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Ex situ storage of seeds, pollen and *in vitro* cultures of perennial woody plant species

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Ex situ storage of seeds, pollen and *in vitro* cultures of perennial woody plant species

FAO FORESTRY

Based on the work of
Ben S. P. Wang
Pierre J. Charest
Bruce Downie

Food and Agriculture Organization of the United Nations

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FOREWORD

Following a review undertaken within the framework of FAO's Regular Programme in the early 1980s on *ex situ* conservation of forest genetic resources, the Seventh Session of the FAO Panel of Experts on Forest Gene Resources, held in December 1989, recommended that an updated document be prepared on the potential role of this strategy as a complement to the more frequently applied *in situ* conservation strategies. This recommendation was made in the light of new methodologies and technology available for the storage of seeds, pollen and tissue of plant species.

While recognizing the value of live collections and *ex situ* conservation stands, the present document, which is based on the work of a team of scientists of the Petawawa National Forestry Institute in Canada and discussions and contacts with a great number of scientists, worldwide, concentrates on *ex situ* storage methodologies. In this rapidly changing and developing field, it reports on the status and prospects as perceived at the present time (July 1993).

We hope to complement this document in the near future with published information on the establishment and management of *ex situ* conservation stands, which together with storage is considered a useful complement to the *in situ* conservation of long-lived, outbreeding and highly heterozygous forest species.

J.P. Lanly
Director
Forest Resources Division
Forestry Department

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Cover photo:
Micropropagation of douglas fir (*Pseudotsuga menziesii*)
at AFOCEL, France. Photo: C. Palmberg-Lerche, FAO.

EXECUTIVE SUMMARY

Drought, climate change, and deforestation lead to soil erosion, desertification, and loss of biological diversity. The consequence is a steady decline in the ability of the land in many areas of the world to produce the goods and services necessary for human survival (FAO 1989). To reverse the current trends of forest depletion, degradation of ecosystems, and loss of genetic resources an integrated strategy for resource management and maintenance of biological diversity is essential and urgent. This is especially true in tropical and subtropical regions, where complex and species-rich ecosystems are being rapidly destroyed or altered, and in the arid and semi-arid regions where fragile environments are threatened by increasing stress from human populations, domestic animals, and fluctuating climates (Palmberg 1987).

In agriculture most crop species are conserved by *ex situ* means using seed banks, field banks, and, in certain cases, tissue culture. In contrast, in forestry, because of the long regeneration time required by trees, the preferred conservation approach is to incorporate *in situ* conservation principles into sustainable forest management (Yeatman 1987), increasing the areas of managed forest reserves and strictly protected areas and complementing these with *ex situ* gene banks and conservation stands. Also seed orchards, botanic gardens, and arboreta can, to a certain degree, meet conservation needs and complement other conservation activities. Despite urgent needs, genetic conservation applied within a framework of forest resource management has been slow to develop and is generally limited to the establishment of national parks and a few *ex situ* seed and conservation stands, seed orchards, and arboreta.

In 1975, FAO/UNEP conducted a pilot study on the methodology for long-term conservation of forest genetic resources within a global context (Roche 1975). The report of the study recommended that the complementary role of *ex situ* seed banks in genetic resource conservation be further considered, and identified the need to establish research programmes especially in the testing, storage, and regeneration of tropical tree seeds. A study of the actual and potential role of *ex situ* conservation

methods was carried out for the Fifth Session of the FAO Panel of Experts on Forest Gene Resources held in 1981 (FAO 1985). At the Seventh Session of the FAO Panel of Experts on Forest Gene Resources, held in December 1989, the Panel recommended that earlier reviews on *ex situ* storage of seeds, pollen, and *in vitro* culture of forest tree species used as complementary measures to *in situ* conservation strategies be updated (Anon. 1989).

In response to the Panel's recommendation, FAO contracted the Petawawa National Forestry Institute, Canada, to conduct such a review.

Seed storage under controlled conditions is the most commonly used short- (3-5 years) to medium term (30 years or longer) *ex situ* conservation strategy for forest trees. Generally, this strategy is used to complement long term (several decades or a century) *in situ* and other *ex situ* conservation methods (e.g. *ex situ* conservation stands, clone banks, seed stands, seed orchards). The success of seed storage as well as that of pollen storage and *in vitro* culture is governed by the inherent longevity and physiological storage behaviour of species (i.e. orthodox, recalcitrant, or intermediate); by initial quality, such as moisture content of the material stored; and storage method and conditions.

Species with true orthodox seeds (e.g. *Pinus, Picea* spp.) have been successfully stored for up to 50 years under normal operational storage conditions of +5°C with a loss of germinability of 14-34%. With improved protocols for seed collection, handling, processing, and storage the longevity of orthodox seeds could be extended to 75-100 years. This can be achieved using cryopreservation. On the other hand, despite two decades of research towards developing effective storage methods for conserving seeds of recalcitrant perennial woody species, a group which includes many important tropical trees (e.g. many species of Dipterocapaceae) and industrial and fruit crop species (e.g. *Hevea brasiliensis, Durio zibethinus, Mangifera indica*), only short term storage methods have been developed, which extend storability from a few days or a few weeks, to 8-12 months (e.g. *Hevea brasiliensis, Theobroma cacao*). These results were achieved by cryopreserving excised embryos,

followed by regeneration using *in vitro* techniques. Such methods are too expensive for operational-scale activities, especially in developing countries. More research is required to develop protocols for possible storage of ultra-dry seeds without refrigeration.

Maintaining an efficient seed bank requires effective techniques for monitoring germinability of the seed stock. Reviewed reports recommend sequential germination tests; non-destructive techniques of viability testing are being researched. Although the IBPGR Storage Committee (1983) recommended a regeneration standard at 85% viability for most crop species, there is no generally applied standard for forest tree seeds.

It is important to note that although storage of seed, as such, is possible over extended periods of time for many tree species, regeneration of seed stocks will pose almost unsurmountable difficulties due to the long vegetative period most tree species undergo prior to producing seed, as well as the large areas needed to regenerate these long-lived, outbreeding, and heterozygous species. It is therefore clear that, at the present time, seed storage can only be considered a complement to *in situ* conservation or to live collections of various age classes established in the field and available for the collection of seed (and other reproductive materials) on a continuing basis.

In many respects pollen storage is affected by the same factors as seeds. The physiological storage behaviour of bicellular and tricellular pollen is comparable to that of orthodox and recalcitrant seeds, respectively. One of the possible limitations of using pollen storage for genetic conservation is the rapid loss of viability of seeds produced from stored pollen observed in some cases. Until the phenomenon is further confirmed to be true, pollen storage could be used in complement with seed storage as the vehicle for *ex situ* conservation. In future research, consideration should be given to optimal moisture content for pollen storage, the mode of desiccation and rehydration, the temperature and atmosphere of storage, and the necessity and type of cryoprotectant used in cryopreservation.

Ex situ storage using tissue culture and the development of artificial seeds are newly developed techniques. Although there have been numerous publications on plant tissue culture methods, tissue culture as a conservation method has been applied only to plant species that are of actual, proven, economic importance. There is also a lack of experience in germplasm preservation using tissue culture methods on a large scale. However, tissue culture appears to be an appropriate alternative conservation method for several classes of plants; these include plants that are only propagated vegetatively (e.g. bananas, cassava, potatoes), species with recalcitrant seeds (many tropical timber, industrial and fruit crop species), species that undergo a lengthy vegetative period prior to producing seeds, and sterile individuals possessing important, desirable, characteristics (e.g. embryo rescue in hybrids). Tissue culture further provides a useful tool in operations such as the procurement of propagules outside of the seed harvesting season and in disease eradication.

Although tissue culture of perennial woody species for conservation purposes seems to offer many advantages, it also has many disadvantages such as resistance to culturing of some species, uncontrolled somaclonal variation, and early maturation of cultured plants. However, recent research has developed guidelines to limit somaclonal variation and innovative, alternative techniques such as micrografting (i.e. *in vitro* grafting of adult meristems on juvenile rootstocks) affords a possibility for application to mature material. Tissue culture protocols can be developed for practically any plant species; however, the long term storage of tissues still present severe problems. Cryopreservation (including vitrification) is the only long term technique to preserve such propagules. However, each species or species group being preserved will need its own storage protocol, further raising the costs of this expensive method.

The report stresses the need to integrate *ex situ* and *in situ* approaches in an overall scheme of genetic diversity conservation, and to duly consider the advantages of the range of available methods. If used judiciously, the methods surveyed in the present document should contribute significantly to the conservation of valuable genetic resources of woody perennials.

Further development of culturing and cryopreservation techniques, *in vitro* tissue culture, and storage of artificial seed where the material is desiccated to moisture levels comparable to orthodox seed dried for storage, is needed to upgrade these methods to serve intermediate and long term conservation needs. Tissue culture research should be extended to the development of protocols for propagating a range of non-commercial species and focused on the development of inexpensive conservation methods, such as artificial seeds.

When evaluating the effectiveness and limitations of presently available *ex situ* methods for genetic conservation of perennial woody species it becomes clear that, with present knowledge, the storage of pollen, the storage of recalcitrant seed, and *in vitro* culture are only viable as short term measures. The only secure, medium- to long term *ex situ* conservation method presently available for large-scale use in forestry is the storage of orthodox seeds. Orthodox seeds can generally be conserved in the long term, through the application of cryopreservation methodologies. In all these cases, however, the problems related to regeneration of seedstocks mentioned above should be kept in mind. Storage per se may not be the most difficult problem in long term *ex situ* conservation strategies.

Future research should be directed to the following areas:

1. The development of proper protocols for screening and clear identification of truly recalcitrant seed species. To achieve this, we should examine rates of desiccation in relation to subsequent rates of germination, with particular attention to desiccation techniques that do not involve the use of heated air.

2. If seeds cannot be dried without injury, then their tolerance to cryopreservation should be examined. This process will involve evaluating temperature gradients of tissues in time and space, the penetration of cryoprotectants, the possibility of using excised embryos, and the feasibility of freeze-drying techniques.

3. For improving cryopreservation efficiency, the effects of cryogenic storage temperature and seed moisture content on induced dormancy should be studied.

4. Development of improved storage methods is required to prolong the longevity of seed of species that have been identified as having "intermediate storage behaviour".

5. Attempts should be made to improve conventional storage methods for moist seeds at ambient or sub-ambient temperatures, including the development of ways to control microbial attack, inhibiting germination with chemicals, and assessing oxygen requirements.

6. In view of the high cost involved in operating refrigerated seed banks (a problem of special relevance to developing countries), there is an urgent need to develop methods for the storage of ultra-dry seeds at ambient or sub-ambient temperatures.

7. Alternative strategies for the storage of recalcitrant seed should be investigated, such as the establishment of seedling banks, *ex situ* conservation stands, and seed orchards.

8. Development of tissue culture methods for non-commercial species and finding the critical factors determining the success of tissue culture in order to permit transfer of the techniques from species to species.

9. Establishment of inexpensive tissue culture preservation protocols that are suitable for long term conservation of trees. The exploration of natural processes, such as zygotic embryo desiccation during seed maturation, and its duplication *in vitro*, could possibly show the way to inexpensive storage.

Chapter one
INTRODUCTION

Drought, climate change, and deforestation lead to soil erosion, desertification and loss of biological diversity. The consequence is a steady decline in the ability of the land in many areas of the world to produce the goods and services necessary for human survival (FAO 1989a). To reverse the current trends of forest depletion, degradation of ecosystems, and loss of genetic resources, an integrated strategy for resource management and maintenance of biological diversity is essential and urgent. This is especially true in tropical and subtropical regions, where complex and species-rich ecosystems are being rapidly destroyed or altered, and in the arid and semi-arid regions where fragile environments are threatened by increasing stress from human populations, domestic animals, and fluctuating climates (Palmberg 1987).

The two commonly used strategies for conserving plant genetic resources are *in situ* conservation, which allows evolution to continue within the area of natural occurrence, and *ex situ* conservation, which conserves plants out of their natural habitat. *Ex situ* conservation involves a higher degree of protection and, consequently, greater isolation of germplasm than *in situ* conservation (Withers 1990c).

In agriculture most crop species are conserved by *ex situ* means using seed banks, field banks and, in certain cases, tissue culture. In contrast, in forestry, because of the long regeneration time required by trees, the preferred conservation approach is to incorporate *in situ* conservation principles into sustainable forest management (Yeatman 1987), increasing the areas of managed forest reserves and strictly protected areas, and complementing these with *ex situ* gene banks and conservation stands. Also seed orchards, botanic gardens, and arboreta can, to a certain degree, meet conservation needs and complement other conservation activities. Despite the urgent need, genetic conservation applied within a framework of forest resource management has been slow to develop and is generally limited to the establishment of national parks and a few *ex situ* seed and conservation stands, seed orchards, and arboreta.

In the tropics, *ex situ* conservation of forest genetic resources has become common practice due to the alarming rate of deforestation and loss of species and provenances. Practically all such conservation measures are in the form of conservation stands of species and provenances of proven value (Palmberg 1987).

In 1975, FAO/UNEP conducted a pilot study on the methodology for long- term conservation of forest genetic resources within a global context (Roche 1975). Its report recommended that the complementary role of *ex situ* seed banks in genetic resource conservation be further considered, and identified the need to establish research programmes especially in the testing, storage, and regeneration of tropical tree seeds. A study of the actual and potential role of *ex situ* conservation methods was carried out for the Fifth Session of the FAO Panel of Experts on Forest Gene Resources held in 1981 (FAO 1985). At the Seventh Session of this panel, held in December 1989, it was recommended that earlier reviews of *ex situ* storage of seeds, pollen, and *in vitro* culture of forest tree species be updated (FAO 1989b). In response, the FAO contracted the Petawawa National Forestry Institute, Chalk River, Ontario, Canada, to conduct such a review.

The present report is the result. Only *ex situ* storage techniques of seeds, pollen, and *in vitro* culture of perennial woody plant species are reviewed here, with particular emphasis on the relative advantages and disadvantages. Arboreta, seedling banks and *ex situ* conservation stands are not discussed in detail in this report.

Chapter two
EX SITU STORAGE USING SEEDS

INTRODUCTION

Seed storage under controlled conditions is the most commonly used short- (3-5 years) to medium term (30 years or more) *ex situ* conservation strategy for forest trees. Generally, this approach is used to complement both long term *in situ* conservation and other *ex situ* methods (e.g. *ex situ* stands, clone banks, seed orchards). This is due to the practical difficulty of regenerating stored seeds of woody species when their germinability declines to below certain levels compared with agricultural crops. However, long term seed storage for the equivalent of one rotation is possible for many woody species, and it could become a viable prospect for *ex situ* conservation in general (Bonner 1990). Maintaining an efficient seed bank requires effective techniques for monitoring germinability of the seed stock. Reviewed reports recommended sequential germination tests; non-destructive techniques of viability testing are being researched. Although the IBPGR Storage Committee (1983) recommended a regeneration standard at 85% viability for most crop species, there is no generally applied standard for forest tree seeds. The advantages and disadvantages of seed storage as a supplement to woody species gene conservation have been reviewed by Wang (1975), King and Roberts (1979), Chin and Roberts (1980), Yeatman (1987), Chin (1988), and Bonner (1990). The present paper reviews recent developments in seed storage requirements and storage technology, and discusses the possibilities and limitations of seed storage as a potential long term germplasm conservation measure for woody species.

The objectives of seed storage for conserving germplasm of woody species is to maintain the initial genetic and physiological quality of the seeds until they are used or can be regenerated. Achieving this requires consideration of major genetic and environmental factors affecting seeds of woody species in storage.

GENETIC FACTORS

The life span of seeds, pollen, or tissue of tree species in storage is genetically controlled and varies with species, even under optimum storage conditions. Until recently, seeds were grouped into two classes according to their storage behaviour: orthodox and recalcitrant (Roberts 1973). More recently a third class, represented by arabica coffee (Coffea arabica L.), with storage behaviour intermediate between those of orthodox and recalcitrant seeds, has been discovered by Ellis *et al.* (1990). Bonner (1990) classified seeds of woody species into four categories based on storage physiology: (1) true orthodox, (2) sub-orthodox, (3) temperate recalcitrant, and (4) tropical recalcitrant.

True orthodox seeds tolerate desiccation and low temperatures and retain viability for long periods under dry, cool storage. Among tree genera having such seeds are *Abies, Betula, Acacia, Eucalyptus, Fraxinus, Larix, Paraserianthus, Picea, Pinus, Platanus, Prosopis, Prunus, Pseudotsuga,* and *Tsuga*. Seeds of this group have a long storage life and can remain viable for up to 50 years. As pointed out by Bonner (1990), good data on long term storage of true orthodox seed of woody species are scarce. Our Table 1 expands Bonner's (1990) Table 1 with additional unpublished data from the National Tree Seed Centre at the Petawawa National Forestry Institute.

TABLE 1
Longevity of some true orthodox seeds

Species	Seed moisture (%FW)	Storage temperature (°C)	Storage period (years)	Viability loss (%)	Reference
Abies balsamea	10	2-4	15	21	Wang unpubl. data
A. lasiocarpa	4.9	2-4	9	9	Wang unpubl. data
A. procera	9	0	7	11	Allen 1957
Acacia leptopetala	-	20-25	18	1	Doran et al. 1983

TABLE 1 continued

Species	Seed moisture (%FW)	Storage temperature (°C)	Storage period (years)	Viability loss (%)	Reference
A. mangium		4-8	1.2	6	Tap & Wong 1983
A. pruinocarpa	-	20-25	16	20	Doran et al. 1983
Acer rubrum	8.5-11.6	2-4	14-15	100	Wang unpubl. data
A. saccharum	10	-10	5.5	5	Carl 1976
Albizia (Paraserianthus) falcataria	-	4-8	1.5	10	Tap & Wong 1983
Alnus crispa	5.7-7.2	2-4	4	0-34	Wang unpubl. data
A. rubra	5.3-8.5	2-4	4	0-13	Wang unpubl. data
Araucaria cunninghamii	16-23	-15	8	little	Shea & Armstrong 1978
	7	19	0.1	0	Tompsett 1982
Betula alleghaniensis	8.5-13.2	2-4	15	54-100	Wang unpubl. data
	9-14.3	-18	15-23	38-43	Wang unpubl. data
Casuarina equisetifolia	16	-3	2	0-5	Jones 1967
C. torulosa	8-12	20-25	18	6	Turnbull, Martensz 1982
Eucalyptus spp.	4-8	3-5	5-20	-	Boland 1986
Larix decidua	7.5	2-4	14	27	Wang unpubl. data
L. gmelini	6.2	2-4	15	5	Wang unpubl. data
L. laricina	5.5-9.8	2-4	17-18	32-52	Wang unpubl. data
L. leptolepis	12.1	2-4	23	66	Wang unpubl. data
L. sibirica	6	2-4	13	0	Wang unpubl. data
Liquidambar styraciflua	5-10	3	9	3	Bonner 1987
Picea abies	5.4-9.8	2-4	21-27	9-83	Wang unpubl. data

TABLE 1 continued

Species	Seed moisture (%FW)	Storage temperature (°C)	Storage period (years)	Viability loss (%)	Reference
P. engelmannii	4.2	2-4	28	31	Wang unpubl. data
P. glauca	3.6-5.5	2-4	17-20	0-14	Wang unpubl. data
	6.4-7.9	2-4	20-24	1-68	Wang unpubl. data
	8.5-9.0	2-4	21-34	47-81	Wang unpubl. data
P. mariana	6.5-13.5	2-4	13-15	1-4	Wang unpubl. data
	5.2	2-4	37	38	Wang unpubl. data
	5.6-13.5	-18	13	26-36	Wang unpubl. data
P. rubens	8.8-9.1	2-4	23-30	0-2	Wang unpubl. data
P. sitchensis	6.8-9.5	2-4	13-24	0-11	Wang unpubl. data
Pinus banksiana	11.2	2-4	17-18	0-8	Wang unpubl. data
	4.8-5.0	2-4	21	0-15	Wang unpubl. data
P. caribaea var. *hondurensis*	-	8	2.7	±16	Yap & Wang 1983
P. contorta var.	3.6-4.8	2-4	29	52	Wang unpubl. data
latifolia	9.2-10	2-4	20	1-3	Wang unpubl. data
P. elliottii	10	4	50	30	Barnett & Vozzo 1985
P. merkusii	8+	4-5	4	0	Pousujja et al. 1986
P. ponderosa	8	0	7	0	Allen 1957
	6.3	2-4	12	0	Wang unpubl. data
P. resinosa	5.4-9.1	2-4	13-20	0-3	Wang unpubl. data
	8.0-10.0	0-2	42	0	Eliason & Heit 1973
P. strobus	5.6-6.5	2-4	15-20	32-77	Wang unpubl. data
	8.0-8.7	-18	15-20	0-18	Wang unpubl. data
Tectona grandis	±12	0-4	7	0	Keiding 1985
Tsuga heterophylla	8	5-18	2	0	Barton 1954

Some sub-orthodox seeds contain a very high lipid content (*Juglans nigra* and some *Carya* species) and others are small-sized with thin seed coats (e.g. *Populus* spp., *Salix* spp.). Seeds of this group can be stored under the same conditions as true orthodox seeds, but only for 0.6 to 6 years. Loss of viability ranges from 0 to 34% when stored at subfreezing temperatures from -5 °C to -20 °C and moisture contents between 5% and 10% (Bonner 1990). See Table 2.

TABLE 2
Longevity of some sub-orthodox seed species

Species	Seed moisture (%FW)	Storage temperature (°C)	Storage period (years)	Viability loss (%)	Reference
Citrus limon	5	-20	9.6	±5	King et al. 1981
Fagus silvatica	10	-10	5	34	Suszka 1975
Gmelina arborea	6-10	-5	2	10	Woessner and McNabb 1979
Malus domestica cv. 'Antonowka'	6.7-8.9	-1 to -18	3.1	0	Grezeskowiak et al. 1983
Populus deltoides	6-10	-20	6	21	Tauer 1979
P. deltoides var. *occidentalis*	8.4-13.5	-18	10	24-100	Wang 1982
P. grandidentata	10.8-14.8	-18	12	14-29	Wang 1982
Prunus avium	8.9-10.4	-1 to -3	2	0	Grezeskowiak et al. 1983
P. cerasifera var. *divaricata*	7.6-11.9	-1 to -3	2	0	Grezeskowiak et al. 1983
Pyrus cancasia	8.6-10.5	-1 to -18	3.1	0	Grezeskowiak et al. 1983
Salix glauca	6-10	-10	1.2	0	Zasada 1977

Temperate recalcitrant seeds are desiccation-sensitive but can be stored at near freezing temperatures. This group includes *Acer saccharinum*, *Quercus* spp., and *Aesculus hippocastanum*. They can be dried to relatively high seed moisture contents of 35 - 50% (fresh weight) and stored safely at temperatures between 3°C and -3°C (Bonner 1973, Suszka and Tylkowski 1981, 1982). The seed longevity of this group lies between 12 and 30 months if relatively high moisture contents are maintained and gas exchange is possible.

Tropical recalcitrant seeds are usually large in size and are mainly found in (a) tree species of moist forests belonging to the families Dipterocarpaceae and Araucariacea, (b) important tropical plantation crops such as rubber, cocoa, and coconut, and (c) tropical fruit crops such as mango, mangosteen, durian, rumbutan, and jack fruit (Chin 1988a). Seeds of this group are desiccation-sensitive and chilling-sensitive, with many intolerant to freezing temperatures (e.g. *Hevea brasiliensis* and *Nephelium lappaceum*). Some cannot tolerate temperatures of 4°C (*Shorea roxburghii* (=*talura*)) or even 15°C (*Theobroma cacao*) (Chin 1988a).

According to the most recent review of the literature on storage of *Dipterocarpus* seeds (Tompsett 1987, 1989), the longevity of 79 tropical recalcitrant species ranged from 14 days for *Shorea dasyphylla* to 365 days for *Hopea hainanensis*. It is interesting to note that, although both temperate and tropical recalcitrant seeds are desiccation-sensitive and low temperature sensitive, the apparent differences between these two groups seem to be the relatively high sensitivity to low temperature and shorter longevity of seed of tropical species. It should be pointed out that the longevity of seeds in their various groups as reported above is based on present knowledge; it could change from one group to another as techniques in desiccation and storage advance.

ENVIRONMENTAL FACTORS

Initial seed quality

In order to maintain the maximum longevity of seed of different groups in storage, a problem associated with gene banking is the initial quality of

seeds before they are placed in storage (IBPGR 1982). All seed harvested from designated populations require careful control of their original genetic and physiological quality throughout the phases of collection, handling, processing, testing, and storage. The detrimental effects of collecting viable immature seed on seed viability in storage has been well documented and reviewed (Wang 1974). For example, for recalcitrant seeds of *Shorea roxburghii* (=*talura*), the final 3 weeks on the tree are crucial for achieving maximum germinability (Sasaki 1980). Although Berjak *et al.* (1990) reported that recalcitrant seeds do not go through maturation drying as do orthodox seed, research results from Thailand suggest they do, at least in the case of *Shorea siamensis* which saw germinability increase when moisture content was lowered from 40% to 30% (Panochit *et al.* 1986). In a similar study Panochit *et al.* (1984) found that *Shorea roxburghii* (=*talura*) seed increased germinability from 33% to 92% as its moisture content dropped from 59% to 49%.

Initial seed quality is also influenced by post-harvest ripening. Recent research findings suggest that post-harvest ripening is important to the seed germinability of *Picea glauca* (Caron *et al.* 1989, 1993) and the germinability, vigour, and storability of *Pinus palustris, P. strobus,* and *P. taeda* seeds (Bonner 1991). The requirements for post-harvest ripening in recalcitrant seed were first reported by Tang and Tamari (1973) for *Hopea helferi* and *H. odorata* seeds. Panochit *et al.* (1986) found that germination of immature seed of *Shorea roxburghii* (=*talura*) increased when the moisture content was reduced from 40% to 30% over 4 days. In seed processing it was found recently that extended periods of exposure of Pinus contorta seeds to 60°C kiln temperature could affect seed vigour negatively through membrane damage (Wang *et al.* 1992).

Because seed dormancy is recognized as a significant contributing factor to extended storage life, it is essential to protect the seedcoat and other physical and physiological characteristics that affect seed dormancy (Barton 1961, Harrington 1972). The importance of initial seed quality to seed longevity in storage was demonstrated by Smith (1992). This recognition was underlined by its inclusion as a major factor in the mathematical viability formulae developed by Roberts (1972) and Ellis and

Roberts (1980) in which the percentage viability of a seed population is tied to storage temperature, seed moisture content, and initial seed quality. Tompsett (1986, 1989) has successfully applied the viability formulae to seeds of tropical trees such as *Araucaria columnaris, Dipterocarpus alatus, Entandrophragma angolense, Swietenia humilis, Terminalia brassii,* and *Ulmus carpinifolia,* and has shown that the longevity of the seeds was closely influenced by initial seed quality.

Seed moisture content

Moisture content, together with storage temperature and oxygen, are the three critical factors affecting seed longevity in storage. Roberts and Ellis (1989) found that moisture content had (a) little or no effect on longevity at low levels (1.5-2.5%) where true orthodox seeds are likely to be stored, (b) a negative effect on longevity in the central moisture range (2.6-14.7%) where most true and sub-orthodox seeds are stored for the long term and where oxygen is detrimental to longevity, and (c) a critical effect when the presence of oxygen was in the higher moisture content range (15-45%) where both temperate- and tropical-recalcitrant seeds are stored.

For long term genetic conservation of both true- and sub-orthodox seeds, it has been recommended that moisture content be reduced to 5 ± 1% (IBPGR 1976). This standard was challenged by Cheng *et al.* (1990) who proved that the moisture content of seed of *Brassica pekinensis* could be reduced from 8.6% to 1.6% by freeze-drying and who considered such ultradry storage potentially useful and cost effective for long term germplasm conservation.

For seed of most true- and sub-orthodox woody species, moisture content is usually reduced to below 10% (fresh weight) at 20-35 °C for short term to medium term storage. For long term gene conservation, the moisture content may have to be reduced further to 5 ± 1% or less (IBPGR 1976). To achieve such a moisture content, drying facilities with a controlled temperature of 15 °C and relative humidity of 10-15% or a two-stage drying system are recommended by Cromarty *et al.* (1982). A lower moisture content (less than 5%) for woody orthodox seeds can be

achieved by freeze-drying, although initial critical moisture contents have to be determined for the species in question. Suber *et al.* (1973) successfully reduced *Picea abies* seeds from 8% to 2.4% moisture content (fresh weight) by freeze-drying without injury and, subsequently, stored the seeds at 25°C for 6 years with a 9% loss of germinability.

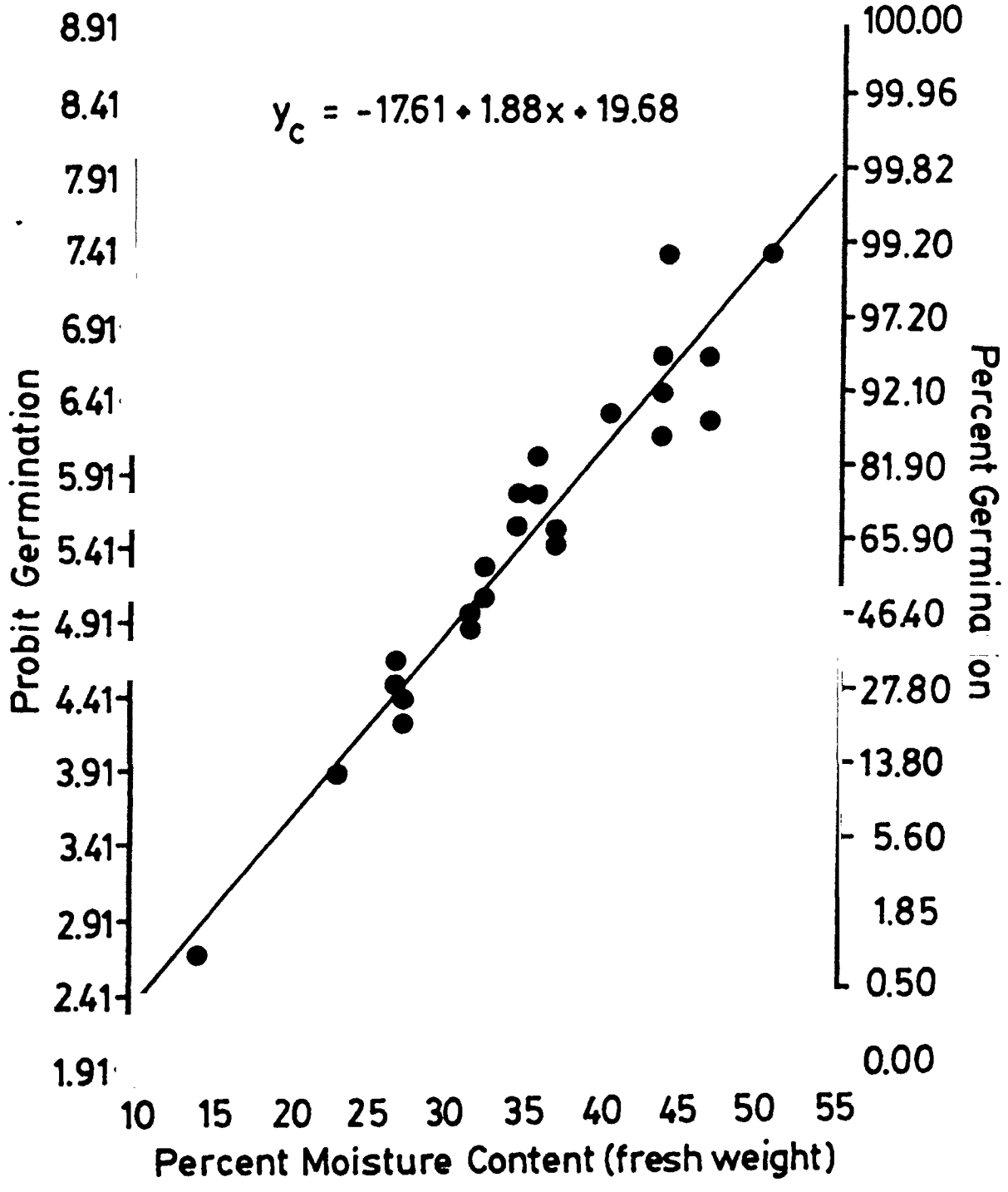
Recalcitrant seeds, on the other hand, cannot be dried below a relatively high moisture content; their longevity is increased with a rise in seed moisture content in the presence of oxygen (King and Roberts 1979). In order to prolong longevity of recalcitrant seeds from both temperate and tropical regions, one approach suggested by King and Roberts (1979) is to examine the conditions for desiccation and rates of desiccation. The relationship of seed moisture content and germinability of *Acer saccharinum* over a 30-day period can be seen from Figure 1 (Wang *et al.* 1991, unpubl. data). Berjak *et al.* (1990) used a flash-drying technique, involving a stream of compressed air passed through two serial columns of activated silica gel, to successfully reduce the moisture content of recalcitrant embryo axes of *Landolphia kirkii* from 49% to 16% moisture content (dry weight) without diminished viability or vigour.

When air drying it should be noted that, because of differences in the chemical composition of seeds, different species do not attain the same equilibrium moisture content when exposed to the same air temperatures and relative humidities (Harrington 1972). For example, under conditions of 4-5°C and 95% relative humidity, starchy acorns of *Quercus alba* attained an equilibrium moisture content of 50%, whereas lipid-rich acorns of *Quercus nigra* gave a 29% reading (fresh weight). This was because starch was more hygroscopic than fat (Bonner and Vozzo 1987).

Wang (1974) reviewed critical moisture content in different species, above or below which there is a rapid loss in seed germinability, and considered the data useful for safe storage. Terminology such as "lowest safe moisture content value" (LSMC) was used for Dipterocarpus seeds by Tompsett (1987). Stanwood (1985) used "high moisture freezing limit" (HMFL) to guide cryopreservation storage that avoided chilling injury. A summary of LSMC values determined from published results for *Dipterocarpus* seed species are presented by Tompsett (1987).

FIGURE 1

Quantitative relationships between moisture content as percent of fresh weight and germinability expressed as percent germination and probit germination of silver maple (*Acer saccharinum*) seeds air-dried at ambient temperature and relative humidity in a cone shed for 30 days (Wang, Saelim, and Downie, unpubl. data 1991)



Storage temperature

Storage temperature is the other major factor affecting longevity of seed. The importance of storage temperature in maintaining seed viability of woody species has been well reviewed (Holmes and Buszewicz 1958; Barton 1961; Heit 1967a, b; Wang 1974; King and Roberts 1979, 1980; Stanwood 1985; Tompsett 1989; Bonner 1990). In general, it has been recommended that long term storage of orthodox seeds of woody species for gene conservation purposes can be achieved by drying to 1-5% moisture content (fresh weight) and storing in sealed containers at -18°C (IBPGR 1976, Roberts and Ellis 1977). However, seed deterioration and loss of viability can still occur at such a low temperature with increased time in storage resulting in a loss of valuable genetic material (Stanwood 1985). According to a paper by Dickie *et al.* (1990), the relationship between comparative seed longevity and temperature suggests that the lower the storage temperature, the longer the potential seed storage life. From this relationship, cryopreservation appears to be a preferred strategy for long term storage of valuable genetic material. At such cryostorage temperatures (-145° to -196°C) all metabolism would effectively be suspended (Stanwood and Bass 1981, Withers 1990c).

Storage method

Proper storage of given species varies with storage behaviour. For true and sub-orthodox seeds, dry storage is recommended in sealed containers with little or no air exchange (Wang 1974). This technique will maintain a constant moisture content of the seeds, reduce respiration as carbon dioxide increases and oxygen decreases, and protect seeds from insects and diseases (Harrington 1972, Wang 1974). In a study comparing the effects of sealed atmospheres of air, carbon dioxide, nitrogen, and a vacuum on germinability of *Pinus radiata* seeds stored with 8% moisture content (fresh weight) at 5° to 35°C temperature for a year, it was found that germination, energy, and seedling vigour were best maintained by storage in nitrogen followed by carbon dioxide, a vacuum, and air, regardless of storage temperature (Shrestha *et al.* 1985). However, there are a number of physiological advantages in storing imbibed orthodox seeds, as suggested by Villiers (1974) and Villiers and Edgcumbe (1975). These are that the enzyme, cell membrane, and DNA repair mechanisms

all remain intact, eliminating damage as it occurs, and that membrane integrity ensures the proper compartmentalization of enzymes and metabolic processes.

Recalcitrant seeds require a relatively high moisture content and will be injured by prolonged sealed storage (Wang 1974). In general, the most successful storage has been achieved by methods that control desiccation, microbial contamination, and germination in storage, providing there is an adequate oxygen supply (King and Roberts 1980). Storage in moist media such as peat, sawdust, or sand has been reported to be successful for seeds of oak (Korstian 1930, Gardener 1939), *Aleurites fordii* (Large *et al.* 1947), rubber (Ang 1976), *Castanea* spp. (Texeira 1955, Jaynes 1969), coffee (Leon 1974), *Citrus* spp. (Honjo and Nakagawa 1978), rambutan (Chin 1975), and cocoa (Evans 1953). As pointed out by King and Roberts (1980b), storage in moist media is only adequate for short term storage of a few months and is unlikely to be effective for the long term. So far, the most successful method of storing recalcitrant seeds has been sealed storage in polyethylene bags. Temperate-recalcitrant acorns of oak species have been successfully stored with 30% moisture content (fresh weight) in 4-10 mil thick polyethylene bags at +3° to -3°C for up to 5 years (Bonner and Vozzo 1987). Longer storability of acorns has been reported in carbon dioxide-enriched storage atmospheres (Vozzo 1976, Tylkowski 1976). A promising method currently used for storing tropical recalcitrant seeds involves partial drying of the seeds for a few hours, treating with fungicide, sealing in thin polyethylene bags, and storing at around 15°C (e.g. cocoa, *Dryobalanops aromatica, Hopea helferi, H. odorata*) (King and Roberts 1980a).

CRYOPRESERVATION

Cryopreservation commonly refers to the use of ultra-low temperatures (-80° to -196°C) for preserving biological materials (Towill 1985), and in the context of this paper it is to preserve seed. As pointed out by Stanwood (1985), physiological preservation of seeds at -40° to -196°C has not been proven to be significantly better to that at -20°C. However, estimates of longevity from the viability equation (Ellis and Roberts 1980)

suggest that the lower the storage temperature, the longer the potential storage time (Stanwood 1985, Dickie *et al.* 1990).

The potential advantages of cryopreservation over conventional techniques are an absence of complicated temperature and humidity controls, the freedom from damage by pests and diseases, and indefinite longevity with little or no genetic damage (Styles *et al.* 1982).

Not all species of seed can be stored at cryogenic temperatures because of differences in chemical composition and storage physiology. Therefore, it is essential to consider the various factors affecting the success of seed cryopreservation. Stanwood (1985) prefers liquid nitrogen (LN2) as a cryopreserving medium to mechanical refrigeration systems because of its relative reliability and ease of operation. He grouped seed of different species into three categories according to their response to LN2 exposure: (1) desiccation-tolerant and LN2-tolerant, (2) desiccation-tolerant and LN2-sensitive, and (3) desiccation-sensitive and LN2-sensitive.

Cryopreservation has been recognized as the most promising approach to storage of recalcitrant seeds (Roberts *et al.* 1984, King and Roberts 1979, 1980, Bonner 1990) and much progress has been made in the development of new and improved storage techniques (Chin 1988a, King and Roberts 1979, 1980a, Farrant *et al.* 1988). In the most recently published status report on recalcitrant seeds, Chin (1988a) stated that so far there has been no success with truly recalcitrant seeds and stressed the importance of developing cryostorage techniques for excised embryos of recalcitrant species.

Desiccation-tolerant and LN2-tolerant seeds

Included in this category are seeds of the most common agricultural and horticultural species, conifers, and small-seeded hardwoods, and they can generally be characterized by their storage behaviour as true- and sub-orthodox seeds. There has been a considerable success in cooling such seeds to the LN2 temperature of -196°C and rewarming them to an ambient temperature of 20°C without loss of viability (Stanwood 1985). Table 3, lists such seeds (see Stanwood and Bass 1981, Stanwood 1985,

TABLE 3
Seeds of some tree species tolerant to LN_2 storage

Species	Seed moisture content (%FW)	Storage period (days)	Viability loss (%)	Reference
Abies alba	-	6	5	Ahuia 1986
Abies concolor	9.8	365	9	Stanwood 1985
A. procera	8.9-10.1	365-1095	1-12	Stanwood 1985
Fagus sylvatica	-	6	100	Ahuia 1986
Larix decidua	-	6	5	Ahuia 1986
Picea abies	-	6	-	Ahuia 1986
P. glauca	3.8-6.7	4	0	Wang & Hay unpub. data
	14	14	30	Wang & Hay unpub. data
P. mariana	4.8-6.7	14	2	Wang unpub. data
Pinus banksiana	6-8	14	10	Wang unpub. data
P. contorta var. *latifolia*	11	14	4	Wang unpub. data
P. echinata	-	112	0	Engstrom 1966
P. lambertiana	6.6	365	10	Stanwood 1985
P. ponderosa	4.7	1095	0	Stanwood 1985
Populus tremula x *P. tremuloides*	-	6	1	Ahuia 1986
Pseudotsuga menziesii	6.8	1095	0	Stanwood 1985
Thuja plicata	7.0	1095	1	Stanwood 1985
Tsuga heterophylla	8.2	1095	0	Stanwood 1985
Ulmus americana	6.4	1095	7	Stanwood 1985
U. pumila	-	112	0	Engstrom 1966

Bajaj 1976, Brown and Escombe 1897, Wang and Hay, unpublished data 1989), all of which are true orthodox.

For successful cryopreservation, an optimal range of moisture content is critical for the cooling and rewarming processes. Stanwood (1985) developed a high moisture freezing limit (HMFL), defined as the threshold moisture content above which loss of seed viability occurs during the cooling/rewarming process. The HMFL value is usually a narrow range of moisture content within a species, but varies between species (Stanwood 1985). For date palm (*Phoenix dactylifera*), the HMFL was found to be between 16.1% and 22.3% (fresh weight). Tompsett (1986) reported the HMFL for *Ulmus carpinifolia* and *Terminalia brassii* seeds to be 19-22% and 17-20%, respectively, when they were stored at -75°C for 1 week.

Although seed of *Abies, Picea, Pinus, Pseudotsuga, Thuja, Tsuga,* and *Ulmus* spp. were successfully cryopreserved in LN2 for 1-3 years using uncontrolled cooling and a rewarming rate of 30°C/min, seed of *Picea glauca* and *P. mariana* were safely stored in LN2 for 4 days without controlling the rate of cooling or thawing (Wang and Hay, unpublished data). Although the rate of cooling and rewarming are not considered important, each species should be tested to avoid loss of viability.

Cryoprotectant chemicals sometimes help lessen injuries incurred by seeds during freezing by decreasing the rate of cooling and/or the temperature at which freezing occurs within the cells (Kausrin and Stushnoff 1985). There are a number of such cryoprotectant substances. The most popular among them are dimethyl sulfoxide (DMSO), 1, 2-propanediol, and glycerol (DeBoucaud and Cambecedes 1988). While DMSO has been the most widely used cryoprotectant, it tends to be toxic whether used alone in aqueous solution (Kausrin and Stushnoff 1985) or mixed with other chemicals (Gazeau 1980). However, its toxic effect, particularly when less than ultra-pure, is generally outweighed by its beneficial effects (L.A. Withers, personal communication).

Physical alteration of seeds due to storage in LN2 can be beneficial, neutral, or harmful. The beneficial action can be traced to increased

permeability due to slight seedcoat cracking. Harmful physical alteration is such that it causes deformity or death of the seeds. This injury may be related directly to freezing in LN2 such as when differential rates of tissue contraction/expansion cause cotyledons to shear from their petioles (Pritchard *et al.* 1988). The injury may also be caused indirectly by imbibitional damage suffered when the increased rate of imbibition through a more permeable seedcoat harms seeds susceptible to imbibitional stress (Spaeth 1986). Wang and Hay (unpublished data) working with *Picea mariana* and *Picea glauca* found that direct immersion of seed in LN2 resulted in complete stripping of the seedcoat in about 1% of seed of both species.

Exposure to cryogenic temperatures of -150°C or lower are not only tolerated but result in the removal of hardseededness in *Lotus corniculatus* (clover) and several other species (Pritchard *et al.* 1988). It may be possible to apply this technique to remove seedcoat dormancy of many tropical legumes. Of the more than 150 species tested, only a few suffered damage from cooling and subsequent rewarming other than to the surface of the seedcoat (Stanwood 1985). For those species suffering physical damage, the possibility of slower cooling rates and/or multi-stage freezing to alleviate seed problems should be examined.

Desiccation-tolerant and LN2-sensitive seeds

Seeds of this class are exemplified by many fruit and nut crops such as *Corylus, Juglans,* and *Prunus* species, as well as forest trees such as *Fagus silvatica, Gmelina arborea,* and *Populus* species. They can be characterized by desiccation tolerance to moisture content below 10% (fresh weight) and a storability of less than 5 years (Stanwood 1985). They are, however, sensitive to temperatures lower than -40°C. These seeds are usually rich in storage lipids, which may go as high as 60-70% in some species (Bewley and Black 1983). Seed of Corylus species lost their viability at temperatures lower than -40°C, apparently due to solidification of the oils (Stanwood 1985). However, the toxicity of LN2, particularly when less than ultra-pure, is generally outweighed by its beneficial effects (L. Withers, personal communication). Recent research shows good survival and normal growth of cryopreserved embryo axes of *Juglans*

(Pence 1990, de Boucand *et al.* 1991), *Carya* and *Corylus* (Pence 1990), *Aesculus hippocastanum* (Pence 1992), and *Quercus* (Gonzalez-Benito and Perez-Ruiz 1992, Jorgensen 1990). More research is needed to develop optimal media for the growth of isolated embryo axes recovering from desiccation and freezing into healthy plants (Pence 1990).

Desiccation-sensitive and LN2-sensitive seed

Seeds of this class represent both temperate and tropical recalcitrant seed, including those of many economic crops such as rubber (*Hevea brasiliensis*), areca, palm, and cocoa (*Theobroma cacao*), tropical fruits such as mango (*Mangifera indica*), jack fruit (*Artocarpus heterophyllus*), and rambutan (*Nephelium lappaceum*), and many timber species of the families Dipterocarpaceae and Araucariaceae (Chin 1988a). These seeds are characterized by their large size, short lifespan, and sensitivity to desiccation and freezing temperature (Chin 1988a). Seed of some species in this class were previously identified as recalcitrant but are now known to be tolerant to both desiccation and LN2 exposure (e.g. passion fruit, paraceara rubber, common guava, papaya) (Stanwood 1985).

There are two principal techniques for preserving recalcitrant seeds cryogenically; either the embryos are excised, dried, and stored in LN2 or the whole seed is used (De Boucaud and Cambecedes 1988). A third method, introduced by Grout (1979), uses imbibed tomato seeds as a model for storing recalcitrant seeds in LN2. Seed with moisture contents exceeding 70% (fresh weight) preserved in LN2 did not germinate, but shoot-meristem explants dissected from such seeds were viable following storage and produced clones through tissue culture. Use of this technique, however, requires (a) that some somatic tissue of the seed in question remains viable after LN2 storage and (b) that tissue culture techniques exist for the species.

Grout *et al.* (1983) pointed out that storage of large recalcitrant seeds of woody species such as rubber, cocoa, coconut, and oil palm could take up a lot of space if the whole seed were stored, increasing the cost of cryopreservation. Thus, storage of germplasm in alternative forms (e.g. excised embryo axes, cell suspensions, tissue culture callus, etc.) may also

be more economical than whole seed storage.

The major problem associated with the storage of recalcitrant seeds is their sensitivity to desiccation. Usage of subfreezing storage temperatures causes intracellular freezing and death (Fu 1951, Harrington 1972, Sasaki 1976, Chin 1978). Whether such lethal desiccation injury occurs rapidly at or below the critical moisture content or loss of viability occurs gradual due to physiological deterioration at low moisture contents is not known (King and Roberts 1979). Another possibility is that a reduction in moisture content results in membrane damage, with resultant effects upon membrane function and organelle integrity, which is manifested in the extreme as nuclear disintegration. This has been demonstrated in sun-dried rubber seeds (Chin *et al.* 1981). Meryman and Williams (1985) cited literature from a wide variety of plants in which hydrated cells survived the loss of 55-57% of cell water prior to irreversible injury. Injury is hypothesized to be due either to the loss of membrane integrity and entry of extracellular ice into the cell or to the irretrievable loss of membrane material causing cell membrane rupture upon rehydration.

Mumford and Grant (1979) found that fresh citrus lemon seed at 90.1% moisture content (dry weight) could not be stored in LN2 without complete loss of viability. Drying intact seed of this species resulted in decreases in viability from 75% (at 90% moisture content) to 30% (approximately 35% moisture content). Subsequent storage of the dried seeds in LN2 had no effect on viability. However, upon removing the testa of the seeds, it was possible to dry the seed to 1.2% moisture content (dry weight) without any decrease in viability while maintaining 95% germination. These seed survived LN2 storage, although some failed to produce radicles upon rewarming. Pritchard and Prendergast (1986) and Grant *et al.* (1983) found that, if embryos of recalcitrant *Araucaria hunsteinii* and oil palm (*Elaeis guineensis*) were excised and dried to about 20% moisture content, they survived storage in LN2, and subsequently grew *in vitro*. Surface sterilization prior to cryopreservation of the embryos reduced root meristem survival. Grant *et al.* (1983) found that the excised embryos of oil palm had a 48.3% moisture content and could be dried successfully to 20% without any reduction in viability (88% germination). However, further

drying to 10.4% moisture content was accompanied by a slight reduction in *in vitro* germination to 75%.

Such desiccated embryos could be frozen and stored in LN2 for at least 8 months with no further loss in viability. According to Chin (1988b), those species that did not survive LN2 storage were subsequently tested with cryoprotectants such as DMSO, glycerol, L-proline, and various enriched media. At present, embryos of *Artocarpus heterophyllus, Nephelium lappaceum, Cocos nucifera,* and *Dryobalangs aromatica* have survived cryopreservation and have regrown in enriched media. Such techniques could be useful in improving the storage life of other recalcitrant species, although some refinement is needed.

Following a review on strategy for future research into the storage of recalcitrant seeds, King and Roberts (1980b) considered long term low temperature storage of recalcitrant seeds feasible. They suggested three approaches to the problem of attempting to increase the storage period of recalcitrant seeds: (1) identifying truly recalcitrant seed species; (2) examining the rates of drying and subsequent rates of germination, particularly focusing on drying techniques which do not involve heated air; and, (3) trying to improve the traditional techniques of storing moist seeds at ambient or sub-ambient temperatures if the approach in (2) proved impossible.

ADVANTAGES AND DISADVANTAGES OF SEED STORAGE

Because of irregular seed production in woody species, especially forest trees, storage is essential to afforestation programs for continuous seedling production. Seed storage at low temperature and humidity could be used to facilitate and complement other strategies of conservation of genetic resources (Hawkes 1980, Bonner 1990). Indeed, with the present knowledge of seed storage behaviour and storage technology, there are many advantages in including seed storage as a component in systems of germplasm maintenance and genetic conservation of tree species. For example:

1. True orthodox seeds of valuable woody species can be desiccated to

low moisture content and stored in LN2 for an indefinite period beyond rotation age with little or no genetic change.

2. Compared to other conservation approaches, seed storage is cost effective and can provide facilities for preserving all three types of diversity — clinal (geographical), inter-population, and intra-population.
3. Good seed conventional storage facilities (excluding LN2 storage) are available worldwide and this can assist widespread international exchange of valuable germplasm material.
4. Seed storage can serve as a repository in the short term for recalcitrant seeds, in the intermediate term for sub-orthodox seeds, and , for true orthodox seeds, also as an important complement within long term genetic conservation strategies.

However, we should remember some disadvantages of storing seeds for long term genetic conservation such as:

1. Seeds of many trees, especially the all-important tropical recalcitrant and temperate recalcitrant species, cannot be stored safely using present techniques.
2. Large-sized seeds of some woody species, particularly the recalcitrants such as cocoa, jack fruit, and mango take up considerable storage space, increasing the cost of seed bank management.
3. Concern has been expressed about the potential hazards of aging-induced genetic changes in stored seeds and their progeny (Roos 1982). Although the evidence is that chromosomal and non-chromosomal mutations are largely eliminated during the sexual process and thus are not passed on to the next generation, the IBPGR (1976) recommends, as a precaution, regenerating stored germplasm when germinability decreases by 5-10%. This will make it necessary to regrow stored germplasm stocks more frequently, with a consequent increase in cost.
4. Unlike agricultural crops, seeds of woody perennial species require long regeneration cycles (15-80 years depending on species) from seed sowing to sexual maturity, which are actually longer than the storage cycles (Hawkes 1980). Regeneration of stored seedlots will therefore pose at times unsurmountable problems.

Chapter three
EX SITU STORAGE USING POLLEN

INTRODUCTION

If the choice is available it is better to use seeds instead of pollen as a vehicle of genetic conservation. Pollen storage for *ex situ* conservation of alleles is important when storing species that are difficult to conserve by other *ex situ* methods, e.g. those that have recalcitrant seeds and exhibit poor response to tissue culture methods. The germinability of fresh pollen, or that which is stored for a short term, is principally determined by the integrity of the vegetative cell membrane (Shivanna and Heslop-Harrison 1981, Hoekstra and van der Wal 1988). The condition of this membrane is influenced by both handling and manipulation of pollen moisture content prior to and during storage. Long term storage increases the risk that other factors, such as loss of enzyme activity, depletion of storage reserves, etc. would cause a loss of germinative capacity (Stanley and Linskens 1974). The major factors that determine pollen longevity in storage are commonly recognized as moisture content and temperature (Towill 1985). These two parameters affect pollen viability for both short- and long term storage and, therefore, determine what proportion of the alleles in the original population are passed on to the next generation. Hence they are physiological constraints on the lifespan and functionality of pollen.

It is of cardinal importance to maintain the largest possible proportion of pollen grains in a viable state by optimizing storage conditions. Callaham (1967) reported that pollen lots diluted with dead pollen did not decrease seed set except for lots with less than 30% viable pollen. However, if a pollen lot has a considerably decreased germinability, it may not represent the genetic composition of the original lot (Towill 1985). This would imply storage at cryogenic temperatures to arrest, insofar as possible, metabolic processes and subsequent death of the pollen grains. Other forms of conservation, at higher temperatures, may be adequate for short term conservation, but they are not sufficient for genetic conservation. The discussion to follow will concentrate on the problems associated with cryo-preservation of pollen.

Unfortunately, there is a dearth of information on the longevity of propagules, be they seed, tissue culture products, or pollen at ultra-low temperatures. A study conducted for 8 years on the viability of tree pollen stored in LN2 has shown that, while most lots did not decrease in viability, others did, the cause not necessarily being connected to species or with variation in moisture content (Ichikawa and Shidei 1972c). Vagaries in testing, however, could account for some of this variation. Furthermore, decreases in viability of maize (*Zea mays* L.) (Nath and Anderson 1975) and wheat (*Triticum aestivum* var. *erytrospermum*) pollen stored at -196°C over short periods has been reported (Andreica *et al.* 1988), although this may have been an artifact of desiccation.

Cryogenic preservation usually necessitates partial desiccation in order to avoid lethal intracellular ice formation. Such desiccation is generally feasible for bicellular pollen grains which have one generative cell and one vegetative cell at the time of dispersal. However, there exists a second category of pollen which is tricellular when disseminated, the generative cell having undergone mitosis to produce two sperm cells prior to anther dehiscence (Brewbaker 1967). These pollen grains typically have very short lifespans and do not usually tolerate desiccation to moisture contents low enough to permit exposure to sub-zero temperatures (Towill 1985).

The bicellular group is the larger of the two, at least for the angiosperms (Brewbaker 1967), and may be subdivided into those with complete or incomplete mitochondria at the time of dehiscence (Hoekstra and Bruinsma 1980). Bicellular pollen can generally withstand desiccation to moisture contents of less than 10% fresh weight (FW) (11.1% dry weight) (Towill 1985). Evidence supporting Schürhoff's hypothesis (1926) that tricellular pollen is more advanced phylogenetically continues to accumulate (Brewbaker 1967, Ottaviano and Mulcahy 1989). The distinction between bi- and tricellular pollen has been correlated to physiological differences. Bicellular pollen has an extended lifespan and shows prolonged growth *in vitro*, traits lacking in tricellular pollen. As well, the site and type of the incompatibility reaction in self-incompatible species differs between the two groups (Brewbaker 1967). Hoekstra and Bruinsma (1975a) found that tricellular pollen grains have an higher respiratory quotient and overall

respiration rate than does bicellular pollen. This is explained by the fact that tricellular pollen is dispersed at dehiscence with fully-developed mitochondria, while biçellular pollen has incomplete mitochondria (Hoekstra 1979).

Detailed discussions of the differences between these two groups are found in Brewbaker (1967), Mäkinen and Brewbaker (1967), and Stanley and Linskens (1974). Systematic classification of the groups based on geographic trends seems futile. Brewbaker (1967) points out that tricellularity appears to have arisen independently in many widely divergent plant families worldwide. However, classification based on life history strategy suggests that male gametophytic selection will alter several biosynthetic processes necessary prior to commencement of tube growth from the post-pollination period (development on the style) to the pre-pollination period (development prior to dehiscence). When pollen grains land on a style they can commence germination immediately and, therefore, compete more effectively (Ottaviano and Mulcahy 1989). Such differences that depend on site of development are documented for bi- and tricellular pollen. For an excellent review of these aspects of angiosperm pollen evolution please consult Ottaviano and Mulcahy (1989, pages 41-43).

ENVIRONMENTAL FACTORS

Initial Quality

It is of extreme importance to develop a method for testing pollen viability that correlates well and consistently with the pollen's ability to set seed (Schoenike and Bey 1981). Owens and Blake (1985) and van Buijtenen (pers. comm.) point out that, although the observation of germination usually correlates well with a pollen lot's ability to set seed, this is not always the case. The "population effect", for example, whereby aggregated pollen grains germinate better than do isolated grains (Ahlgren and Ahlgren 1978), decreases the reliability of the germination test. It should also be recognized that accidental damage to pollen exines may produce artifacts that appear as germinating pollen. This is especially true with tricellular pollen (Hoekstra and Bruinsma 1975a). Tube identification

has been facilitated by use of the aniline blue stain to detect callose under an UV microscope (Hoekstra and Bruinsma 1975a). Furthermore, the optimal sucrose, boron, and/or calcium concentration and/or pH level of the germination medium is often species specific (Cauneau-Pigot 1988, Hall and Farmer 1971, Hoekstra and Bruinsma 1975b). Aged pollen often requires higher concentrations of sugar (Vasil 1962) and/or boron (Visser 1955) in the medium than fresh pollen for optimal germination. It has been hypothesized for *Pinus* and *Picea* pollen exhibiting this response that the permeability of stored pollen to sugar decreases (Kühlwein and Anhaeusser 1951). Desiccated and/or stored pollen has been shown to take longer to germinate *in vivo* (Barnabás and Fridvalszky 1984, Heslop-Harrison 1979) than fresh pollen. Hence Barnabás and Fridvalszky (1984) urge pollination with stored pollen only under optimal environmental conditions. Artificial pollination in Douglas-fir with pollen stored in LN2 has been implied to increase conelet abortion relative to fresh pollen (Copes 1985). Although this trend is not significantly different, it suggests that the stored pollen elicited a higher rate of conelet abortion than did fresh pollen. Data implying a similar reaction has been published by Ganeshan (1986b) although this was not statistically analyzed.

Germination *in vitro* has been stimulated when exposed to electromagnetic fields. Such conditions have resulted in increased pollen germinability and germination vigour *in vitro*, and decreased germination time (Alexander and Ganeshan 1990). The techniques may improve correlations between *in vitro* viability and seed set *in vivo*. However, when preserving pollen for genetic conservation, it is not enough to simply predict the extent of seedset (Callaham 1967). *in vitro* pollen germinability may be low while seed set remains high, possibly resulting in progeny that contain proportions of paternal contributions of DNA that are much altered from the original gametic population. This is because *in vitro* pollen tests randomly sample and sum the germinability of individual grains, while fertilization of a megagametophyte requires but one viable pollen grain.

Experiments using electrical conductivity tests and infrared optical density analysis of pollen leachate have given conflicting results. Ching and Ching (1976), Foster and Bridgewater (1979), and Goddard and Matthews

(1981) reported high correlations with pollen germinability *in vitro* while Moody and Jett (1990) found little or no correlation.

A variety of staining techniques have been used in an effort to predict seed set. Acetocarmine has been used but the results do not correlate well with actual viability (Johri and Vasil 1961). Many other staining reactions, most concentrating on the vegetative cell, have been used which attempt to predict pollen viability, but they have been inconclusive. Furthermore, attempts to correlate vegetative cells enzymatic activity to viability have had poor results due to retention of enzyme activity in recently dead pollen (Heslop-Harrison and Heslop-Harrison 1970, King 1960). Even tetrazolium tests may give ambiguous results (Oberle and Watson 1953). Alexander (1980) developed a multipurpose stain for use with pollen that, according to Ganeshan (1986a, 1986b), distinguishes successfully between germinating and non-germinating pollen grains. However, no attempt was made to assess whether the non-germinating grains were indeed dead.

Heslop-Harrison and Heslop-Harrison (1970) have concentrated on the membrane integrity of the vegetative cell of pollen grains as a test of viability. They hypothesize that if this cell exhibits normal permeability then the cell is, in all likelihood, viable. Cell permeability was tested by staining with fluorescein diacetate, a non-fluorescent, non-polar chemical that acts as substrate for the cell's esterase enzyme complex (Rotman and Papermaster 1966). Hydrolysis within the cell produces fluorescein which does not permeate out of intact, viable cells. Thus fluorescein accumulation in cells seems generally to be highly correlated with viability, at least for short storage durations (Shivanna and Heslop-Harrison 1981, Heslop-Harrison *et al.* 1984).

Ideally, pollen viability should be tested by dusting stigmas of the same species and by observing pollen germination *in vivo*. Holman and Brubaker (1926) were of the opinion that there may be some "substances" in the stigma that stimulate tube formation and aid fertilization. It may be possible to homogenize stigmas and styles of the species of interest and to include the homogenate in standard germination testing medium. Knowlton (1922) found that *Antirrhinum* pollen, initially incapable of

germination on artificial medium, was stimulated to do so when a piece of the stigma was introduced onto the medium. More precise identification of factors essential to or promoting pollen germination from plant tissues was pioneered by Hodgkin and Lyon (1986). They separated tissue homogenate by thin layer chromatography, smeared pollen of the same species across the thin layer strip, and documented the bands on which pollen germinated vigorously.

Moisture content (MC)

Although bicellular pollen is generally less prone to lethal damage from decreases in moisture content than is tricellular pollen, reduction of MC to near 0% has been shown to be either lethal or result in greatly decreased longevity (Towill 1985). Bramlett and Matthews (1991) have shown that for loblolly pine (*Pinus taeda* L.) pollen, frozen at temperatures that ranged from -4 to -20° C for a year, *in vitro* germinability was 94 and 91% for pollen at 9 and 7% moisture content FW, respectively, while for pollen at 3% moisture content, germinability was 73%. The total number of seeds produced and the percentage of filled seeds were highest for the lots stored at 9 and 7% MC and lowest for the lot stored at 3%. Pollen of tricellular species of the genera *Tulipa, Clivia, Aesculus, Parnassia,* and of *Plantago media* cannot be dried to moisture contents of less than 40% dry weight (approximately 28.5% moisture content FW) and pollen of all Gramineae species must be kept at high MC to prolong viability for even a few days (Stanley and Linskens 1974). However, Shi and Tian (1989) dried tricellular rye pollen as low as 6% MC and maintained viability for one year in LN2.

Towill (1985) points out that the lower limit of desiccation tolerance is species dependant, and that the initial MC of pollen prior to desiccation may affect survival. Purportedly, for bicellular pollen the lower the initial MC the better the pollen is able to survive further desiccation. This has been found to be the case for Japanese pear (*Pyrus serotina*) (Akihama *et al.* 1978) and western white pine (*Pinus monticola* Dougl.) (Ching and Ching 1964). The viability of pollen grains in storage of Japanese pear and Japanese persimmon (Diospyros kaki) was inversely proportional to their predrying MC (Akihama and Omura 1986). However, the contention that

the lower the initial MC of the pollen the better it survives further dessication is confounded in these reports with the mode of dessication, i.e. freeze-drying. The lower the initial MC of the pollen, the shorter its exposure to vacuum, and vacuum-drying is injurious to pollen (Livingston and Ching 1967; Davies and Dickenson 1971). A better designed experiment by Hoekstra and van der Wal (1988) has shown that pollen with very low MCs (approx. 5-8%) are invariably damaged by further drying. Barnabás and Rajki (1981) working with trinucleate maize pollen and air-drying found that the range of MCs optimal for low temperature storage survival and subsequent seed set was extremely wide (9.8-25.6%). However, when they examined their results, based on the percent of original moisture lost, a clear optimum resulted — maximum survival was upon loss of 30% of original water content. Losses less than or exceeding this value resulted in much decreased seed set. It is difficult to reconcile these results with current theories involving the integrity of both the plasmalemma of the vegetative cell and pollen mitochondria (Shivanna and Heslop-Harrison 1981, Crowe *et al.* 1989a,b). The importance of the mode of rehydration in maintaining pollen viability cannot be overestimated, and one must realize that all studies cited above are complicated by the manner of rehydration. Furthermore, the study by Barnabás and Rajki (1981) was on tricellular pollen; their physiological response to desiccation and rehydration may differ from that of bicellular pollen.

Hoekstra and Bruinsma (1975b) found that pollen viability of different species of Compositae varied inversely with temperature and relative humidity (RH) at the time of collection. For *Chrysanthemum cinerariaefolium* pollen collected at 24° C, viability was maintained at approximately 85% only in relative humidities less than or equal to 60%. Above this level a sharp decrease in viability was noted. Presumably the pollen equilibrates to the relative humidity. This affects the respiration rate of both tri- and bicellular pollen, with little CO2 evolution occurring at relative humidities below 77% (Hoekstra and Bruinsma 1975a). Therefore, due to the confusion regarding the optimum amount of original moisture to lose prior to storage, and the necessity of desiccation prior to subzero storage, it would be prudent to collect pollen during periods of low relative

humidity (Barnabas and Fridvalszky 1984).

Mode of desiccation/rehydration

Disruption of the plasmalemma due to desiccation (Hoekstra and van der Wal 1988, Crowe *et al.* 1989a) and/or imbibitional chilling injury when dry pollen imbibes cold water quickly (Caffrey *et al.* 1987) are possible causes of decreased pollen germination. Furthermore, the two types of injury are not necessarily different (Hoekstra and van der Wal 1988, Sack *et al.* 1988). It is thought that some type of disruption occurs during transition of the phospholipids from a relatively rigid gel phase, at low moisture content or temperature, to a fluid phase upon water imbibition. Injury is independent of the degree of saturation of the hydrophobic tails of these lipids (Hoekstra and van der Wal 1988). Furthermore, for membranes possessing large amounts of phosphatidyethanolamine (PE) and other phospholipids capable of forming inverted hexagonal (HII) tubes (Simon 1974, Crowe *et al.* 1989a, Quinn 1985), injury may be sustained upon rehydration by leakage prior to the phospholipids reverting to the lamellar form (Crowe *et al.* 1989a). Additionally, injury can be caused by free radicle generation which can attack unsaturated bonds in lipids as well as de-esterify the phospholipid head group of the fatty-acid and leave free carbon tails in the plasmalemma (Niehaus 1978). This changes membrane fluidity.

Imbibitional chilling damage to the membranes of Typha pollen resulted in ultrastructural changes relative to viable pollen (Sack *et al.* 1988). The most obvious difference between viable pollen and pollen killed by imbibitional damage is the inability of dead pollen to dilate upon hydration (Gilissen 1977, Hoekstra and van der Wal 1988). Therefore, the ability of dried membranes to preserve their structural organization is essential to the recovery of dried pollen (Shivanna and Heslop-Harrison 1981). This, in turn, is regulated to some extent by the properties of the constituents of the membrane (characteristics of the polar heads, length of the hydrocarbon chains, presence of stabilizing solutes, etc.).

Crowe *et al.* (1989a,b) show that the stability of the liquid-crystal, lamellar form of pollen phospholipid membranes breaks down at low MCs.

Their stability at low MCs can be enhanced by the addition of sugars such as sucrose and trehalose, which may replace water around the polar phosphate heads and expand the bilayer, thus decreasing the van der Waal forces among the tails. Hoekstra and van der Wal (1988) also point out that certain carbohydrates can maintain phospholipids in a liquid state at a lower MC than normal and that mature pollen usually has high levels of sugars. Carpenter and Crowe (1988) showed that carbohydrates (sucrose) maintained lactate dehydrogenase in its native configuration under conditions that would usually cause denaturation. Many solutes, among them sugars, are preferentially excluded from contact with proteins in aqueous solution. This leads to a thermodynamically unstable state due to high relative concentrations of the solute in the bulk solution compared to conditions around the protein. Denaturation of the protein would lead to an increase in its surface area, augmenting the entropically unfavourable state. Some solutes, excluding DMSO, which protect proteins from solution-induced perturbations are also used as cryoprotectants where they may serve the same function (Carpenter and Crowe 1988).

Jain and Shivanna (1987) advocate the use of non-polar solvents for pollen storage. Upon examining a wide range of different compounds, they found that the less polar a solvent the higher the post-storage germinability of the pollen. They hypothesize that the non-polar solvents allowed retention of an osmotically active (fluid crystal) plasmalemma in the pollen grains after rehydration. Pollen was collected from Crotalaria retusa L. and was desiccated to 5.2% MC prior to storage in solvents. However, this pollen was not damaged by desiccation and the experiment does not shed any light on the probable cause of pollen death due to membrane disruption at low MCs. It would be appropriate to use this system for dessication-sensitive pollen storage; the pollen could be rehydrated in a moist atmosphere and tested for germinability immediately.

One of the most widely used methods for desiccating pollen for *ex situ* storage has been freeze-drying. However, this technique affects pollen viability and is fraught with difficulties. Drying under vacuum was shown to be the cause of altered viability, respiration, and permeability in lily (*Lilium longiflorum* cv. Ace) (Davies and Dickinson 1971). There is some

evidence to suggest that the rate of electron transport is limiting to respiration in freeze-dried pollen, impairing oxidative phosphorylation, ATP production and, therefore, viability (Davies and Dickinson 1971). Livingston and Ching (1967) found that freeze-drying Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) pollen resulted in decreased viability. Cauneau-Pigot (1988) has shown that fragile pollen (thin exine pollen) cannot withstand desiccation by freeze-drying. Hence, she concludes that the desiccation/preservation technique employed may be dictated by pollen morphology.

Many of the problems in correlating *in vitro* pollen germination with that *in vivo* are due to control of pollen rehydration. Numerous cases have been documented where pollen germination *in vitro* was successful only after rehydration in a moist atmosphere prior to testing, while pollen used for *in vivo* testing germinated well without rehydration prior to being placed on the stigma (Dereuddre and Gazeau 1986, Hecker et al 1986).

Tricellular pollen can be stored longer at higher relative humidities but the longevities vary considerably (Towill 1985). The rate at which pollen dries varies with the thickness of the exine, but this factor has no relationship to the desiccation sensitivity of the pollen (Towill 1985).

Pre-desiccation treatment

Cold conditioning prior to dessication has been shown to benefit pollen longevity in some species. Douglas-fir pollen chilled at 0°C for 36 days while concurrently undergoing air drying exhibited the best survival when subsequently freeze-dried (Livingston and Ching 1967). Cold conditioning prior to freeze-drying also prolonged longevity in western white pine pollen (Ching and Ching 1964). Even for pollen with very high moisture contents (70%), high survivability was attained by a prefreezing technique (Ching and Slabaugh 1966). Success with this pretreatment is not limited to freeze-drying. Exposure to prefreezing temperatures from -15° and -30°C for between 1 and 5 hours resulted in high survivability of pollen fast-cooled to either -80° or -196°C (Ichikawa and Shidei 1972a,b). The prefreezing temperature and duration of exposure that was most effective in promoting survivability varied among species. But pre-dessication cold

treatment had no effect on coconut pollen (Whitehead 1965). Akihama and Omura (1986) found no differences in pollen survival of six species if the pollen had been cooled prior to freeze-drying.

Temperature

Prior to the 1970s, much of the literature on pollen storage examined storage for short durations from 1 to 2 years (Towill 1985, see also Table 4). This allowed the use of temperatures from slightly above 0°C to just below -30°C for the duration of storage and allowed maintenance of viability at a high level until the pollen was used in breeding programs, etc. However, such temperatures are not sufficient for long term conservation to safeguard the genetic diversity present in a pollen sample. Ching and Slabaugh (1966), working with bicellular coniferous pollen, studied ice crystal formation during freezing by X-ray diffraction analysis. Ice crystals were not formed in pollen at 10% MC (FW) but they were at 36% MC. Detectable ice crystals were formed at about -25°C and pollen grains with detectable ice were not viable. Some pollen of Pinus monticola at about 35% moisture was killed prior to intracellular ice formation, decreasing germinability of the control (irradiated pollen) from about 95% prior to freezing down to about 68% after cooling to -25°C. It was concluded that non-membrane piercing events caused pollen death, and it was observed that frozen pollen that did survive took longer to germinate than unfrozen pollen.

As with recalcitrant seeds, tricellular pollen presents us a quandary with regards to storage. The pollen at a high MC respires at a high rate and declines quickly in viability. Freezing of high MC pollen is lethal due to the events occurring during intracellular ice formation. At the same time the MC cannot usually be decreased to allow subzero storage; such desiccation will also kill the pollen. Long term storage of such pollen will probably require cryogenic temperatures. Treatment of the pollen is going to be complex, and will probably be lethal to portions of the pollen sample.

Number of cooling/rewarming cycles

Koopowitz *et al.* (1984) found that *Gladiolus* pollen germinability was dependant on the number of freeze-rewarm cycles it went through.

TABLE 4

Effect of pollen storage on seedset (total number seeds/flower), filled seed weight, number of filled seeds/flower, seed germinability, and seed storability relative to fresh pollen

Source	Species	Temp. of storage	Storage duration	Seed set	Seed weight	Seed germinability	Seed storage
Akihama et al. 1980	peach	-20°C	9 years	Same	----	----	----
	+						
	pear	-20°C	6 years				
Bramlett & Mathews 1991	loblolly pine	-18°C	3 years	Lower	----	----	----
	loblolly pine	-18°C	10 years	Lower?	----	----	----
Crisp & Grout 1984	broccoli	-196°C	5 minutes	Same	Same	----	Poorer
Ganeshan 1986b	papaya	-196°C	300 days	Higher flower abortions with stored pollen ?	----	----	----
Ganeshan 1986a	onion	-196°C	360 days	Same	----	----	----
Haunold & Stanwood 1985	hop *(Humulus)*	-196°C	1 year	Same	Same	Same	----
Hecker et al. 1986	sugar beet	-196°C	1 year	Lower	----	Same	----
Ichikawa & Shidei 1972c	*Pinus*, *Larix*, *Cryptomera*	-196°C	1 year	Same	Same	Same (*Larix*) others not tested	----
Lee et al. 1985	jojoba	-196°C	2 years	Same	----	----	----

Papaya pollen at an unreported MC could tolerate rapid freezing and rewarming for at least six cycles without decreasing in viability (Ganeshan 1986b). Ganeshan (1986a) also reported results that appear to show that onion pollen, at an unreported MC, could be frozen and partially rethawed at least four times without significantly reducing germinability. Hecker *et al.* (1986) found that tricellular beet pollen at approximately 12% MC could be partially thawed and immediately refrozen without affecting germinability. Preferably, samples of propagules used for germplasm conservation should not be cooled or rewarmed more than once. Samples used for periodic monitoring can be premeasured and stored separately in different vials under the same conditions as the bulk of the pollen lot.

Use of cryoprotectants

Bicellular pollen, which can usually withstand desiccation to MCs that permit cryopreservation, would not benefit much from the addition of cryoprotectants. However, because tricellular pollen cannot normally be desiccated without loss of membrane integrity and the capacity to germinate, it cannot be frozen without lethal damage due to intracellular ice formation. Hence, tricellular pollen might be frozen and thawed without decreased viability if it can be cryoprotected.

Research has been conducted on cryoprotectant chemicals and their mode of action, which allows hydrated tissue to be subjected to low temperatures without allowing the free water in the cells to form ice crystals (Meryman 1966, King and Roberts 1980). MacFarlane and Forsyth (1990) studied a host of different cryoprotectant chemicals used to vitrify aqueous solutions. Vitrification, the formation of "amorphous ice" or "glass", occurs when cooling progresses so rapidly that water solidifies without orienting itself into crystals. The best cryoprotectants are those that act as moderately strong bases, disrupting the ability of water to form a highly hydrogen-bonded network at low temperature. Secondly, they must increase solution viscosity greatly, thereby decreasing the rates of nucleation and crystallization. Finally, strong bases resulted in strong solute-water interactions, which led to an increase in the glass transition temperature. 2,3-butanediol was found to posses all the characteristics identified above (MacFarlane and Forsyth 1990). Carpenter and Crowe

(1988) have shown how a variety of solutes protect lactate dehydrogenase from denaturation during freezing (see above). Due to the small size of pollen grains and the high permeability of tricellular pollen exines generally, infusion with cryoprotectants as well as their efflux after thawing should be easy relative to seeds and other large multicellular bodies used in tissue culture. Unfortunately, no account of the use of cryoprotectants and subsequent freezing of tricellular pollen is available, something which should be remedied by research.

Storage atmosphere

Storage in a vacuum or in a nitrogen atmosphere prolonged maize pollen longevity compared to storage at the same temperature and humidity in air (Nath and Anderson 1975). The current hypothesis as to why storage in oxygen containing atmospheres is more deleterious than in anoxic atmospheres is that oxygen causes the formation of free radicles in dried tissues (Heckly 1978). These free radicles can attack unsaturated bonds in lipids, particularly membranes lipids (Lehninger 1982, Niehaus 1978) and de-esterify polar heads from carbon tails. Storage in LN2 or its vapor should preclude such degradation.

GENETIC FACTORS

Intraspecific variation

Variation in tolerance to manipulation has been documented on a strictly genetic level. Dereuddre and Gazeau (1986) state that intra-/interspecific variability in response to freezing and/or drying, and the physiology of tricellular pollen, pose the greatest hindrances to pollen conservation. Ockendon and Gates (1976a,b), working with onion (*Allium cepa*), found that pollen viability varied among anthers on the same flower and among flowers within the same umbel. Dietze (1973) points out that there is clone-related sensitivity to freeze-drying in tree species, with some individuals losing germinative capacity while others of the same species maintaining it. Major variation in germination among trees of the same species occurs in the same stand (Farmer and Hall 1975). Furthermore, some variation in tolerance to manipulation can be due to environmental and genetic factors. Ahlgren and Ahlgren (1978) found that pollen viability

varies annually regardless of abundance. Dereuddre and Gazeau (1986) have documented differences in resistance to drying and freezing among species of the genus *Prunus*, as well as differences among cultivars of the same species. However, Hecker *et al.* (1986), working with sugar beet pollen, could find no indication of genetic effects regarding tolerance to storage.

POLLEN CONSERVATION

Possibilities and limitations

The record of using pollen as a vehicle of gene conservation is mixed:

Advantages

1. When the species to be preserved has recalcitrant seeds and exhibits poor response to tissue culture methods, pollen, if it could be stored for a long term, would be a preferred mode of *ex situ* conservation.
2. Controlled crossing is feasible at a later time. Pollen is easily shipped and, upon thawing and rehydration, is ready for use. Controlled crosses can be done directly with pollen whereas, with seeds, one would have to wait for the seedling to grow to maturity (Towill 1985).
3. Pollen is more economical to store due to the small size of grains.
4. Pollen banks could represent large populations and haploidy is potentially useful in developing numerous clones of androgenic plants of known genetic makeup (Ottaviano and Mulcahy 1989).

Disadvantages

1. Only half the genome will be preserved and these genes may diverge widely from those desired. Namkoong (1981), however, points out that alleles can be recombined in future breeding generations if they persist in the preserved pollen. Nonetheless, the screening and breeding programs necessary for such recombination are daunting.
2. The method of pollen storage can select for specific genotypes, altering gene frequencies in the progeny produced. Because the Hardy-Weinburg Law breaks down if there is selection at the gametophytic level (Pallais *et al.* 1986), this development could lead to a permanent loss of rare alleles.

3. Advantages to be derived from pollen storage are dependant on the survival and fertility of females of the species or their survival in storage as tissue or seeds and subsequent growth to maturity. Furthermore, even after successful pollination, many tree species (Pinus, for example) have seed development times of 18 to 24 months during which insect damage, flower abortion, etc. can result in poor yield (Goddard and Matthews 1981). As the use of stored pollen will usually imply the production of seeds, the use of seeds themselves as the vehicle of genetic conservation would seem to be a preferable strategy, when this is possible.

Such considerations impact on the strategic decision to use pollen in gene conservation. Once the strategic decision has been made, tactical decisions regarding how the pollen is to be stored must be addressed. Both the strategic decision to use pollen and the tactical decisions on how best to store it depend on the type of pollen produced by the species of interest.

Bicellular pollen that have completely developed (Type I) or incompletely developed (Type II) mitochondria at the time of anther dehiscence are relatively easy to store due to their tolerance of desiccation, which permits freezing. Tricellular pollen (Type III) are generally intolerant of desiccation and cannot be frozen due to their high MCs. The physiology of Type III pollen is such that longevities are much lower compared to bicellular pollen.

Future research needs

Tactical considerations that must be investigated further include the moisture content at which to store the pollen, the mode of desiccation if the pollen can survive drying, the mode of rehydrating dried pollen, the temperature of storage (this will depend on the duration of storage), the storage atmosphere, and the necessity and type of cryoprotectant used.

Stored pollen produced seeds that were not different in terms of filled seed weight, empty seed percentage, or germinability (Table 4). However, Crisp and Grout (1984) found that such seeds, although initially

indistinguishable, rapidly lost viability relative to seeds from fresh pollen when seeds from both pollen sources were stored. This phenomenon, if generally valid, presents far reaching consequences for the use of pollen as a means of genetic conservation. It necessitates the cultivation of seeds produced from stored pollen immediately upon harvesting, which may be impractical or impossible.

Chapter four
EX SITU STORAGE USING TISSUE CULTURE

INTRODUCTION

There have been numerous publications (Bajaj 1986, Bonga and Durzan 1987a,b,c, Hanover and Keathley 1988, Ahuja *et al.* 1988, Kartha 1985b, Sharp *et al.* 1984a,b, George *et al.* 1987,1988, Evans *et al.* 1986, Ammirato *et al.* 1984, 1990, Von Arnold and Wallin 1988, Borman *et al.* 1991) on plant tissue culture methods and related aspects. However, there is very little literature on germplasm preservation using tissue culture methods in global terms. Furthermore, little information exists to date on plant regeneration from tissue culture for many plant species that are not of economical importance.

There are three central aspects of germplasm preservation using tissue culture-derived propagules:

1) *in vitro* propagation methodologies;

2) problems associated with *in vitro* tissue culture, such as somaclonal variation and early maturation of tissue culture-derived plants that can have significant detrimental consequences on germplasm conservation; and,

3) techniques of long term "storage" of the tissue culture material.

METHODOLOGIES

Tissue culture preservation of germplasm is an appropriate alternative conservation method for several broad classes of plants (Benson and Withers 1988, Withers 1980, Scowcroft 1984, Biondi 1986, Withers 1990a,b,c; Ford-Lloyd and Jackson 1991; Roca 1989) such as:

1. species that are only propagated vegetatively e.g. bananas, cassava, potatoes, etc.;
2. long life-cycle species where the production of seeds takes a long time;
3. species with recalcitrant seeds that will die if desiccated (e.g., most tropical species: cacao, mango, coconut, avocado);
4. sterile individuals that possess important characteristics (e.g., through embryo rescue);
5. propagules that have to be collected out of the seed shedding season;

6. species where disease elimination is desired for successful conservation and subsequent propagation.

Plant tissue culture or *in vitro* culture can be defined as the process in which plant cells, tissues, organs, or any parts thereof are grown in aseptic conditions in an artificial environment (Scowcroft 1984). Tissues cultured *in vitro* are divided into two categories. First, explants that retain their developmental integrity and have a definite organization, such as meristems (shoot tips and axillary buds). Second, explants that dedifferentiate to a more or less organized state such as somatic embryos, gametogenic embryos, and adventitious buds. The dedifferentiation process can be complete or incomplete, ranging from a disorganized callus to embryoid formation. Somatic embryos were reported for the first time in 1958 by Steward *et al.* and Reinert from carrot cells. They are referred to as "somatic" because they originate from somatic cells and not from gametogenic fusion (Ammirato 1989) and "embryos" because they are structurally similar to zygotic embryos. The process of somatic embryogenesis is also called adventive embryogenesis.

Gametogenetic embryos are derived from immature pollen or non-fertilized ovules and give rise to haploid plants. Adventitious buds are created by the process of organogenesis in which dedifferentiated plant tissues cultured *in vitro* are induced to produce new meristems. Table 5 compares the different methods. Cell suspensions and protoplasts are often derived from other tissues grown *in vitro* and their use is for very specialized purposes such as secondary metabolite production (cell suspensions) and genetic transformation (protoplasts). Cell suspensions can be used for micropropagation. Protoplasts, on the other hand, are more difficult to use for this purpose because of their fragility and the prevelance of specificity of the protocols for each genotype.

Different types of tissue culture methods have been developed suitable for different species. However, certain species are "recalcitrant" to tissue culture and this is a major obstacle in using tissue culture for germplasm conservation. In general, tissue culture methods have been developed mostly for dicotyledons and within this group the Solanaceae and

TABLE 5

Comparison of tissue culture methods available for tree and shrub germplasm conservation.

Explants	Advantages	Disadvantages
Existing meristems (Apical and axillary buds)	- Lowest frequency of somaclonal variation - Easy to propagate - True to type of parent material - Genotype independent - Full genome present - Little work required	- Potential for early maturation - Rooting problems in certain species
Adventitious meristems (Organogenesis)	- Full genome present - True to type of parent material	- Highest frequency of somaclonal variation - Rooting problems in certain species - Genotype dependent - Labour intensive
Somatic embryos (Adventitious embryogenesis)	- Low frequency of somaclonal variation in most species - Root and aerial system well developed - True to type from starting tissue	- Genotype dependent - Labour intensive
Gametogenic embryos (Adventitious embryogenesis)	- Bypass fertilization process - Need only male or female organs	- Haploid genome - Genotype dependent - Not true to type; results of meiosis but true to type in the same line - Only from gametes - Labour intensive - Only a few species of trees and shrubs worked on
Zygotic embryos (excised from seeds, not tissue cultured per se)	- Genotype independent - No early maturation - Little work required	- Seeds must be available - Not true to type; result of sexual process

TABLE 5 continued

Explants	Advantages	Disadvantages
Cell suspension (Embryogenic or organogenic)	Refer to embryogenesis or organogenesis for regeneration	
	- Potential to scale up using fermenter technology	- Higher frequency of somaclonal variation
Protoplasts (Embryogenic or organogenic)	Refer to embryogenesis or organogenesis for regeneration	
	- Allows initiation of tissue culture from a single cell	- Highest frequency of somaclonal variation - Methods have to be developed for each genotype - Extremely labour intensive
Isolated DNA	- Requires minute quantity of plant material - Exact copy of parental DNA - Can be amplified artificially - Little work required - From live or dead tissues	- No possibility of whole plant regeneration - Only conserves genetic information of parent tree - A last resort - Only a few genes could be transferred at a time into a different host genome

Cruciferae contain model species. Monocotyledones are more recalcitrant to tissue culture, particularly the Graminae; however, extensive work has been done with Palmales and Iridales (e.g. *Musa* spp). With the gymnosperms, most of the work has been with the conifers within which the genus *Pinus* is relatively recalcitrant to tissue culture. Outside the conifer group, cycads have been successfully regenerated via somatic embryogenesis while the ginkgos are difficult to work with. Fern trees have

not yet been used for tissue culture experiments.

The tissue culture of tree and shrub species for conservation offers the following advantages (Engelmann 1991):

- multiplication rates are high and allow fast production of material to be preserved;
- plants are grown in aseptic conditions where pathogens are absent;
- the space needed is relatively small;
- allows the production of haploid plants;
- permits the rescue of zygotic embryos which would normally abort;
- allows conservation of species possessing recalcitrant seeds and tricellular pollen that could not be preserved otherwise.

There are also some disadvantages. In addition to the problem of tissue culture recalcitrance discussed above:

- tissue culture is labour intensive and time consuming for initial stock preparation and maintenance;
- sampling of an adequate number of representative trees for conservation is a problem (see below);
- *in vitro* conditions (tissue culture media, environmental parameters) have to be worked out for each species (and sometimes genotype) that has to be preserved;
- there are problems associated with somaclonal variation and early maturity of the plants regenerated (see below);
- in general, there is a loss of morphogenic potential with time (Benson and Withers 1988, Benson and Harding 1991).

The major problems for germplasm conservation by tissue culture are recalcitrance, somaclonal variation, and early maturity in regenerated woody plants. These points are discussed next.

SOMACLONAL VARIATION OF TISSUE CULTURE-DERIVED MATERIAL

The expression "somaclonal variation" was first used by Larkin and

Scowcroft (1981) to identify the genetic variation found in "somaclones", plants regenerated from any type of *in vitro* tissue culture. Somaclonal variation is perceived as the major obstacle in using plant tissue culture for large scale propagation and germplasm conservation. However, genetic variation occurs in nature (Walbot 1985, Orton 1984, Poethig 1989) and this could contribute to somaclonal variation. Natural genetic variation must, therefore, be taken into account when evaluating tissue culture methodologies for their potential in germplasm conservation. This will be highlighted at the end of this section.

Numerous papers have been published on somaclonal variation and they indicate that it is widespread among plant species. It is not the goal of this review to provide a comprehensive account; however, it is important to outline the status of our knowledge in this area and to relate this phenomenon to germplasm conservation.

Variation is often observed in plant tissue culture but not all of this is somaclonal. Four characteristics have to be present if it is (De Klerk 1990). First, the variation has to be transmitted to the progeny through meiosis. Second, it must not be reversible. Third, it must be found in regenerated plants and, fourth, the variation has to be random. Somaclonal variation can be confounded by epigenetic changes, reversible and predictable, which are found in plants regenerated from all types of meristems (adventitious, apical, and axillary) and which are due to physiological mechanisms. A good example of epigenetic variation is habituation of tissue grown *in vitro* (Binns 1981). The habituated tissues are able to grow without growth regulators and this characteristic can be lost following shoot regeneration.

Somaclonal variation can be associated with the process of dedifferentiation of plant tissues cultured *in vitro* (Scowcroft 1984, Karp and Bright 1985). Generally, explants that dedifferentiate and go through the process of somatic embryogenesis or organogenesis to form adventitious meristems give rise to plants showing frequent somaclonal variation (Karp 1989). Explants that retain their developmental integrity, such as shoot tips and axillary buds (apical and axillary meristems), very

rarely give rise to plants showing somaclonal variation. Factors affecting frequencies of somaclonal variation range from the developmental state and the level of ploidy of the explant source to the physical factors affecting the explant in culture and the age and number of tissue culture cycles (De Klerk 1990, Karp and Bright 1985, Orton 1984, Larkin 1987). Generally, tissues kept partly differentiated (somatic embryos) will show less variation than tissues propagated as dedifferentiated callus. Table 6 summarizes these factors. Genetic variability is generally more frequent and better tolerated in polyploids than in monoploids or diploids. However, it is postulated that multiple gene copies in polyploids buffer for imbalances caused by a genetic change, whereas monoploids show immediate effects of detrimental changes. The number of tissue culture cycles and duration in culture often contribute to somaclonal variation.

Characterization of somaclonal variation has been at various levels (Orton 1983, Larkin 1987). Initially, phenotypic changes were recorded but these observations proved not to be reliable. Other tools such as cytology, biochemical markers (isozymes) and, recently, restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPD) have been used to study somaclonal variation. However, most data available on somaclonal variation deals with the study of the chromosome content of cells in tissue culture. Examples of observed changes at the chromosome level are aneuploidy, polyploidy, mitotic aberrations, loss of satellite DNA, translocations, deletions, centric fusion, inversions, duplications, breakages, and interchanges. The major problem in analyzing somaclonal variation was the nature of the observations. The only conclusive data at the genetic level were obtained with RFLP markers and fingerprinting (Orton 1983) which allows identification of changes at the DNA level. Examples of this have been found for deamplification and amplification of genes, single gene mutations, mobilization of transposable elements (Evans 1989, Larkin 1987), and mitochondrial DNA rearrangements (Evans 1989).

An important question is the origin of somaclonal variation. It is believed that somaclonal variation is either an initial character of plant cells or is created de novo during tissue culture (Orton 1984). This is especially critical for the evaluation of tissue culture as a conservation strategy. If

somaclonal variation in tissue culture has its equivalent in nature then it becomes less a disadvantage for conservation. Recently, some evidence supporting this has been published (Edwards *et al.* 1990). The plant cell, when undergoing mitosis, has an intrinsic error rate which is the central source of natural selection. Under normal *in vivo* conditions, the differentiation process is very stringent and strongly selects against survival of abnormal cells whereas under *in vitro* conditions, the stringency is low and perpetuation of genetic variation is thus more frequent. In both cases, the genetic variation due to the error rate is cumulative and could be accentuated by the tissue culture process.

The phenomenon thus likely has its origins as a natural process in the life strategy of plants (Walbot 1985, Poethig 1989). Plants, unlike animals, cannot move to avoid environmental stress. Changes in their physiology have to occur for the plants to survive. An important anatomical feature of plants is the apical meristem which continuously produces new organs and meristems, including reproductive system tissues, such that in most plants the gametes differentiate at the periphery of the plant following extensive growth and the passage of time. Somatic mutation within the meristem can thus have tremendous consequences in the subsequent branches that will be formed and in sexual and asexual progeny. This is potentially an important mechanism in producing genetic variability that can contribute to survival of a plant species within a dynamic environment. For instance, resistance to the insect Anoplognathus montanus in some branches of the same Eucalyptus trees has been identified as a somatic change "established" by increased selection pressure (Edwards *et al.* 1990), a characteristic which can be passed on to progeny. Another example is the formation of bud sports in fruit trees; some are transmitted to the progeny, whereas others will cause flower sterility and cannot be transmitted naturally (Walbot 1985). This natural genetic mosaic formation in plants shows that somatic variation is not confined to *in vitro* tissue culture. This counterbalances the view that only tissue culture provokes high frequency genetic changes in plants.

Somaclonal variation represents a problem for true-to-type clonal propagation of a species. Therefore, the choice of tissue culture system

chosen should thus address this question by attempting to use an approach with the least chance of inducing genetic change (see Table 6). However, for germplasm conservation, the induction of somaclonal variation could be less critical because of similar random changes that occur in nature. As well, the process of regeneration will eliminate most detrimental mutations. It has been found that many of the genetic changes due to somaclonal variation are detrimental to regeneration *in vitro*. This process can thus act to eliminate many detrimental changes (Wershun 1989) without losing tissues because only normal cells will regenerate. The same is true for gametogenesis, fertilization, and embryo development.

TABLE 6

Generally accepted relative levels of somaclonal variation in plant tissue culture

Tissue culture methods	Relative variation level
Meristems (apical + axillary)	LOW
Embryogenic massess - somatic embryos - gametogenic embryos	
Disorganized callus - adventitious buds	
Cells suspensions	
Protoplasts	HIGH

Taking into account the state of knowledge on somaclonal variation during tissue culture, the following guidelines have been suggested to limit the impact of this problem (Karp 1989):

- the tissue source should be selected from stable genotypes and preferably diploid species. Old or specialized tissues should be avoided;
- the duration in "active" culture should be as short as possible and regeneration systems inducing the least variation should be used. (For

instance, it was demonstrated in *Solanum tuberosum* that slow growth induced more variation than cryopreservation (Harding 1991));
- components in the tissue culture media that are potentially mutagenic (e.g. 2,4-D) should be avoided.

EARLY MATURATION IN TISSUE CULTURE DERIVED MATERIAL

The phenomenon of early maturation is often observed with woody plants derived from tissue culture material. Early maturation stunts the growth of planted trees and induces characteristics such as reduced growth rate, reduced or complete lack of rooting, early flowering, and plagiotropy (Pierik 1990, Bonga and von Aderkas 1993). Generally, these unwanted attributes have been associated with the use of mature tree tissues as source of explants; however, the phenomenon has also been reported from plants derived from juvenile material (Bonga *et al.* in Hanover and Keathley 1988). It has been postulated that the phenomenon of early maturation is due to a deficient root system causing "reduction" or "elimination" of cytokinin production.

With tree and shrub species, it is critical that the tissue culture process causes rejuvenation to help avoid the problems of early maturation. Somatic embryogenesis as a tissue culture process has this positive characteristic. However, it has not yet been possible to apply somatic embryogenesis to mature plant material in conifer species. Meristem culture, although a difficult technique, is also a good mechanism of rejuvenation and has been used in numerous woody species (Pierik 1990). The process of adventitious meristem formation *in vitro* has been used for rejuvenation, but its success appears dependant on the number of subculture cycles tolerated by *in vitro* grown tissue. Adventitious meristems are probably most prone to the problem of early maturation. Interestingly, the technique of micrografting, or grafting of adult meristems on juvenile rootstocks *in vitro*, has been used successfully in rejuvenating mature material and is another possible avenue for *in vitro* conservation of trees and shrubs from mature material (Misson and Giot-Wirgot 1984, Tranvan and David 1985, Monteuuis 1986).

Early maturation is a detrimental phenomenon in woody plants that impairs the use of tissue culture as a conservation strategy. It is also a difficult problem to tackle since it manifests itself after the tissue culture process is completed.

METHODS OF TISSUE CULTURE CONSERVATION

Once plant tissues have been established *in vitro*, an effective method is required for long term storage. Although this can be partially achieved by regular subculturing on fresh tissue culture media, such subculturing is not practical for long term or medium term germplasm conservation due to danger of microbial contamination, equipment failure, and the labour and costs involved. The problems of somaclonal variation and loss of morphogenic capacity are also prevalent. Two other approaches which reduce or eliminate these problems are slow growth and cryopreservation. Another approach, artificial seeds, is in development and could be combined with germplasm conservation for specific applications.

Slow growth methods refer to any tissue culture procedure aimed at reducing the growth rate of plant tissues *in vitro* (Withers 1990a,b, 1991, 1989, Engelmann 1990, 1991). Synonyms such as growth limitation, inhibition of growth, and minimal growth are also found in the literature. The goal of this method is to obtain the longest period of subculture without detrimental effects to the plant tissues (Engelmann 1990, Withers 1991). Slow growth is achieved by using one or all of the following techniques (Withers 1990a,b,c, 1991; Engelmann 1991):

- reducing temperature;
- addition of growth inhibitors;
- reduction of O2 concentration;
- reduction of light intensity;
- decreasing the nutrient content of tissue culture media;
- choosing small explants;
- using a small tissue culture vessel;
- adding chemicals with osmotical properties.

Although the slow growth technique is widely used (Engelmann 1990, Roca 1989), certain factors such as a reduction of temperature cannot be used as efficiently and drastically with tropical plant species because of their higher temperature growth habit (Withers 1991). Furthermore, this method does not alleviate other problems associated with tissue culture such as somaclonal variation. There is also a risk of unwanted selection pressure, due to the stress associated with this method, changing allelic frequencies in the material conserved.

Cryopreservation is potentially the most appropriate for long term storage of plant tissue culture material. For extensive reviews of cryopreservation techniques, the reader should consult Kartha (1985b). In addition, although extensive cryopreservation work has been done on excised explants of woody plants by Sakai (Sakai 1984, 1986) this will not be discussed here because it is not tissue culture per se. In theory, plant tissues can be stored indefinitely when cryopreserved; however, in animal cells it was estimated that natural radiation would cause irreversible damage after 10 000 years (Ashwood-Smith and Friedman 1979).

Cryopreservation of plant tissues involves several steps that have to be optimized for each species and for each type of tissues (Engelmann 1991, Kartha 1985a). These are:

1. Choice of the plant material
   Preferably rapidly growing young material more resistant to freezing because of smaller size, possession of fewer or smaller vacuoles, and the presence of a dense cytoplasm.
2. Pretreatment
   Dehydration of the tissues and protection of cell membranes. The method of pretreatment varies with the plant species and the tissue culture system.
3. Freezing
   Needed to avoid damage through ice formation. If cooled slowly, cells lose water to equilibrate increased solute concentration outside the cell produced by extracellular ice formation. Rapid freezing to cryogenic temperatures produces only residual water and leads to very little

damage through internal ice formation. Cryoprotectants such as DMSO and sorbitol are used to protect the cell by lowering the freezing temperature and altering the pattern of ice crystalization. Rapid cooling can also be used that allows microscopical ice formation without recrystallization. However, this procedure has been found less efficient (Sakai *et al.* 1978, Kartha *et al.* 1980).

4. Storage
   The method of storage itself is not critical as long as storage temperature is kept below -140°C, at which point the metabolism is supressed.
5. Thawing
   Thawing is performed in such a manner as to avoid harm being done to the cell by formation of intracellular ice crystals.
6. Post-treatment
   This procedure dilutes the toxic effect of cryoprotectants and to reduces osmotic shock.

Recently, a simplified cryopreservation procedure has been developed that does not involve the use of a sophisticated programmable freezer (Lecouteux *et al.* 1991). It consists of 1) pre-treatment of somatic embryos with sucrose as a cryoprotectant, 2) freezing in a domestic freezer at -20°C for 24 hours, and 3) immersion in liquid nitrogen. This method allowed an high survival rate (80%) and could be a solution to the problem of a lack of expensive equipment in laboratories.

Generally, the cryopreservation of plant tissues is easier with unorganized cell cultures and more difficult with highly organized structures (Kartha 1985a). Tissues with elaborate structural organization means heterogeneous populations of cells that each react differently to the procedure. For instance, meristems are difficult to cryopreserve and stuctural damage is often observed (Withers 1990). On the other hand, zygotic and somatic embryos and somatic embryogenic tissue have been cryopreserved successfully (Pence 1990, Engelmann 1990, 1991, Klimaszewska *et al.* 1992). The difficulty with embryos lies at an organizational level. This requires the absence of damage critical to recovery of the whole structure and safeguarding of the regenerative cells

to insure regrowth after freezing.

A modification of the cryopreservation technique, namely vitrification, can be used for organized structures. It consists of ultra-rapid cooling of the tissues at rates of -1800°C to -4800°C per minute in a solution of high concentration of cryoprotectants such as DMSO, glycerol, and ethylene glycol (Towill 1990, Sakai *et al.* 1990, Langis and Steponkus 1990, Langis *et al.* 1989, Uragami *et al.* 1989). Tissues are rewarmed as rapidly for their regrowth. This process does not allow ice formation but rather glass formation within the cells, thus avoiding intracellular damage. This technique is still in development but could be a viable solution for the preservation of organized structure such as meristem explants and somatic embryo-derived plantlets.

A major problem with plant tissue cryopreservation is the sudden arrest of metabolic activities at all levels. The implication is that cell division and DNA replication are interrupted before completion. This can have negative consequences at the cellular level and could cause genotypic changes (DeVerno *et al.* 1992).

A promising method of germplasm conservation is through the creation of artificial seed. The techniques are still being developed, but they could contribute in future to global conservation schemes. A prerequisite for this method is the feasibility of somatic embryogenesis for the subject species. Consequently, it is based on other tissue culture technologies. Most of the work in this direction has been done with species such as carrot, lettuce, celery, and alfalfa (Redenbaugh 1990, Redenbaugh and Ruzin 1989, Redenbaugh *et al.* 1987, 1988). The aim has been to mimic the natural seed, which consists of a zygotic embryo with nutritive and protective envelopes. The initial approach coated somatic embryos with a hydrogel. Such envelopes prevent embryo desiccation and can provide nutrients and protective agents. The technique can be used with both orthodox and recalcitrant seeds. Recently, artificial seeds of carrot have been cryopreserved (Dereuddre *et al.* 1992). This opens the door for the integration of this technology into conservation strategies.

A variation is to desiccate somatic embryos and then coat them with a protective envelope containing nutrients and protective agents. This could be used for germplasm conservation but can only be applied to orthodox seeds where desiccation of the embryos is possible and does not cause damage. This long term survival of dessicated embryos has been demonstrated in alfalfa (McKersie *et al.* 1989). Furthermore, encapsulated apical meristem can be cryopreserved and used for germplasm conservation (Eucalyptus; Poissonnier *et al.* 1992).

In summary, artificial seed technology has the potential for germplasm conservation but requires development for a wider range of species. Table 7 compares the different conservation methods. Based on this comparison, the method of plant tissue conservation that presently has the most potential is cryopreservation.

CONSERVATION OF DNA

Few researchers have described the use of DNA as a method of last resort for germplasm conservation in plants. DNA is the basis of all genetic information contained in a plant and, through techniques of gene sequencing, this information can be read and stored. The genes themselves can also be stored as DNA and used for germplasm conservation. This option has to be considered seriously where no other alternatives are available. Furthermore, this method is currently used in Australia (Mattich *et al.* 1992) and USA (Giannasi 1992). For reviews, the reader should consult Peacock (1989), Ford-Lloyd and Jackson (1991), and Adams and Adams (1992).

The factors that make possible the use of DNA for germplasm preservation are the quantity of material needed and the possibility of reintroducing the DNA by genetic transformation methods into related genotypes or species of the original plant. With new developments of molecular biology such as polymerase chain reactions combined with gene cloning, minute amount of tissues can be used to provide a comprehensive collection of all the DNA of a plant genome (White *et al.* 1989). Moreover, DNA can be isolated from dead tissues, giving access to information that

TABLE 7

Comparison of conservation methods for tissue culture material.

Methods	Advantages	Disadvantages
Subculture	- Tissue ready to be propagated - All types of tissues	- Short term approach (1 week to 2 months) - Labour intensive - Space required - High frequency of somaclonal variation - High risk of loss to human error (e.g. contamination) - Loss of morphogenic capacity
Slow growth	- Tissues ready to be propagated - All types of tissues	- Short term to middle term approach (2 months to 2 years) - Somaclonal variation - Risk of loss to human error - Methodology different according to species - Loss of morphogenic capacity (less probable than with subculture)
Cryopreservation	- Minimum space required - Long term approach (in theory indefinite) - Tissue is in an arrested state; low somaclonal variation (Harding 1991)	- Sophisticated equipment required (except if simplified protocol used) - Tissues need regrowing before propagation - Not well developed for organized tissues - Methodology has to be developed on a species by species basis
Vitrification	- Same as cryopreservation and more suited for organized tissues	- Sophisticated equipment required - Tissues need regrowing before propagation - Methodology has to be developed on a species by species basis
Artificial seeds	- Propagules ready to be sowed - Tissue in a latent state, low somaclonal variation - Minimum space required	- Methodology in development - Not possible for all types of tissue culture material (embryo-like structure) or meristems - Time range for storage is unknown

would be lost if only living tissues were used (Pyle and Adams 1989, Rollo *et al.* 1991, Pääbo *et al.* 1989). This capability of isolating DNA in small amounts can be combined with more powerful and repeatable methods of genetic transformation. To date, over 40 different plant species have been genetically transformed with foreign DNA (Charest and Michel 1991) and this number is increasing. Techniques could be developed in future that would allow introduction of whole chromosomes. This is not the same as transferring a whole genome but it would allow the rescue of some genes that would otherwise have been lost.

Chapter five
CONCLUSION

The present report stresses the need to integrate *ex situ* and *in situ* approaches in an overall scheme of genetic diversity conservation and to duly consider the advantages of the range of methods available, as shown in summarized form in Table 8. If used judiciously, the methods surveyed should contribute significantly to the conservation of valuable genetic resources of woody perennials.

Further development in culturing and cryopreservation techniques for recalcitrant seeds, *in vitro* tissue culture, and storage of artificial seed, all of which involve desiccation to moisture levels comparable to true orthodox seed dried for storage, are necessary to upgrade these methods to contribute to intermediate and long term conservation needs. Tissue culture research should be extended to the development of key protocols for the propagation of a range of non-commercial species and focused on the development of inexpensive preservation methods.

When evaluating the effectiveness and limitations of presently available *ex situ* methods for genetic conservation of perennial woody species, it becomes clear that, with present knowledge, the storage of pollen, the storage of recalcitrant seed, and *in vitro* culture are only viable as short term measures. The only secure, medium term *ex situ* conservation method presently available for large-scale use in forestry is the storage of orthodox seeds. True orthodox seeds can generally also be conserved in the long term, through the application of cryopreservation methodologies. In all these cases, however, the problems related to regeneration of seedstocks should be kept in mind. Storage, per se, may not be the most difficult problem in long term *ex situ* conservation strategies.

Future research should be directed to the following areas:

1. The development of proper protocols for screening and clear identification of truly recalcitrant seed species. To achieve this, we should continue by examining rates of desiccation in relation to

subsequent rates of germination, with particular attention to desiccation techniques that do not involve the use of heated air.

2. If seeds cannot be dried without injury, then their tolerance to cryopreservation should be examined. This process will involve evaluating the temperature gradients of tissues in time and space, the penetration of cryoprotectants, the possibility of using excised embryos, and the feasibility of freeze-drying techniques.
3. For improving cryopreservation efficiency, the effects of cryogenic storage temperature and seed moisture content on induced dormancy should be studied.
4. Consideration should be given to optimal moisture content for pollen storage, the mode of desiccation and rehydration, the temperature and atmosphere of storage, and the necessity and type of cryoprotectant used.
5. Improvements should be made to conventional storage methods for recalcitrant seeds at ambient or sub-ambient temperatures, including the developing of ways to control microbial attack, inhibiting germination with chemicals, and assessing oxygen requirements.
6. In view of the high cost involved in operating refrigerated seed banks (a problem of special relevance to developing countries), there is an urgent need to develop methods for storing ultra-dry seeds at ambient or sub-ambient temperatures.
7. Alternative strategies for the storage of recalcitrant seed should be investigated, such as the establishment of seedling banks, *ex situ* conservation stands, and seed orchards.
8. Tissue culture methods should be developed for non-commercial species. There should also be a determination of the critical factors tied to the success of tissue culture to permit transfer of the techniques from species to species.
9. There should be inexpensive tissue culture protocols for tree species suitable for long term conservation. The exploration of natural processes, such as zygotic embryo desiccation, during seed maturation and its duplication *in vitro* could possibly show the way to inexpensive storage of artificial seeds.

TABLE 8

Comparison of *in situ* and *ex situ* conservation methods

Conservation strategy	Advantages	Disadvantages/ Difficulties
In situ: Protected Areas and Managed Resource Areas	- Conserves genetic resources in their natural habitat, maintains interactions with other species and organisms; - Conservation of intra-specific variation can be combined with various degrees of conservation of inter-specific variation; - Applicable to species with orthodox and recalcitrant seeds, and to vegetatively propagated species.	- Space required; - Resources prone to loss by accident, pests, diseases; - A network of spatially separated conservation areas is required to capture provenance (clinal, geographical) variation of target species; - Information required on silviculture and management interventions needed to meet specified conservation objectives.
Ex situ: Seed Banks	- Propagules readily available for use; - Minimum work; little space required (small seeds); - Provenance (clinal, geographical), variation can be conserved provided species range adequately sampled.	- Not applicable to species with recalcitrant seeds nor to vegetatively propagated species; - Space required (large seeds); - Does not conserve associated species in the ecosystem; - Regular regeneration of seedlots poses at times insurmountable problems.

TABLE 8 continued

Conservation strategy	Advantages	Disadvantages/ Difficulties
Ex situ: Pollen Banks	- Minimum space required; - Applicable to species with recalcitrant and orthodox seeds; - Provenance (clinal, geographical), inter- and intra-specific variation can be conserved provided species range adequately sampled.	- Only half of the genome conserved; - Tri-cellular pollen storage extremely difficult; - Needs female flowers for conventional propagation; - Propagules not readily available; - Does not conserve associated species.
Ex situ: Tissue Culture Banks (*see* Table 5, page 43, for additional information)	- Minimum space required; - Genetic erosion reduced if methods such as cryopreservation are used; - Applicable to species with recalcitrant and orthodox seeds and to vegetatively propagated species; - Provenance (clinal, geographical), inter- and intra-specific variation can be conserved provided species range adequately sampled; - Aseptic conservation (minimizes disease risk); - Time required to produce propagules for use is short.	- Sampling problems (within tree and sampling of adequate number of representative trees); - Protocols are species and at times genotype-specific; - Problems of somaclonal variation and early maturation; - Does not conserve associated species.

TABLE 8 continued

Conservation strategy	Advantages	Disadvantages/ Difficulties
Ex situ: DNA Banks	- Minimum space required, large numbers of samples can be handled; - Applicable to species on the verge of extinction (or extinct). - Applicable to all kinds of plants.	- Impossible at this time to get whole genome into another living organism/plant; - Not a practical germplasm conservation method *per se*; - Last recourse.
Ex situ: Seedling Banks, *Ex Situ* Conservation Stands	- Applicable to species with recalcitrant and orthodox seeds; - Provenance (clinal, geographical), inter- and intra-specific variation can be conserved provided species range adequately sampled.	- Space required; - Requires spatial isolation to conserve provenance identity; - Does not conserve associated species; - Generally not a preferred alternative for species with orthodox seeds, or those without actual socio-economic value, due to economic considerations.
Ex situ: Arboreta	- Applicable to species with recalcitrant and orthodox seeds and to vegetatively propagated species; - Provenance (clinal, geographical), inter- and intra-specific variation can be conserved provided species range adequately sampled; - Useful method for unique phenotypes/ genotypes (e.g. mutants, variants, sterile trees).	- Space required; - Resources prone to loss by accident, pests, diseases; - Does not conserve associated species; - Not apt for the conservation of provenance (clinal, geographical) variation; - Requires a minimum number of individuals larger than needed to conserve inter-species variation which is generally the purpose of arboreta.

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The document discusses the role of *ex situ* conservation for woody perennial species in the light of new methodologies and technology available for the storage of seeds, pollen and tissue. Based on a detailed review, it concludes that with current knowledge the storage of pollen, recalcitrant seeds and *in vitro* cultures is only viable as a short-term measure for these species. The only technically secure, medium–to long-term *ex situ* conservation method available at present for large-scale use in forestry is storage of orthodox seeds. Such seeds can usually be stored in the long term, through the application of cryopreservation methods. However, in this case the problems related to regeneration of seedstocks will present often insurmountable difficulties. Storage *per se* may thus not be the most difficult problem in long-term *ex situ* conservation strategies for woody perennials. Such strategies should continue to be considered as complements to rather than substitutes for *in situ* conservation.

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