo-Lett., 12: 31–36.

Chin, H.F. and Roberts, E.H., 1980. Recalcitrant Crop Seeds. Tropical Press SDN/BDH, Kuala Lumpur, Malaysia.

Crowe, J.H., Carpentier, J.F., Crowe. L.M. and Anchordoguy, T.J., 1990. Are freezing and dehydration similar stress vectors? A comparison of mode of interaction of stabilizing solutes with biomolecules. Cryobiology, 27: 213–219.

Crowe, J.H., Crowe, L.M., Carpentier, J.F., Rudolph, A.S., Wistrom, C.A., Spargo, B.J. and Anchordoguy, T.J., 1988. Interaction of sugars with membranes. Biochem. Biophys. Acta, 947: 367–384.

Dickie, J.B., Ellis, R.H., Kraak, H.L., Ryder, K. and Tompsett, P.B., 1990. Temperature and seed storage longevity. Ann. Bot., 65: 197–204.

Dumet, D. and Berjak, P., 1995. Desiccation tolerance and cryopreservation of embryonic axes of recalcitrant species. In: Proc. Vth Internat. Workshop on Seeds. University of Reading, UK, 10–15/09/95.

Dumet, D., Engelmann, F., Chabrillange, N. and Duval, Y., 1993a. Cryopreservation of oil palm (*Elaeis guineensis* Jacq.) somatic embryos involving a desiccation step. Plant Cell Rep., 12: 352–355.

Dumet, D., Engelmann, F., Chabrillange, N., Duval, Y. and Dereuddre, J., 1993b. Importance of sucrose for the acquisition of tolerance to desiccation and cryopreservation of oil palm somatic embryos. Cryo-Lett., 14: 243–250.

Dumet, D., Engelmann, F., Chabrillange, N., Dussert, S. and Duval, Y., 1994. Effect of various sugars and polyols on the tolerance to desiccation and freezing of oil palm polyembryonic cultures. Seed Sci. Res., 4: 307–313.

Ellis, R.M., Hong, T.D. and Roberts, E.H., 1990. An intermediate category of seed behaviour? 1—Coffee. J. Exp. Bot., 41: 1167–1174.

Ellis, R.M., Hong, T.D., Roberts, E.H. and Soetisna, U., 1991. Seed storage behaviour in *Elaeis guineensis*. Seed Sci. Res., 1: 99–104.

Engelmann, F., 1992. Cryopreservation of embryos. In: Y. Dattée, C. Dumas and A. Gallais (Eds.), Reproductive Biology and Plant Breeding. Springer-Verlag, Berlin, pp. 281–290.

Engelmann, F., Duval, Y. and Dereuddre, J., 1985. Survie et prolifération d'embryons somatiques de palmier à huile (*Elaeis guineensis* Jacq.) après conservation dans l'azote liquide. Compte Rendus de l'Académie des Sciences, Paris, 301 (III): 111–116.

Engelmann, F., Chabrillange, N. and Duval, Y., 1995. Cryopreservation of zygotic embryos and kernels of oil palm (*Elaeis guineensis* Jacq.). Seed Sci. Res., 5: 81–86.

Farrant, J.M., Pammenter, N.W. and Berjak, P., 1992. Development of the recalcitrant (homoiohydrous) seeds of *Avicennia marina:* anatomical ultrastructural and biochemical events associated with development from histodifferentiation to maturation. Ann. Bot., 70: 75–86.

Farrant, J.M., Pammenter, N.W. and Berjak, P., 1993. Seed development in relation to desiccation tolerance: A comparison between desiccation-sensitive (recalcitrant) seeds of *Avicennia marina* and desiccation-tolerant types. Seed Sci. Res., 3: 1–13.

Finch-Savage, W.E., 1996. The role of developmental studies in research on recalcitrant and intermediate seeds. In: A.S. Ovédraogo, K. Poulsen and F. Stubsgaard (Eds.), Intermediate/Recalcitrant Tropical Forest Tree Seeds. IPGRI, Rome, pp. 83–97.

Franks, F., 1990. Freeze drying: from empiricism to predictability. Cryo-Lett., 11: 93–110.

Fu, J.R., Xia, Q.H. and Tang, L.F., 1993. Effects of desiccation on excised embryonic axes of three recalcitrant seeds and studies on cryopreservation. Seed Sci. Techol., 21: 85–95.

Fu, J.R., Zhang, B.Z., Wang, X.P., Qiao, Y.Z. and Huang, X.L., 1990. Physiological studies on desiccation, wet storage and cryopreservation of recalcitrant seeds of three fruit species and their excised embryonic axes. Seed Sci. Technol., 18: 743–754.

Gamene, S., Kraak, H.L. and van Pijlen, J.G., 1994. *Azadirachta indica* seeds from Burkino Faso: Intermediate storage behaviour? Proc. Internat. Workshop on Desiccation Tolerance and Sensitivity of Seeds and Vegetative Plant Tissues. Kruger National Park, South Africa, p. 23.

Hatanaka, T., Yasuda, T., Yamaguchi, T. and Sakai, A., 1994. Direct regrowth of encapsulated somatic embryos of coffee (*Coffea canephora*) after cooling in liquid nitrogen. Cryo-Lett., 15: 47–52.

Hatanaka, T., Arawaka, O., Yasuda, T., Uchida, N. and Yamaguchi, T., 1991. Effect of plant growth regulators on somatic embryogenesis in leaf cultures of *Coffea canephora*. Plant Cell Rep., 10: 179–182.

Hendry, G.A.F., 1993. Oxygen free radical processes and seed longevity. Seed Sci. Res., 3: 141–153.

IBPGR., 1976. Working Group Report on Engineering, Design and Cost Aspects of Long-term Storage Facilities. Rome, Italy.

Kartha, K.K. (Ed.)., 1985. Cryopreservation of Plant Cells and Organs. CRC Press, Boca Raton, Florida, 276 pp.

Kartha, K.K. and Engelmann, F., 1994. Cryopreservation and germplasm storage. In: I.K. Vasil and T.A. Thorpe (Eds.), Plant Cell and Tissue Culture. Kluwer, Dordrecht, pp. 195–230.

Koster, K.L., 1991. Glass formation and desiccation tolerance in seeds. Plant Physiol., 96: 302–304.

Koster, K.L. and Leopold, A.C., 1988. Sugars and desiccation tolerance in seeds. Plant Physiol., 96: 829–832.

Krogstrup, P., Baldursson, S. and Nørgaard, J.V., 1992. *Ex situ* genetic conservation by use of tissue culture. Opera Botanica, 113: 49–53.

Leopold, A.C., 1990. Coping with desiccation. In: R.G. Alsher and J.R. Cuming (Eds.), Stress Response in Plants: Adaptation and Acclimation Mechanisms. Wiley-Liss, Inc., New York, pp. 37–56.

Mazur, P., 1984. Freezing of living cells: Mechanisms and application. Am. J. Physiol., 247, Cell Physiol, 16: C125–C142.

Morel, G. and Wetmore, R.M., 1951. Fern callus tissue culture. Am. J. Bot., 38: 141–143.

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15: 473–497.

Mycock, D.J. and Berjak, P., 1990. Fungal contaminants associated with several homoiohydrous (recalcitrant) seed species. Phytophylactica, 22: 413–418.

Mycock, D.J., Wesley-Smith, J. and Berjak, P., 1995. Cryopreservation of somatic embryos of four species with and without cryoprotectant pre-treatment. Ann. Bot., 75: 331–336.

Nitsch, C. and Nitsch, J.P., 1967. The induction of flowering *in vitro* in stem segments of *Plumbago indica* L. The production of vegetative buds. Planta, 72: 355–370.

Normah, M.N. and Vengadasalam, M., 1992. Effects of moisture content on cryopreservation of *Coffea* and *Vigna* seeds and embryos. Cryo-Lett., 13: 199–208.

Normah, M.N., Chin H.F. and Hor, Y.L., 1986. Desiccation and cryopreservation of embryonic axes of *Hevea brasiliensis* Muell.-Arg. Pertanika, 9: 299–303.

Pammenter, N.W., Vertucci, C.W. and Berjak, P., 1991. Homeohydrous (recalcitrant) seeds: Dehydration, the state of water and viability characteristics in *Landolphia kirkii*. Plant Physiol., 96: 1093–1098.

Pammenter, N.W., Vertucci, C.W. and Berjak, P., 1993. Response to dehydration in relation to non-freezable water in desiccation-sensitive and -tolerant seeds. In: D. Côme and F. Corbineau (Eds.), Proc. IV Internat. Workshop on Seeds: Basic and Applied Aspects of Seed Biology. AFSIS, Paris, pp. 867–872.

Pannetier, C., Arthuis, P. and Liévoux, D., 1981. Néoformation de jeunes plantes cultivées *in vitro* d'*Elaeis guineensis* à partir de cals primaires obtenus sur fragments foliaires. Oléagineux, 36: 119–122.

Pence, V.C., 1991. Cryopreservation of immature embryos of *Theobroma cacao*. Plant Cell Rep., 10: 144–147.

Roberts, E.H., 1973. Predicting the storage life of seeds. Seed Sci. Technol., 1: 499–514.

Sacandé, N., Van Pijlen, J.G., de Vos, C.H., Hoekstra, F.A. and Groot, S.P.C., 1995. Improvement of storage behaviour of neem seeds from Burkina. In: Proc. Vth Internat. Workshop on Seeds. University of Reading, UK, 10–15/09/95.

Schemske, D.W., Husband, B.C., Ruckelshaus, M.H., Goodwillie, C., Parker, I.M. and Bishop, J.G., 1994. Evaluating approaches to the conservation of rare and endangered plants. Ecology, 75: 584–606.

Smith, M.T. and Berjak, P., 1995. Deteriorative changes associated with the loss of viability of stored desiccation-tolerant and -sensitive seeds. In: J. Kigel and G. Galili (Eds.), Seed Development and Germination: Applied Aspects. Marcel Dekker, New York, pp. 701–746.

Tessereau, H., 1993. Développement d'une méthode simplifiée de cryoconservation de tissus et d'embryons somatiques végétaux et étude de l'acqusition de la tolerance à la congélation. Thèse de l'Université Pierre et Marie Curie, Paris, 247 pp.

Tisserat, B., 1984. Clonal propagation: Palms. In: I.K. Vasil and F.L. Orlando (Eds.), Cell Culture and Somatic Cell Genetics of Plants. Vol. 1: Laboratory Procedures and Their Applications. Academic Press, London, pp. 74–81.

Vertucci, C.W. and Roos, E.E., 1993. Theoretical basis of protocols for seed storage, II. The influence of temperature on optimal moisture level. Seed Sci. Res., 3: 201–213.

Vertucci, C.W., Berjak, P., Pammenter, N.W. and Crane, J., 1991. Cryopreservation of embryonic axes of an homeohydrous (recalcitrant) seed in relation to calorimetric properties of tissue water. Cryo-Lett., 12: 339–350.

Wesley-Smith, J., Vertucci, C.W., Berjak, P., Pammenter, N.W. and Crane, J., 1992. Cryopreservation of desiccation-sensitive axes of *Camellia sinensis* in relation to dehydration, freezing rate and the thermal properties of tissue water. J. Plant Physiol., 140: 596–604.

Williams, R.J. and Leopold, A.C., 1989. The glassy state in corn embryos. J. Plant Physiol., 89: 977–981.

Withers, L.A., 1990. Tissue culture in the conservation of plant genetic resources. In: Conservation of Plant Genetic Resources through *in vitro* Methods. Proc. MNCPGR/CSC Internat. Workshop on Tissue Culture for Conservation of Biodiversity and Plant Genetic Resources, Malaysia, May 1990.

Dumet Dominique, Berjak P., Engelmann Florent. (1997).

Cryopreservation of zygotic and somatic embryos of tropical species producing recalcitrant or intermediate seeds.

In : Razdan M.K. (ed.), Cocking E.C. (ed.) Conservation of plant genetic resources in vitro : volume 1. General aspects.

New Hampshire : Science Publishers, 153-172. ISBN 1-886106-76-2