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CONSERVATION OF PLANT MATERIALS BY CRYOPRESERVATION - A REVIEW

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ABSTRACT
Plant biotechnology provides new options for collection, multiplication and short to long term conservation of plant diversity, using *in situ* and *ex situ* culture techniques. Significant progress has been made to conserve the plant, animal diversity etc., one such technique used for conservation of plant materials is cryopreservation. Cryopreservation is a perfect method for long term conservation of plant genetic resources, using very low temperature (liquid nitrogen) at -196°C. during cryopreservation all the biochemical activities are significantly reduced and biological deterioration are stopped. Metabolic and most physical process are stopped at this temperature. Thus, plants can be store for a very long period and the problem such as genetic instability and the risk of loose accessions due to contamination or human error due to subculture overcome. Techniques like cryopreservation helps to collect and store plant genetic resources, especially plants with low seed capability. Formation of ice crystal during cryopreservation leads to physical damage to the cells. Nowadays, conservation of plant genetic materials has altered from slow cooling to vitrification. The availability or development of simple, reliable,and cost effective strategies and the subsequent regeneration of plants are basic requirements for germplasm conservation. Cryopreservation of shoot tip is also being applied to eradicate systemic plant pathogens, a process termed as cryotherapy.

KEYWORDS: Cryopreservation, Vitrification, Vegetatively Propagated Species, Recalcitrant Seed Species, Germplasm Conservation.

INTRODUCTION
Conservation of plant genetic materials is necessary for food security and agro-biodiversity. Genetic diversity provides options to develop through selection and breeding of new and more productive crops, resistant to biological and environmental stresses. Conservation of plant diversity can be performed *in situ* or *ex situ.*

Advances in plant biotechnology, especially those associated to *in vitro* culture and molecular biology, have also provided powerful tools to support and improve conservation and management of plant diversity. At present, biotechnological methods have been used to conserve the endangered, ornamental, medicinal and forest species, allowing conservation of pathogen-free material, elite plants and genetic diversity for short-, medium- and long term. *in vitro* conservation is especially important for vegetatively propagated and for non-orthodox plant species.

In vitro technique offer a same mean to internationally exchange plant material, enable the establishment of extensive collections using minimal space, allow supply of valuable material for wild population recovery and facilitate molecular investigations and ecological studies.

Cryopreservation techniques
Some materials such as, orthodox seeds or dormant buds, display natural dehydration process and can be cryopreserved without any pretreatment. However, most of the experimental systems employed in cryopreservation (cell suspensions, calluses, shoot tips, embryos, etc.,) contain high amount of cellular free water and are thus extremely sensitive to freezing injury since most of them are not inherently freezing-tolerant. Cells have thus to be dehydrated artificially to protect them from damage caused by crystallization of intracellular water into ice. The techniques employed and the physical mechanism upon which they are based are different in classical and new cryopreservation techniques.

Classical cryopreservation techniques
Classical cryopreservation techniques involves slow cooling down to a defined prefreezing temperature, followed by rapid immersion in liquid nitrogen. With temperature reduction, during slow cooling, cells and the external medium initially supercool, followed by ice formation in the medium. The cell membrane acts as a

barrier and prevents the ice from seeding the cell interior and the cells remain unfrozen but supercool. Since cells remain supercooled and their aqueous vapour pressure exceeds of that of the frozen external compartment, cells equilibrate by loss of water to external ice. In optimal conditions, most or all intracellular freezable water is removed, thus reducing or avoiding detrimental intracellular ice formation upon subsequent immersion of the specimen in liquid nitrogen. Thawing should be as rapid as possible to avoid the phenomenon of recrystallization in which ice melts and reforms at a thermodynamically favorable, larger and more damaging crystal size.

Classical freezing procedures includes the following successive steps: pregrowth of samples, cryoprotection, slow cooling to a determined prefreezing temperature, rapid immersion of samples in liquid nitrogen, storage, rapid thawing and recovery.

Long term conservation using cryopreservation

Cryopreservation is the only technique which ensures the safe and long term conservation of various categories of plants, including non orthodox seed species, vegetatively propagated plants, rare and endangered species and biotechnological products. In all cryopreservation process water removal plays a major role in preventing freezing injury and in maintaining post-thaw viability of cryopreserved material. There are two methods of cryopreservation protocols that differ in their physical mechanisms: classical cryopreservation procedures, in which cooling is performed in the presence of ice; and the procedures based on vitrification, in which cooling normally takes place without ice formation. In cryopreservation methods subculture is not needed and the somoclonal variations are reduced. Advantages of cryopreservation is that germplasm can be kept for theoretically indefinite term with low cost and little space. Besides its used for conservation of genetic materials, cryopreservation can be used for the safe storage of plant cell with specific characteristics. Different types of plant cells, tissues and organs can be cryopreserved. Cryopreservation is the most suitable long term storage method for genetic resources of vegetatively maintained crops. For vegetatively propagated species, the best organs are shoot apices excised from *in vitro* plants. Shoot apices or meristem cultures are suitable because of virus-free plant production, clonal propagation, improving health status, easier recovery and less mutation. Cryopreservation offers a good method for conservation of the species, especially woody plant germplasm. Cryostorage of seeds in LN was initially developed for the conservation of a large number of species at low cost, significantly reducing seed deterioration in storage. New cryobiological studies of plant materials has made cryopreservation a biological tool for long-term storage, for tropical species, which are not intrinsically tolerant to low temperature and dessication, has been extensively investigated. Number of species, which can be cryopreserved has rapidly increased over the last several years because of the new techniques and progression of cryopreservation research. The vitrification/one-step freezing and ED methods have been applied to an increasing number of species. A new method named encapsulation-vitrification is note worthy. The new techniques have produced high post-thaw and minor modifications. In cryopreservation, information recording such as types, size of explants pretreatment and the correct type and concentration of the cryoprotectants, explants water content, cryopreservation method, rate of freezing and thawing, thawing method, recovery medium and incubation condition is very important. All germplasm requires safe storage because even exotic germplasm without obvious economic merit may contain genes or alleles that may be needed as a new disease, insect, environmental or crop production problems arise. It is also important to record the recovery percentage after short conservation period. A major concern is the genetic stability of the conserved material.

New cryopreservation techniques

In vitrification based procedures, cell dehydration is done prior to freezing by exposure of sample to concentrated cryoprotectant media and/or air dessication. This is followed by rapid cooling. As a result, all the factors that affect ice formation are avoided. Glass transitions during cooling and rewarming have been recorded during thermal analysis. Vitrification based procedures provides practical advantages in comparison to classical freezing procedures techniques. By preincluding ice formation in the system, vitrification based procedures are operationally less complex than classical procedures and have great potential for broad applicability, requiring only minor modifications for different cell types.

Seven different vitrification based procedures can be identified:

(1) Encapsulation-dehydration: It is based on the technology developed for the production of artificial seeds. Explants are encapsulated in alginate beads, pregrown in liquid medium enriched with sucrose for 1-7 days, partially desiccated in air current of laminar air flow cabinet or with silica gel to a water content around 20%, then frozen rapidly.

(2) Vitrification: It includes pre-culture of samples on medium enriched with cryoprotective substances, treatment with loading solutions, dehydration with a highly concentrated vitrification solution such as the glycerol based PVS2 solution, rapid freezing, thawing, removal of cryoprotectants and recovery.

(3) Encapsulation-vitrification: Here the samples are encapsulated in alginate beads and is subjected to freezing by vitrification.

(4) Dehydration: It is the simplest procedure since it consists of dehydrating explants, then freezing them rapidly by direct immersion in liquid nitrogen. It is mainly used with zygotic embryos.

(5) Pregrowth: It consists of cultivating samples in the

presence of cryoprotectants, then forcing them rapidly by direct immersion in liquid nitrogen.

(6) **Pregrowth-dehydration:** Explants are grown in the presence of cryoprotectants, dehydrated at laminar airflow cabinet or with silica gel and frozen rapidly.

(7) **Droplet freezing:** Treated with liquid cryoprotective medium, then placed on an aluminium foil in minute droplets of cryoprotectant and frozen slowly or rapidly in liquid nitrogen.

Cryopreservation of vegetatively propagated species

Several review papers have been published recently, which provides lists of species which have been successfully cryopreserved. For vegetatively propagated species, cryopreservation has a wide applicability both in terms of species coverage. Regeneration is rapid and direct, and callusing is observed only in cases where the technique is not optimized. No ice formation takes place in vitrification-based procedures, thus allowing direct, organized regrowth of frozen explants. Many vegetatively propagated species successfully cryopreserved. In addition, *in vitro* material is 'synchronized' by the tissue cultures and pregrowth procedures. Relatively homogeneous samples in terms of size, cellular composition, physiological state and growth response are employed for freezing, thus increasing the chances of positive and uniform response to treatment. Finally, vitrification-based procedures allow the use of samples of relatively large size which can grow directly without difficulty. Freezing techniques are now operational for large-scale experimentation and commercialization with an increasing number of vegetatively propagated plants. In view of wide range of efficient and operationally simple techniques available, any vegetatively propagated species should be amendable to cryopreservation, provided that the tissue culture protocol is sufficiently operational for this species.

Cryopreservation of recalcitrant seed species

Several review papers have been published in recent years which present extensive lists of plant species whose embryos have been successfully cryopreserved. Freezing of embryos is a routine procedure applicable to numerous species, whatever their storage characteristics. Careful examination of the species mentioned revealed that only a limited number of truly recalcitrant seed species are included. In comparison to the results obtained with vegetatively propagated species, research is still at a very preliminary stage for recalcitrant seeds. A number of reasons explain the limited development of cryopreservation for recalcitrant seed species. Seeds and embryos of recalcitrant species also display very large variations in moisture content and maturity stage between provenances, between and among seed lots, as well as between successive harvests, thus making their cryopreservation difficult.

Seeds of many species are too large to be frozen directly and embryos are thus successfully employed for cryopreservation. Embryos are often of complex tissue composition which display differential sensitivity to dessication and freezing, the root pole seeming more resistant than shoot pole. In some species, embryos are are extremely sensitive to dessication and even a minor reduction in their moisture content leads to structural damage. There is scope for technical improvements in the current cryopreservation protocols for embryos and embryonic axes. Pregrowth on media containing cryoprotective substances may confer increased tolerance to the tissues for further dessication and reduce heterogeneity of the material. Flash drying followed by rapid freezing has also been effective. Attempts to freeze whole embryos have been proven unsuccessful, it has been suggested to use shoot apices sampled from embryos.

Cryopreservation for germplasm conservation

In the case of orthodox seeds species, cryopreservation is used mainly for storing seeds with limited longevity and of rare or endangered species with limited longevity and of rare or endangered species. The national center for genetic resources preservation conserves 37654 accessions over the vapors of liquid nitrogen. The national bureau for plant genetic resources conserves 1200 accessions from 50 different species, consisting mainly of endangered medicinal species. cryopreservation is also applied to intermediate seeds which are tolerant to freezing. In case of dormant buds, the 2200 accessions of the germplasm field collection are duplicated under cryopreservation. Breeders routinely store pollen in liquid nitrogen. In India, the NBPGR conserves cryopreserved pollen of 65 accessions and the Indian institute of horticultural research conserves pollen of 600 accessions.

Finally cryopreservation is being applied in genebanks for long term storage of genetic resources of vegetatively propagated species, using apices from *in vitro* plantlets. Potato cryopreservation is the currently the most advanced. Large scale utilization of cryopreservation implies scaling-up of the amount of material to be handled and stored. Cryopreservation imposes a series of stresses to plant material, which is susceptible to induced modifications in cryopreserved cultures and regenerated plants. No modification has been observed at the phenotypical, biochemical, chromosomal or molecular level which could be attributed to cryopreservation. Studies performed on cost of cryopreservation are still fragmentary, but the first estimates tend to confirm the interest of this technique also form a financial perspective.

Other uses of cryopreservation

Cryopreservation is also employed for other uses other than germplasm conservation. More recently, cryopreservation has been used for cryotherapy, I.e., for eliminating viruses from infected plants, as a substitute or complementary to classical virus eradication techniques such as meristem culture and cryotherapy.

Cryotherapy is based on selective cell destruction by cryopreservation. The differentiated cells of apices which contain viruses also have a high water content; they are killed by the formation of which leads to growth of apices, have a more concentrated cytoplasm and withstand freezing. In large-scale propagation programs of conifers based on somatic embryogenesis, the ability to maintain donor tissue juvenility through cryopreservation represents an immeasurable advantage over propagation programs based on rooted cuttings.

Recovery of cryopreserved germplasm

Long storage can takes place in LN or in vapour phase. For the recovery of the germplasm after cryostorage rapid rewarming is required to prevent recrystallisation. Vials containing germplasm are generally immersed in water bath at 35-40°C. When germplasm are not included in the vials, for example in droplet method, rewarming takes place in liquid medium at room temperature. In many species recovery of the apices cryopreserved with the new method is direct, without callus formation. By contrast with the classical methods, the structural integrity of most cells is well preserved. Some studies have shown the importance of post-thawing culture conditions to enhance organised growth. In many cases selection of suitable growth medium for recovery of germplasm is necessary. Adjustment of growth regulator concentration or even medium salt formation could be required for normal development of frozen germplasm. In some germplasm conservation centres, 20% recovery is considered enough for long term preservation. Others consider that survival should be higher than 40%. it is important that those percentages be reproducible.

CONCLUSION

Even though cryopreservation is still routinely employed in a limited number of cases only, the development of the new vitrification-based freezing techniques has made its applications to a broad range of species possible. A significant advantage of these new techniques is their operational simplicity. For many vegetatively propagated species, cryopreservation are sufficiently advanced to envisage their immediate utilization for large-scale experimentation in genebanks. Research is much less for recalcitrant seed species. However, there are various technical approaches to explore to improve the efficiency and increase the applicability of cryopreservation techniques to recalcitrant species. In addition, research is done worldwide to improve the knowledge of biological mechanisms underlying seed recalcitrant. It is hoped that new findings on critical issues such as understanding and control of dessication sensitivity will contribute significantly to the development of improved cryopreservation techniques for recalcitrant seed species.

A very positive development in the establishment of the cryopreservation protocols is that an increasing number of researches make systematic use of analytical tools which provide a scientific basis to the success/failure of an experimental treatment. Such a rational and scientific approach should greatly facilitate the establishment of freezing protocols, especially for problem species. It is important to stress, however, that cryopreservation is not seen as a replacement for conventional *ex situ* approaches. Cryopreservation offers genebank curators an additional tool to allow them to improve the conservation of germplasm collections placed under their responsibility. In years, cryopreservation will become more frequently employed for long-term conservation of plant genetic resources.

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REFERENCES

1. Assay-Bah, B.; Engelmann, F.Cryopreservation of mature embryos of coconut (*Cocos nucifera* L.) and subsequent regeneration of plantlets. Cryo Letters, 1992; 13: 117-126.
2. Bereetche, J.; Galerne, M.; Dereuddre, J. Efficient regeneration of plantlets from embryonic callus of *Picea abies* (L.) Karst after freezing in liquid nitrogen. C.R. Acad. Sci. Paris ser., 1990; 3 310: 357-363.
3. Chandel, K.P.S.; Chaudhury, R.; Radhamani, j.; Malik, S.K. Dessication and freezing sensitivity in recalcitrant seeds of tea, cocoa and jackfruit. Ann. Bot., 1995; 76: 443-450.
4. Dereuddre, J.; Hassen, M.; Blandin, S.; Kaminski, M. Resistance of alginate coated somatic embryos of carrot (*Daucus carota* L.) to desiccation and freezing in liquid nitrogen: 2. thermal analysis. Cryo Letters, 1991; 12: 135-148.
5. Dussert, S.; Chabrillange, N.; Engelmann, F.; Anthony, F.; Hamon, S. Cryopreservation of coffee (*Coffea arabica* L.) seeds: importance of the precooling temperature. Cryo Letters, 1997; 18: 269-276.
6. Engelmann, F. Importance of desiccation for the cryopreservation of recalcitrant seed and vegetatively propagated species. Plant Genet. Res. Newslett., 1997a; 112: 9-18.
7. Engelmann, F. Plant cryopreservation: progress and prospects. *In vitro* cell. Dev. Biol.-Plant, September-October 2004; 40: 427-433.
8. Engelmann, F. Use of biotechnologies for the conservation of plant biodiversity.
9. *In vitro* Cell. Dev. Biol.-Plant 10.1007/s11627-010-9327-2
10. Engelmann, F. *in vitro* conservation of tropical plant germplasm-a review.
11. *Euphytica*, 1991; 57: 227-243.
12. Engelmann, F. Cryopreservation of embryos. In: Dattee Y.; Dumas C.; Gallais A. (eds) Reproductive biology and plant breeding. Springer, Berlin, 1992; 281-290.

13. Kartha K. k.; Engelmann F. Cryopreservation and germplasm storage. In: Vasil I. K.; Thorpe T. A. (eds) Plant cell and tissue culture. Kluwer, Dordrecht, 1994; 195-230.
14. Keller E. R. J.; Senula A.; Leunufna S.; Grube M. Slow growth storage and cryopreservation-tools to facilitate germplasm maintenance of vegetatively propagated crops in living plant collections. *Int Refrig*, 2006; 29: 411-417.
15. Malik S. K.; Chaudhury R. The cryopreservation of embryonic axes of two wild and endangered *Citrus* species. *Plant Genet Res: Charact Utiliz*, 2006; 4: 204-209.
16. Meryman H. T.; Williams R. J.; Douglas M. S. J. Freezing injury from solution effects and its prevention by natural or artificial cryoprotection. *Cryobiology*, 1977; 14: 287-302.
17. Pence V. C. Cryopreservation of seeds of Ohio native plants and related species.
18. *Seed Sci Technol*, 1991; 19: 235-251.
19. Wesley-Smith J.; Vertucci C.W.; Berjak P.; Pammenter N. W.; Crane J. Cryopreservation of desiccation-sensitive axes of *Camellia sinensis* in relation to dehydration, freezing rate and the thermal properties of tissue water. *J Plant Physiol*, 1992; 140: 596-604.
20. Reed B. M. Plant cryopreservation: a practical guide. Springer, Berlin, 2008.
21. Roberts H. F. Predicting the viability of seeds. *Seed Sci Technol*, 1973; 1: 499-514.
22. Sakai A.; Engelmann F. Vitrification, encapsulation-vitrification and droplet-vitrification: a review. *Cryoletters*, 2007; 28: 151-172.
23. Touchell D. H.; Dixon K. W. Cryopreservation of seed of Western Australia native species. *Biodivers Conserv*, 1993; 2: 594-602.
24. Touchell D.H.; Dixon K. W. Cryopreservation for seedbanking of Australian species. *Ann Bot*, 1994; 40: 541-546.
25. Withers L. A.; Engelmann F. *In vitro* conservation of plant genetic resources. In: Altman A.(ed) Biotechnology in agriculture. Marcel Dekker, New York, 1998; 57-88.
26. Uragami A.; Sakai A.; Magai M. Cryopreservation of dried axillary buds from plantlets of *Asparagus officinalis* L. grown *in vitro*. *Plant Cell Rep.*, 1990; 9: 328-331.
27. United Nations Conference On Environment And Development(UNCED). C*onvention On Biological Diversity;* UNCED: Geneva, Switzerland, 1992.
28. Altman D. W.; Fryxell, P.A.; Koch S.D.; Howell C.R.; *Gossypium* germplasm conservation augmented by tissue culture techniques for field collecting. *Econ. Bot.*, 1990; 44: 106-113.
29. Bunn E.; Turner S. R.; Panaia m.; Dixon K.W. The contribution of *in vitro* technology and cryogenic storage to conservation of indigenous plants. *Austr. J, Bot.* 2007; 55: 345-355.
30. Villalobos V.M.; Engelmann F.; *Ex situ* conservation of plant germplasm using biotechnology. *World J. Microb. Biotechnol*, 1995; 11: 375-382.
31. Fay M.F.; conservation of rare and endangered species using *in vitro* methods. *In vitro. Cell. Dev. Biol. Plant*, 1992; 28: 1-4.
32. Berjak P.; Bartels P.; Benson E.; Harding K.; Mycock D.; Pammenter N.W.; Sershen; Wesley-Smith J. Cryoconservation of South African Plant Genetic Diversity. *In vitro. Cell dev. Biol. Plant.*, 2011; 47: 65-81.
33. Wang Q.C.; Valkonen J.P.T. Cryotherapy of shoot tips: Novel pathogen eradication method. *Trends Plant Sci.*, 2009; 14: 199-122.
34. Paunescu A.; Biotechnology for endangered plant conservation: A critical overview. *Rom Biotech. Lett.*, 2009; 14: 4095-4103.