In Vitro Conservation Including Rare and Endangered Plants, Heritage Plants and Important Agricultural Plants

Krystyna A. Johnson

Department of Environmental Sciences, University of Technology, Sydney
Westbourne Street, Gore Hill, 2065, NSW. Australia
Email: Krystyna.Johnson@uts.edu.au

Abstract

Plant germplasm collection and its conservation are an integral part of ensuring the availability of plant genetic materials for present and future breeding programs of important horticultural and agricultural plant crops; preservation of rare and endangered species, and of heritage plants. This paper reviews current technologies and their implications for future research. The most efficient and economical means of germplasm preservation under normal circumstances is in the form of seeds. However, this kind of storage is not always feasible because:

i) some plants do not produce seeds, therefore, they have to be propagated vegetatively

ii) seeds remain viable only for a limited duration or are recalcitrant

iii) seeds are heterozygous and, therefore, not suitable for maintaining true to type genotypes

iv) seeds of certain species deteriorate rapidly due to seed born pathogens.

To improve germplasm preservation of endangered species, elite genotypes which are multiplied on a large scale in production laboratories, and plant materials with special attributes, eg metabolite-producing cell lines and genetically engineered material, various strategies have been investigated. They include slow growth techniques or medium-term conservation; simple freezing techniques for differentiated materials such as apices and embryos; and long-term conservation (liquid nitrogen, -196°C). This last technology allows us to store plant material without modification or alternation, protects it from contamination, and requires limited maintenance. There are a number of cryopreservation technologies: freezing, ultra rapid freezing, vitrification, encapsulation/dehydration and encapsulation/vitrification. Additional research is needed to investigate existing cryopreservation techniques on a large scale in a genebank context and to develop protocols for additional species. In this paper, all of the above issues are considered and future approaches discussed.

Keywords

biodiversity, genebank, germplasm, cryopreservation, encapsulation-dehydration, vitrification, encapsulation-dehydration

A. Taji and R. Williams (eds)
The Importance of Plant Tissue Culture and Biotechnology in Plant Sciences
2002 University of New England Publications Unit, Australia

Introduction

Conservation of plant genetic resources attracts more and more public interest as the only way to guarantee adequate food supplies for future human generations. However, the loss of biodiversity due to exploitation of natural populations, harvest without permits from the wild, natural hazards, cultural, political and economical issues, pose a great threat to plant genetic resources. Over the last 30 years, there has been significant increase in the number of plant collections and in accessions in *ex situ* storage centres throughout the world.

However, seeds of a number of species, predominantly tropical or subtropical, belong to the recalcitrant or intermediate categories, have a limited longevity which renders their long-term preservation impossible. For example, *Cocos nucifera* (coconut), Theobroma cacao (cacao), and many tree and shrub species have seeds which are physiologically immature when shed, have high moisture content, cannot withstand much desiccation and are sensitive to chilling, therefore cannot be stored in low temperatures. Others, such as *Coffea* spp. and oil palm (*Elaieis guineensis*) can be stored only for a short period of time and long term conservation remains unattainable (Roberts, 1973; Chin and Pritchard, 1988; Ellis *et al.*, 1990, 1991).

Alternatives to Conservation of Seeds

For species which do not produce seeds, or are predominantly vegetatively propagated, conservation in seed form is impossible, or has limited application, e.g. banana and plantain (*Musa* spp.). Other crops, such as potato (*Solanum tuberosum*), yam (*Dioscorea* spp.), cassava (*Manihot* spp.), sweet potato (*Ipomea batatas*) or sugar cane (*Saccharum* spp.), have either sterile genotypes, or produce orthodox seeds which are of no interest if conservation of particular gene combinations is to be conserved (Engelmann, 1997). The most common method of preserving the genetic resources of these species is as plants in the field. There are, however, several serious problems with field genebanks, such as exposure to attacks by pests and diseases, natural hazards, high labour costs for maintenance of collections, and restriction of germplasm exchange due to risks of disease transfer.

Classical (slow growth) *in vitro* conservation techniques have been developed for a wide range of species, including temperate woody plants (Aitken-Christie and Singh, 1987), fruit trees (Sakai, 1986; Withers, 1992), horticultural species (Engelmann, 1991a), as well as numerous tropical species (Dodds, 1991; Engelmann, 1991b). However, a recent FAO survey conducted in 1994 (FAO, 1994), indicatated that only 37,600 accessions are conserved *in vitro* worldwide. Slow growth conservation is used routinely for the conservation of only a few species, including banana, potato and cassava in regional and international germplasm conservation centres such as National Bureau of Plant Genetic Resources (NBPGR), Centro Internacional de la Papa (CIP), Instituto de Investigaciones Fundamentales en Agricultura Tropical (INIFAT) or International Institute for Tropical Agriculture (IITA), and alternative medium term conservation techniques are still at the experimental stage. Cryopreservation procedures have been developed for around 100 different plant species cultured as suspension cell cultures, calluses, shoot apices, zygotic and somatic embryos (Bajaj, 1991; Withers, 1992; Kartha and Engelmann 1994; Engelmann *et al.*, 1995; Withers and Engelmann 1997). However, the number of plant species stored *in vitro* is constantly changing as the conservation techniques are being developed and applied.

Importance of *in vitro* techniques

A major objective of current plant cell and tissue culture work is more efficient exploitation of specific properties of plant genotypes. Progress in each of several areas, for example, the generation of variation, the selective transfer of genes, the identification of desirable traits and the conversion of *in vitro* cultures to plants, contributes to this increased efficiency. However, these efforts are wasted if the products on *in vitro* procedures are prone to variation or, worse, accidental loss. Thus, germplasm preservation must be a component of any *in vitro* program wherein genetic identity and integrity are important (Withers, 1989). This technology has been successfully applied in the field collections for extraction of coconut embryos (Assy-Bah *et al.*, 1987), and cacao budwood (Yidana *et al.*, 1987). It has also been extensively used for the international exchange of germplasm for its obvious advantages, such as quality of the material (virus or disease indexed), small amount of space required, or speed of delivery. Plant cells are inherently totipotent, therefore the use of *in vitro* culture techniques in conjunction with careful manipulation of cryobiological methods can be profitably used for the storage and preservation of important crop species. In 1987, Bigot reported application of *in vitro* culture techniques to more than 1000 different plant species.

Storage of *in vitro* Cultures

Cells and tissues are normally stored in a well-lighted room at 25°C. However, this requires periodic transfer to fresh media, involving not only man-power and high cost, but also the hazards of contamination, and sometimes the loss of the entire germplasm material. Moreover, the cultures retained on periodic subcultures undergo genetic erosion, such as a change in ploidy level, mutations, translocation, gene amplification, etc. (Bajaj, 1991). Therefore, attempts are being made to develop methods that reduce growth rate of cultures, or suspend growth entirely by cryopreservation. The former, along with the cultures in normal growth, are active collections and parallel the *in vivo* field genebank, whereas the latter represents base storage (Withers, 1987).

The *in vitro* Genebank

The flow of material through an *in vitro* genebank and the relationship of the genebank to the field genebank and externally acquired material are shown in Figure 1 (IBPGR, 1986). This plan is aimed specifically at the conservation of clones of vegetatively propagated material, and populations of recalcitrant seed-producing species stored as shoots or embryos, respectively. It provides, however, a model for expansion or modification to embrace other subjects for storage. This scheme can be broken down into a number of areas. These relate to: (i) germplasm acquisition; (ii) disease eradication, indexing and quarantine; (iii) culture establishment and multiplication; (iv) *in vivo* establishment and germplasm exchange; (v) storage by slow growth or cryopreservation; (vi) monitoring stability; (vii) characterisation and evaluation; and ultimately, (viii) utilisation through *in vitro* breeding (Withers, 1989).

All these areas are integral parts of germplasm conservation, and are promoted by the International Plant Genetics Resources Institute (IPGRI) previously known as IBPGR (International Board for Plant Genetic Resources). This institution is an autonomous international scientific organisation operating under the aegis of the Consultative Group of International Agricultural Research (CGIAR). IPGRI's mandate is to advance the conservation and use of plant genetic resources for the benefit of present and future generations. IPGRI works in partnership with other organisations, undertaking research, training and the provision of scientific and technical advice and information. IPGRI has four major strategic objectives: strengthening national programs, contributing to international

collaboration, improving strategies and technologies for conservation and providing an international information service. The few examples of the international collaborations are listed below (Engelmann, 1995):

(i) University of Birmingham collaborates with the Catholic University of Leuven, Belgium, and the International Institute for Tropical Agriculture, Ibadan in Nigeria (IITA), evaluating the possibility of using RAPD to detect off-types of bananas and plantain generated *in vitro*,

(ii) Forest Research Institute of Malaysia, Kepong investigates conservation of species with recalcitrant seeds. The research program aims at developing cryopreservation techniques for excised embryos of dipterocarps,

(iii) Universiti Pertanian Malaysia, Serdang (UPM) works on cryopreservation techniques for tropical fruit trees,

(iv) National Bureau of Plant Genetic Resources, New Delhi, India (NBPGR) investigates biological mechanisms determining the recalcitrance of seeds of tea (*Camellia sinensis*), cacao (*Theobroma cacao*), jackfruit (*Artocarpus heterophyllus*), lychee (*Litchi sinensis*), breadfruit (*Artocarpus altilis*), almond (*Prunus dulcis*), and vegetatively propagated crops, sweet potato (*Ipomoea batatas*), and yam (*Dioscorea* spp.),

(v) Centro Nacional de Investigaciones Cientificas, Havana City (CNIC) in Cuba investigates *in vitro* conservation of vegetatively propagated species of sugarcane (*Saccharum officinarum*) apices,

(vi) and the Instituto de Investigaciones Fundamentales en Agricultura Tropical, Santiago de las Vegas (INIFAT) studies cryopreservation protocol for banana and plantain (*Musa* spp).

(vii) Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) and Institut für Pflanzenbau, Bundesforschungsanstalt für Landwirtscha (FAL) work on refining cryopreservation techniques for potato (*Solanum tuberosum*), the assessment of genetic stability of cryopreserved material, freezing of additional varieties and transfer of the technique to CIP (Centro Internacional de la Papa, Lima, Peru), which has the mandate for conservation of the potato world collection.

Sigaud (2000), reported an establishment of an International group involving FAO, IPGRI, and International Centre for Research in Agroforestry (ICRAF) for the conservation and management of the forest genetic resources.

Applications for Biodiversity Conservation

As indicated above, the *in vitro* conservation techniques were developed previously to protect the genetic resources of food and other economic species. However, there is also an increasing recognition of their usefulness in other aspects of biodiversity conservation. Below are a few examples of an application of *in vitro* technology to the conservation of rare and endangered plant species.

Long term storage of rare and threatened Australian native species *Boronia edwardsii, B. pilosa, Cheiranthera volubilis, Grevillea biternata, Correa volubilis, Prosthanthera eurybioides P. calycina, P. rotundifolia* and *Rhagodia spinescens* was reported by Williams and Taji (1987). *Blandfordia* species can be stored at 10°C for 8 months under dark conditions (Johnson, unpublished). Poissonnier *et al.* (1992) reported procedure for cryopreservation of *in vitro* grown shoot tips of *Eucalyptus gunni.*

Cryopreservation procedures are being currently developed by King's Park and Botanic Gardens (Perth, Western Australia) for the conservation of rare and endangered species of Western Australia. In 1992, Touchell *et al.* reported cryopreservation of shoot tips and axillary buds of *Grevillea*

scapigera using slow-cooling regime. All shoots regenerated from thawed tissues and transferred to soil appeared phenotypically identical to untreated control shoots and plants. Turner *et al.* (2001) reported successful application of cryopreservation via vitrification of shoot apices, of six endangered West Australian species from the Haemodoraceae (or 'bloodroots') family: *Anigozanthos humilis*, *A. kalbariensis*, *A. viridis*, *Conostylis dielsia*, *C. micrantha* and *C.wonganensis*.

Leontopodium alpinum (*Composite*), native to Romania, being extremely ornamental, and also containing precious secondary metabolite has been excessively collected and was on the verge of extinction. The species was successfully micropropagated, and the *in vitro* conservation protocol for the species developed (Zapartan, 1996). Plant tissue culture and conservation techniques have been also recently developed and applied to an endemic and threatened Spanish plant species *Minuartia valentina* (Ibanez *et al.*, 1998), and to a rare Scottish plant *Primula scotia* (Benson Erica *et al.*, 2000).

Major Technologies

Slow Growth

Minimal growth storage is the most direct way of restricting growth and development of *in vitro* materials, and is usually applied to differentiated plantlets and developing meristem cultures. It may also be applied to larger callus masses. The cultures can be induced into a state of slow growth by various means, including culture at reduced temperature in the presence of hormonal growth inhibitors (e.g. abscisic acid), reduction in sucrose concentrations, or the addition of osmotically active additives (e.g. 3% w/v mannitol) (Staritsky *et al.*, 1986; Zandvoort *et al.*, 1994).

Under reduced temperature, a high accumulation of unsaturated lipids on cell membranes causes cell membrane thickening, and retards cell division and elongation. Cold storage in some plant species is done under either low light intensity or complete darkness (Orlikowska, 1992; Zandvoort *et al.*, 1994). At its simplest, lowering the environmental temperature of cultures below their active growing temperature is used to significantly extend the interval between subcultures. Care must be taken, however, to avoid temperatures below freezing, or levels where other temperature related injuries may occur, e.g. chilling injury (Lyons *et al.*, 1979), or denaturation of active macromolecules (Franks, 1985).

Several types of plant material have been investigated for cold storage, e.g. shoot tips, buds, nodal segment and rooted shoots. Meristem derived explants were mostly suggested for their genetic and generative stability (Orlikowska, 1992). However, nodal segment and rooted shoots were used as explants for preservation of citrus germplasm by Aitken-Christie and Singh (1987). Orlikowska (1992) used shoot and nodal segment for cold preservation of apple rootstocks.

The slow growth is one of the major tissue culture techniques used for preservation of *in vitro* grown plants (Dodds and Roberts, 1985; Janeiro *et al.*, 1995; Engelmann, 1997). Their routine use, however, is still restricted to a limited number of crop species. Despite the genetic stability inherent in organised plant structures, such as meristems, there are risks to DNA structure, slow metabolism and low level of viability under conditions of slow growth resulting from the oxidative activity of free radicals (Benson, 1990; Grout, 1995).

As indicated in the Introduction, *in vitro* slow growth is used routinely for conserving germplasm of plants such as banana and plantain (*Musa* species) stored at 15°C (Banerjee and De Langhe, 1985; Van den Houwe and Panis, 2000), cassava at higher than 20°C (Roca *et al.*, 1984), potato stored at under a wide range of conditions (Sarkar and Prakash, 1998; Prematilake and Mendis 1999), and a wide range of *Coffea* species are conserved on standard medium at 27°C (Bertrand-Desbrunais 1991). Temperatures in the range of 0-5°C are employed with cold tolerant species, e.g. apple (*Malus domestica*), plum (*Prunus*) or strawberries (*Fragaria* x *ananassa*) (Mullin and Schlegel 1976; Druart, 1985). Kiwi fruit can be stored at 8°C (Monette, 1986), and taro (*Colocasia*

esculenta) tolerates 9°C (Staritsky *et al.*, 1986). Other examples may be found in the literature and in the IBPGR International Data Base on *In Vitro* Conservation (Wheelans and Withers 1984).

Alternative Techniques

Medium term conservation can be achieved by reducing the growth of the plant materials by using a range of alternative techniques. In mid-term conservation, environmental conditions and/or culture medium have to be modified to induce growth reduction. This is most frequently achieved by temperature reduction (Janeiro *et al.*, 1995). However, there are other alternative techniques that can be applied which aim to induce the changes in the gaseous environment of cultures. Such changes include, for example, the reduction of oxygen available to cultures, by covering the explants with liquid medium, paraffin or mineral oil. This technique was developed by Caplin (1959), who managed to store carrot calluses for 5 months under a layer of paraffin. Such technique has also been successfully applied to other callus cultures, such as grapes and *Catharanthus,* by Augereau *et al.* (1986) and Moriguchi *et al.* (1988). Oxygen content can be also reduced by decreasing the atmospheric pressure of the culture chamber. Tobacco and chrysanthemum plantlets were reported to be stored under low atmospheric pressure (1.3% O2) for 6 weeks (Bridgen and Staby, 1981).

Since the development of synthetic seed technology (Radenbauch *et al.*, 1991), an increasing interest has been paid to the desiccation and/or encapsulation of explants techniques as an alternative technique for storing not only callus or shoot tips, but also somatic embryos (Bomal and Tremblay, 2000; Engelmann, 1997). However, for truly satisfactory long term storage, a method is needed that eliminates renewal operations by suspending growth.

Cryopreservation

The term 'cryopreservation' is used exclusively to cover the storage of living biological material at ultralow temperatures, normally at or near the temperature of liquid nitrogen (-196°C). At this temperature, all cellular divisions and metabolic events are stopped. The plant material can thus be stored without alteration for extended periods, with limited maintenance (George and Sherrington, 1984; Matsumoto *et al.*, 1994; Grospietsh *et al.*, 1999). Except for orthodox seeds, dormant buds and some pollen, higher plant structures cannot generally survive the transition to and from the storage temperature without protection. Samples are submitted to a cryoprotective treatment before freezing, and substances such as dimethylsulphoxide (DMSO), sorbitol, mannitol, sucrose and polyethylene glycol are usually applied. Sakai, in 1960, reported for the first time the survival of mulberry twigs in liquid nitrogen after dehydration mediated by extra organ freezing. In 1968, Qantrano pretreated successfully flax cell cultures with DMSO. This study was followed by successful thawing of carrot cell cultures by Latta (1971). However, the removal of cryoprotectants by washing after thawing, which was widely adapted in the past, has been displaced by a much less stressing technique for removing cryoprotectants, developed by Chen *et al.* (1984).

A typical cryopreservation procedure has been established and consists of the following stages: (i) pregrowth, (ii) chemical cryoprotection, (iii) slow dehydrative cooling (iv) storage in liquid nitorgen, (v) rapid thawing, (vi) recovery. The exact treatments given at each stage will vary with culture system (Withers and Street 1977; Kartha 1985).

However, for each new material to be cryopreserved, optimal conditions have to be defined for each successive step of the protocol (Turner *et al.*, 2001). The physiological state of the material can affect its survival. Cell suspensions are more likely to withstand freezing when they are employed during their expotential growth phase (cells are small and have relatively low water content). A routine method is available for the cryopreservation of cell cultures (Withers and King, 1980). Shoot and embryo cultures are more relevant to genetic conservation and a number of species have been cryopreserved successfully, e.g. shoot tips of cassava (Kartha *et al.*, 1982), or somatic embryos of oilpalm (Engelmann and Dereuddre 1988). However, the situation is far from satisfactory. Problems are experienced in maintaining symplastic continuity in relatively large

unit specimens and such material tends to regenerate after freezing as a callus tissue, which may lead to genetic instability (Withers, 1989).

Recent Developments in Cryopreservation

In recent years, several new cryopreservation procedures have been developed, allowing an application of the procedure to a larger range of tissues and organs (Kartha and Engelmann, 1994; Withers and Engelmann, 1997). New cryopreservation techniques, such as encapsulation-dehydration, vitrification, encapsulation-vitrification and desiccation lengthen the list of species which cannot be stored in low temperatures. By employing this technology, our valuable genetic resources can be conserved. Moreover, it requires only a small space and it is less labour intensive (Villalobos and Engelman, 1995). These procedures include:

1. **Encapsulation-dehydration.** This is a cryopreservation technique which involves encapsulation of explants in beads, then dehydration under laminar airflow, followed by rapid cooling in liquid nitrogen (Niino and Sakai, 1992; Fukai *et al.*, 1994; Grout, 1995; Shatnawi *et al.*, 1999; Wu *et al.*, 1999).
2. **Vitrification.** This is a process whereby plant materials are dehydrated by highly concentrated cryoprotectant, then directly dipped in liquid nitrogen (Sakai *et al.*, 1990; Towill, 1990; Niino *et al.*, 1992; Matsumoto *et al.*, 1994; Engelmann, 1997; Debabrata and Prakash, 1998; Turner *et al.*, 2001).
3. **Encapsulation-vitrification.** This method combines the encapsulation and vitrification techniques and has been established by Engelmann (1997). Such method reduces the injury effect of the explant vitrification (Matsumoto *et al.*, 1995; Engelmann 1997; Ashmore, 1997).

All these new cryopreservation technologies are simple, inexpensive and have high genetic stability of cryopreserved materials. They will become more widely applied once their flexibility and practicality are clearly demonstrated.

Monitoring Stability

The need to maintain genetic integrity in conserved germplasm is implicit. While accepting that absolute stability is not the norm in nature (Scowcroft, 1985), and may not even be desirable in evolutionary terms, *in vitro* procedures will not be acceptable if they introduce risk of genetic instability. Genetic diversity of germplasm and maintenance of genetic stability of conserved germplasm have been investigated using cytological, biochemical (isozymes) and molecular (RAPID, RFLP) techniques (Withers, 1989. Ryynnanen *et al.*, 2001).

Conclusions

This paper attempted to illustrate the developments, trends and ways in which *in vitro* techniques may be used to supplement, enhance and possibly replace conventional conservation practices. There is a gradual accumulation of knowledge about germplasm conservation. In some cases, technological developments are still at the stage of examining a model system, such as in the case of native endangered plant species. In others, such as in the case of agriculturally and horticulturally important crops, there is an increasing number of germplasm storing techniques which can be considered operational. However, their routine application is still restricted to the conservation of cell lines in the research laboratories. Unless they are cost effective, and resolve genuine problems, the extent to which these new techniques will be used will remain to be seen. Close collaboration between breeders, genetic conservationists and *in vitro* technologists will be essential to the appropriate development and implementation of these new techniques for *in vitro* conservation of plant germplasm.

References

Adams, R.P. 1993. The conservation and utilisation of genes from endangered and extinct plant DNA Bank-Net. p. 35-52. In: J.P. Gustafson (ed.), Gene Conservation and Exploitation, Plenum Press, New York.

Aitken-Christie, J. and Singh, A.P. 1987. Cold storage of tissue cultures. p. 285-304. In: J.M. Bonga and D.J. Durzan (eds.), Cell and Tissue Culture in Forestry. Martinus Nijhoff, Dordrecht.

Ashmore, S.E. 1997. Status report on the development and application of *in vitro* techniques for the conservation and use of plant genetic resources. International Plant Genetic Resource Institute, Rome.

Assy-Bah, B., Durand-Gasselin, T. and Pannetier, C. 1987. Use of zygotic embryo culture to collect germplasm of coconut (*Cocos nucifera* L.). FAO/IBPGR Plant Genetic Resources Newsletter 71:4-10.

Augereau, J.M., Courtois, D. and Petiard, V. 1986. Long term storage of callus cultures at low temperatures or under mineral oil layer. Plant Cell Reports 5:372-376.

Bajaj, Y.P.S. 1991. Storage and Cryopreservation of *In Vitro* Cultures. p. 361-381. In: Y.P.S. Bajaj (ed.), Biotechnology in Agriculture and Forestry, Vol. 17, High-Tech and Micropropagation I. Springer-Verlag, Berlin, Heidelberg.

Banerjee, N. and De Langhe, E. 1985. A tissue culture technique for rapid clonal propagation and storage under minimal growth conditions of *Musa* (banana and plantain). Plant Cell Reports 4:351-354.

Benson Erica, E., Danaher, J.E. Pimbley I.M. Anderson, C.T., Wake, J.E, Daley, S. and Adams, L.K. 2000. *In vitro* micropropagation of *Primula scotia*: A rare Scottish plant. Biodiversity and Conservation 9:711-726.

Benson, E.E. 1990. Free Radical Damage in Stored Plant Germplasm. International Board for Plant Genetic Resources, Rome.

Bertrand-Desbrunais, A. 1991. La conservation des ressources genetiques des cafeiers. These d'Universite Paris 6.

Bigot, C. 1987. *In Vitro* Manipulation of Higher Plants: Some Achievements, Problems and Perspectives. In: J. Boccon-Gibod, A. Benbadis and K.C. Shont (eds.), Proceedings of IAPTC French-British Meeting. 8-9 October, Cell Culture Techniques Applied to Plant Production and Plant Breeding, Angers, France, p. 5-17.

Bomal, C. and Tremblay, F.M. 2000. Dried cryopreserved somatic embryos of two *Picea* species provide suitable material for direct plantlet regeneration and germplasm storage. Annals of Botany. 86:177-183.

Bridgen, M.P. and Staby, G.L. 1981. Low pressure and low oxygen storage of *Nicotiana tabacum* and *Chrysanthemum* x *Morifolium* tissue culture. Plant Science Letters 22:177-186.

Caplin, S.M. 1959. Mineral oil overlay for conservation of plant tissue cultures. American Journal of Botany 46:324-329.

Chen, T.H.H., Kartha, K.K., Leung, N.L., Kurz, G.W.G., Caston, K.B. and Constabel, F.C. 1984. Cryopreservation of alkaloid producing cell cultures of periwinkle (*Catharanthus roseus*). Plant Physiology 73:726-731.

Chin, H.R .and Pritchard, H.W. 1988. Recalcitrant seeds a Status Report. International Board for Plant Genetic Resources, Rome.

Debabrata, S. and Prakash, S.N. 1998. Cryopreservation of shoot tips of tetraploid potato (*Solanum tuberosum* L.) clones by vitrification. Annals of Botany. 82:455-461.

Dodds, J.H. 1991. *In vitro* Methods for Conservation of Plant Genetic Resources. Chapman and Hall, London.

Dodds, J.H. and Roberts, L.W. 1985. Experiments in Plant Tissue Culture. Cambridge University Press, Cambridge.

Druart, P. 1985. *In vitro* Germplasm Preservation Technique for Fruit Trees. p.167-171. In: A. Schafer-Menuhr (ed.), *In Vitro* Techniques-Propagation and Long-term Storage. Nijhoff/Junk for CEC, Dordrecht.

Ellis, R..E., Hong, T. and Roberts, E.H. 1990. An intermediate category of seed storage behaviour? I. Coffee. Journal of Experimental Botany 41:1167-1174.

Ellis, R.E., Hong, T., Roberts, E.H. and Soetisna, U. 1991. Seed storage behaviour in *Elaieis guineensis*. Seed Science Research 1: 99-104.

Engelmann, F. 1991a. *In vitro* conservation of horticultural species. Acta Horticulturae 298:327-334.

Engelmann, F. 1991b. *In vitro* conservation of tropical plant germplasm-review. Euphytica 75:227-243.

Engelmann, F. 1995. Brief overview of IPGRI's research activities on *in vitro* conservation of plant species. The Royal Botanic Gardens, Kew Vol. 2.

Engelmann, F. 1997. *In vitro* conservation methods. p.119-150 In: J.A.Callow, B.V.Ford-Lloyd, and H.J. Newbury (eds.). Biotechnology and Plant Genetic Resources, CAB International.

Engelmann, F. and Dereuddre, J. 1988. Cryopreservation of oil palm somatic embryos: importance of the freezing process. Cryo-Letters 9:220-235.

Engelmann, F., Dumet, D., Chabrillange, N., Abdelnour-Esquivel, A., Assay-Bah, B., Dereuddere, J. and Duval, Y. 1995. Cryopreservation of zygotic and somatic embryos from recalcitrant and intermediate-seed species. IPGRI/FAO Newsletter 103:27-31.

FAO. 1994. Survey of Existing Data on ex situ Collections of Plant Genetic Resources for Food and Agriculture. Food and Agriculture Organisation, Rome.

Franks, F. (1985). Biophysics and Biochemistry at Low Temperatures. Cambridge University Press, Cambridge.

Fukai, S., Togashi, M. and Goi, M. 1994. Cryopreservation of *in vitro* grown *Dianthus* by encapsulation-dehydration. Technical Bulletin of Faculty of Agriculture, Kawaga University 46:101-107.

George, E.L.F. and Sherrington, P.D. 1984. Plant Propagation by Tissue Culture. Exogenetics Ltd. Eddington, Weastburg, Wilts England.

Grospietsch, M., Stodulkova, E., Zamecnik, J. 1999. Effect of osmotic pressure on the dehydration tolerance and cryopreservation of *Solanum tuberosum* shoot tips. Cryo-Letters 7:339-346.

Grout, B. 1995. Genetic Preservation of Plant Cells *in Vitro*. Springer Lab Manual. Springer-Verlag, Berlin, Heilderberg. New York.

Ibanez, M.R. and Amo-Marco, J.B. 1998. Promotion by phloroglucinol of micropropagation of *Minuartia valentina*, an endangered and endemic Spanish plant. Plant Growth Regulation 26:49-56.

IBPGR 1986. Design,Planning and Operation of *In Vitro* Genebanks. International Board for Palnt genetic resources, Rome.

Janeiro, L.V., Vieitez, A.M. and Ballester, M. 1995. Cold storage of wild cherry, chestnut and oak. Annales des Sciences Forestieres 52:287-293.

Kartha, K.K. 1985. Cryopreservation of Plant Cell Organs. CRC Press, Boca Raton, FL.

Kartha, K.K. and Engelmann, F. 1994. Cryopreservation and Germplasm Storage. p.195-230. In: I.K. Vasil and T.A.Thorpe (eds.), Plant Cell and Tissue Culture. Kluwer, Doedrecht.

Kartha, K.K., Mroginski, L.A., Pahl, K. and Leung, N.L. 1982. *In vitro* growth response and plant regeneration from cryopreserved meristems of cassava (*Manihot esculanta* Crantz). Zeitschrift fur Planzenphysiologe 107:133-140.

Latta, R. 1971. Preservation of suspension cultures of plant cells by freezing. Canadian Journal of Botany 49:1253-1254.

Lyons, J,M., Raison, J.K. and Steponkus, P.L. 1979. The plant membrane in response to low temperature: an overview. p. 1-24. In: J.M. Lyons, D. Graham, and J.K. Raison (eds.), Low Temperature Stress in Crop Plants-the Role of the Membrane. Academic Press, London.

Matsumoto, T., Sakai, A. and Yamada, K. 1994. Cryopreservation of in vitro grown apical meristems of lily (*Lilium* L.) by vitrification. Cryobiology 31:579-580.

Matsumoto, T., Sakai, A., Takahashi, C. and Yamada, K. 1995. Cryopreservation of apical meristems of wasabi (*Wasabia japonica*) by encapsulation-vitrification method. Cryo-Letters 16:189-196.

Monette, P.L. 1986. Cold storage of kiwi shoot tips *in vitro*. HortScience 21:1203-1205.

Moriguchi, T. Kozaki, I., Matsuta, N. and Yamaki, S. 1988. Plant regeneration from grape callus stored under a combination of low temperature and silicone treatment. Plant Cell Tissue and Organ Culture 15:67-71.

Mullin, R.H. and Schlegel, D.E. 1976. Cold storage maintenance of strawberries meristem plantlets. Horticultural Science 11:100-101.

Niino, T. and Sakai, A., 1992. Cryopreservation of alginate-coated *in vitro*-grown shoot tips of apple, pear and mulberry. Plant Science 87:199-206.

Niino, T., Sakai, A., Enomoto, S., Magosi, J. and Kato, K. 1992. Cryopreservation of *in vitro*-grown shoot tips of mulberry by vitrification. Cryo-Letters 13:303-312.

Orlikowska, T. 1992. Effect of *in vitro* storage at 4°C on survival and proliferation of two apple rootstocks. Plant Cell, Tissue and Organ Culture 31:1-7.

Poissonnier, M., Monod, V., Paques, M. and Dereuddre, J. 1992. Cryopreservation in liquid nitrogen of *Eucalyptus gunni* shoot tips grown *in vitro* following encapsulation and dehydration. Annales de Recherches Sylvicoles. FOCEL, 5-23.

Prematilake, D.P. and Mendis, M.H. 1999. Microtubers of potato (Solanum tuberosum L.): *In vitro* conservation and tissue culture. Journal of National Science Foundation of Sri Lanka 27:17-28.

Quantrano, R.S. 1968. Freeze-preservation of cultured flax cells utilizing DMSO. Plant Physiology 43:2057-61.

Radenbaugh, K., Fujii, J.O. and Slade, D. 1991. Synthetic Seed Technology. p.35-74. In: Vasil, I.K. (ed.), Cell Culture and Somatic Cell Genetics of Plants. Academic Press, San Diego, CA.

Ryynanen, L., Haggman, H. and Aronen, T. 2001. Genetic stability of micropropagated and cryopreserved silver birch. Proceedings of 1st International Symposium on Acclimatisation and Establishment of Micropropagated Plants, Sani-Halkidiki, Greece,19-22. p.70.

Roberts, H.F. 1973. Predicting the viability of seeds. Seed Science Technology 1:499-514.

Roca, W.M., Reyes, R. and Beltram, J. 1984. Effect of various factors on minimal growth in tissue culture storage of cassava germplasm. Proceedings of Sixth Symposium of the International Society for Tropical Root Crops. Lima, Peru 21-26 February. p.441-446.

Sakai, A. 1960. Survival of twigs of woody plants at -196°C. Nature 185:392-394.

Sakai, A. 1986. Cryopreservation of Germplasm of Woody Plants. p. 113-129. In: Y.P.S. Bajaj (ed.), Biotechnology in Agriculture and Forestry, Vol.1 Trees. Springer-Verlag Berlin, Heidelberg.

Sakai, A., Amtsumoto, T., Hirai, D. and Niino, T. 1990. Cryopreservation of nucellar cells of naval orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. Plant Cell Reports 9:30-33.

Sarkar, D. and Prakash, S.N. 1998. Cryopreservation of shoot tips of tetraploid potato (Solanum tuberosum L.) clones by vitrification. Annals of Botany 82:455-461.

Scowcroft , W.R. 1985. Somaclonal Variation: The myth of Clonal Uniformity. p.217-245. In: B. Hohn, and E.S. Denis (eds.), Genetic Flux in Plants. Springer-Verlag, Vienna.

Shatnawi, M., Engelmann, F. Frayttarelli, A. and Damiano, C. 1999. Cryopreservation of apices of in vitro plantlets of almond (Prunus dulcis Mill.) Cryo-Letters 20:13-20.

Sigaud, P. 2000. The Need for an International Framework for the Conservation and Sustainable Utilization of Forest Genetic Resources. p. 217-222. In: C. Matyas (ed.), Forest Genetics and Sustainability, Vol. 63. Kluwer Academic Publishers, The Netherlands.

Staritsky, G., Dekkers, A.J., Louwaars, N.P. and Zandvoort, E.A. 1986. In vitro Conservation of Aroid Germplasm at Reduced Temperatures and Under Osmotic Stress. p.277-284. In: L.A. Withers and P.G. Alderson (eds.), Plant Tissue Culture and its Agricultural Applications. Butterworths, London.

Touchell, D.H., Dixon,K.W. and Tan, B. 1992. Cryopreservation of shoot-tips of *Grevillea scapigera* (Proteaceae): a rare and endangered plant from Western Australia. Australian Journal of Botany 40 305-10.

Towill, L.E. 1990. Cryopreservation of isolated mint shoot tips by vitrification. Plant Cell Reports 9:178-180.

Turner, S.R, Senaratna, T., Bunn, E., Tan, B., Dixon, K.W. and Touchell, D.H. 2001. Cryopreservation of shoot tips from six endangered Australian species using a modified vitrification protocol. Annals of Botany 87:371-378.

Van den Houve and Panis, B. 2000. *In vitro* Conservation of Banana: Medium-term Storage and Prospect for Cryopreservation. p. 225-257 In: M.K. Razdan and E.C. Cocking (eds.), Conservation of Plant Genetic Resources in Vitro, Vol. 2, Applications and Limitations. Science Publishers, Inc. Enfield, USA, Plymouth, UK.

Villalobos, V.M. and Engelmann, F. 1995. *Ex situ* conservation of plant germplasm using biotechnology. World Journal of Microbiology and Biotechnology 11:375-382.

Wheelans, S.K. and Withers, L.A. 1984. The IBPGR International Database on *in vitro* Conservation. Plant Genetic Resources Newsletter 60:33-38.

Williams, R.R. and Taji, A.M. 1987. Effects of temperature, darkness and gelling agent on long term storage *in vitro* shoot cultures of Australian Woody plants. Plant Cell Tissue and Organ Culture 11: 151-56.

Withers, L.A. 1987. *In vitro* methods for germplasm collecting in the field. Plant Genetic Resources Newsletter 69: 2-6.

Withers, L.A. 1989. *In vitro* Conservation and Germplasm Utilisation.p.309-333. In: A.H.D.Brown, O.H. Frankel, D.R. Marshall and J.T. Willimas (eds.), The Use of Plant Genetic Resources. Cambridge University Press, Cambridge, N York, Sydney.

Withers, L.A. 1992. *In vitro* Conservation. p.169-200. In: F. Hammerschlag and R.E. Litz (eds.), Biotechnology of Perennial Fruit Crops. CAB International, Wallingford, Oxon.

Withers, L.A. and Engelmann, F. 1997. *In vitro* Conservation of Plant Genetic Resources. p. 511-515. In: A. Altman (ed.), Biotechnology in Agriculture. Marcel Dekker, New York.

Withers, L.A. and King, P.J. 1980. A simple freezing-unit and a routine cryopreservation method for plant cell cultures. Cryo-Letters 1:213-220.

Withers, L.A. and Street, H.E. 1977. Freeze preservation of cultured plant cells. III. The pregrowth phase. Physiologia Plantarum 39:171-178.

Wu, Y., Engelmann, F., Zhao, Y., Zhou, M and Chen, S. 1999. Cryopreservation of apple shoot tips: Importance of cryopreservation technique and of conditioning of donor plants. Cryo-Letters 20:121-130.

Yidana, J.A., Withers, L.A. and Ivins, J.D. 1987. Development of a simple method for collecting and propagating cocoa germplasm *in vitro*. Acta Horticulturae 212:95-98.

Zandvoort, E.A., Hulshof, M.J.H. and Staritsky, G. 1994. *In vitro* storage of *Xanthosoma* spp. under minimal growth conditions. Plant Cell Tissue and Organ Culture 36:309-316.

Zapartan, M. 1996. Conservation of Leontopodium alpinum using *in vitro* techniques. The Royal Botanic Gardens, Kew, Vol. 2.

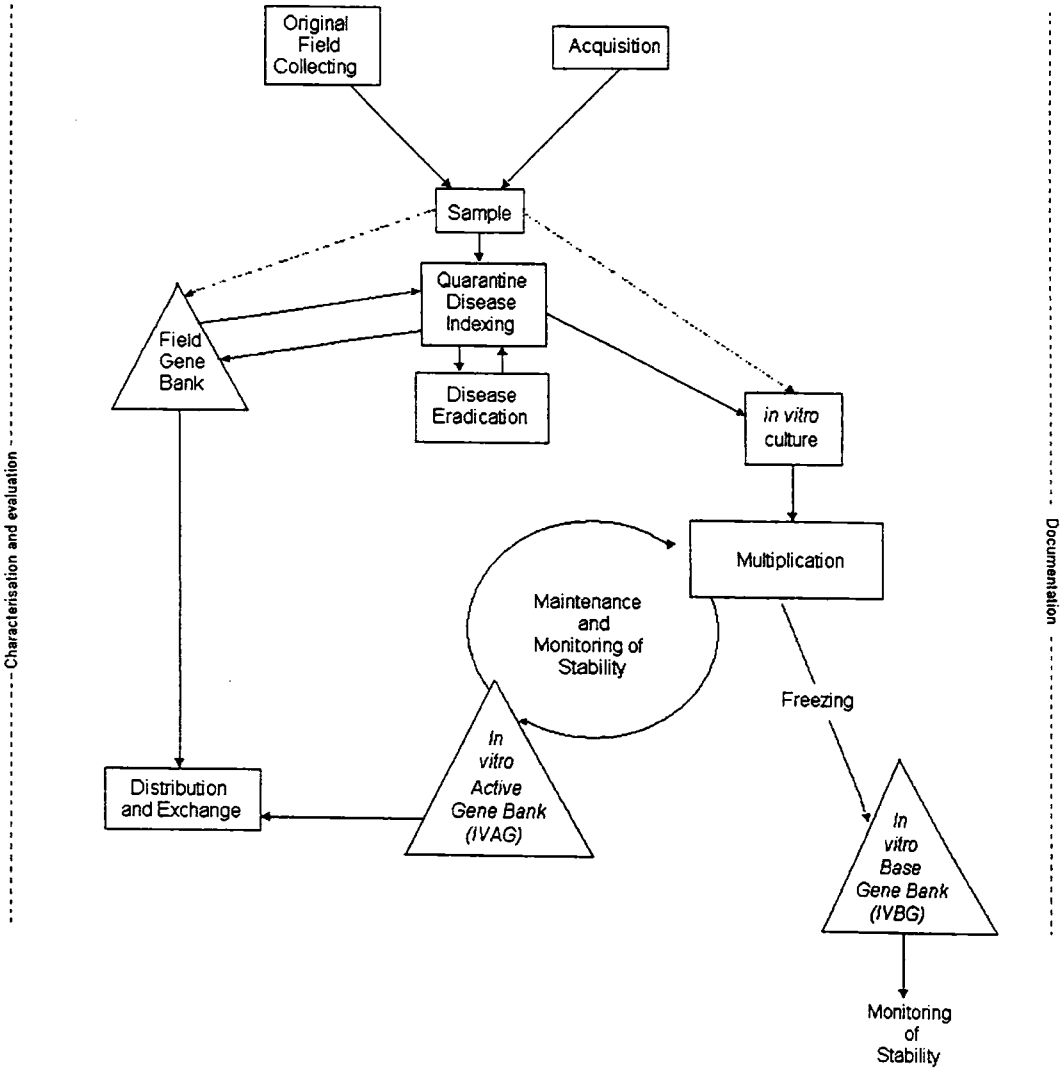

Figure 1: Relationships between accessed germplasm, the field genebank, material for distribution and exchange and the *in vitro* genebank (IBPGR, 1986)

The Importance of Plant Tissue Culture and Biotechnology in Plant Sciences
International Association for Plant Tissue Culture and Biotechnology
(The Australian Region)
Refereed Proceedings of the 7th meeting
University of New England Australia

First published in 2002
University of New England
Armidale, NSW, Australia

National Library of Australia
Cataloguing-in-Publication data:

Taji, Acram M.
Williams, Richard, R.

ISBN 1 86389 781 X

University of New England Publications Unit, Australia