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# Expanding Applications of Cryobanking: Meeting Challenges for Effective Long-term Storage

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ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR) leading the plant cryobanking efforts since 1987 equipped with large capacity cryotanks have successfully banked non-orthodox seeded species, prioritized orthodox seeds, pollen, dormant buds and genomic resources at ultra-low temperatures with retesting data generated for 28 years of cryostorage. Expansion of activities while extending the cryobanking facilities to ICAR-National Bureau of Agriculturally Important Microbes (ICAR-NBAIM) has led to microbes cryobanking. Wide scope exists for expansion of basic cryo-principles to store endangered exceptional species that produce few seeds, tropical and sub-tropical oilseeds especially orchids, species with inherently short-lived seeds (Type I seeds), despite being orthodox in nature, neglected and underutilised plant species (NUS), with focus on species of biodiversity hot-spots in the moist tropics as well as biotechnologically important cell lines and transgenics. Co-cryopreservation of orchid seed and fungal symbionts/mycorrhizal fungi for the conservation of endangered orchids successfully demonstrated in few studies needs to be taken up. With the concept of cryopreservation of phyto-diversity encompassing algae and cyanobacteria besides the higher plants, greater expansion is envisaged. With the state-of-the art cryo-technologies existing for aquatic species and veterinary animals, as recently reviewed in International Conference, “Low Temperature Science and Biotechnological Advances” held by ICAR-NBPGR, India in April 2015, expansion of large scale cryobanking is imminent. Studies on cellular impact of cryoprotocols and changes in gene expression in relation to cryopreservation are ongoing in different labs of the World. Broad range of technical solutions to permit establishment of more efficient and user-friendly protocols for cryobanking of ‘hydrated sensitive’ materials are now available and work is being expanded.

## Cryobanking the Plant Genetic Resources

Cryopreservation as a strategy to extend viability of conserved biological materials, for theoretically ‘indefinite’ periods, has been widely acknowledged. Germplasm/species/ organisms selected and prioritised for cryostorage are determined by each Cryogenebank keeping in view the technical, practical and economic aspects of cryopreservation. The priority for cryobanking depends upon the designed infrastructure, scope of expansion, networking with other institutes with common goals and policy statement of the organisation or the country. Several countries of the world have adopted large scale banking for long-term conservation of germplasm as zygotic seeds and its components, pollen, dormant buds, *in vitro* raised explants with each institute/ organisation focusing on each type separately and in few cases all the forms in the same bank. National Center for Genetic Resources Preservation (NCGRP), USA, besides being a seed bank, is also a repository for animal genetic resources in the form of semen and plant genetic resources as graftable buds or *in vitro* explants. ICAR-NBPGR, New Delhi is yet another Institute cryoconserving, plant origin explants, microbes and genomic resources.

## Plant Cryobanking at NBPGR: Seeds, Embryos, Embryonic Axes, Pollen, Dormant buds and Genomic Resources

With proven wide applicability of cryotechniques in plant sciences, diverse germplasm of national and international importance is being cryopreserved in National Cryogenebank at ICAR-NBPGR with emphasis on non-orthodox spp. (intermediate and recalcitrant species). Over a span of 28 years, cryoprotocols have been developed, in most cases on species-specific basis, using explants like seeds, embryos, embryonic axes, pollen and dormant buds of the difficult-to-store types.

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Orthodox seed species belonging to threatened and endangered plant species with critically small population size, wild and weedy relatives of crop plants, genetic stocks including core collections of crop diversity have been selectively cryostored. The Cryogenebank Facility with six cryotanks of extra large capacity (1000 lits LN) can hold quarter million samples. Presently it holds about 42,000 containers (cryovials of various capacities, polyolefin tubings of different diameter) storing more than 11,700 accessions belonging to about 794 plant species (Anon., 2015). Cryopreservation protocols have been standardised primarily for tropical species of horticultural, plantation, agro-forestry and industrial importance. Base collection of tropical and temperate fruits, plantation crops and agroforestry species e.g. of *Citrus* (25 species), *Piper nigrum*, *Buchanania lanzan, Prunus armeniaca*, *P. dulcis*, *Morus* (13 spp.), *Mangifera*, *Jatropha, Pongamia* and *Azadirachta* germplasm has been established in various forms like seeds, embryos, embryonic axes, dormant buds, pollen and genomic resources (Chaudhury and Malik, 2014). Cryobanking of non-orthodox (recalcitrant and intermediate) seeds is intensively undertaken (Chaudhury and Malik, 2010). The aim had been to recover plants from seeds, embryos, embryonic axes after various storage periods, bring back to room temperature without any damages-structural and physiological and obtain plantlets without an intervening callus to ensure genetic integrity of the conserved germplasm. Techniques have already been optimised to recover plantlets without an associated callus. Despite best efforts not all species under threat and those with recalcitrant seeds can be assisted by conservationists. Specialised techniques of desiccation-freezing, vitrification, encapsulation-dehydration and step-wise slow freezing are applied to difficult-to-store species.

Basic cryobiological investigations involving biochemical, biophysical and ultra-structural markers of desiccation and freezing sensitivity are being pursued (Chaudhury *et al*., 2011; Malik *et al*., 2011). Cryoconserved samples randomly tested for various periods upto 25 years (Choudhary *et al*., 2013; Chaudhury and Malik, 2014) and after 28 years storage (pers. comm.) have shown retention of viability values comparable to that in initial samples. Recently cryobanking of genomic resources comprising mainly cloning vectors, cloned genes and promoters, different DNA libraries, BAC, YAC, PAC clones etc. of plants, animals and microbes has been initiated. In addition, HRD training component is executed through the Centre of Excellence (CoE), recognised by ICAR and Bioversity International, for conduct of international trainings on *in vitro* conservation and cryopreservation. Seven international trainings have been conducted and so far more than 100 trainee participants of the World have been trained with three in collaboration with Royal Botanic Gardens, Kew, UK.

## Microbes Cryobanking at NBPGR

Cryopreservation of microbes leads to extended viability (several years) and prevents genetic and physiological changes expected during long-term culture of actively growing organisms. For microorganisms which are prone to cryogenic-injury, specific protocols have been designed to ensure optimal cryopreservation (Smith and Ryan, 2012). Microbes are reportedly successfully cryobanked including 4,000 species of fungi, belonging to over 700 genera. However, some microbes e.g. bacteria *Helicobacter*, fungi e.g. members of *Basidiomycota* and *Chromists* etc. are found to be preservation recalcitrant. To carry out research on the interactions between microbes in plants and algae, whether beneficial or pathogenic, requires utilisation of cryopreserved microbial cultures. Throughout the world most of the reputed microbial resource centres (eg. ATCC, BCCM-LMG, CABI, NES-MCC, etc) have adopted the cryopreservation technology.

At NBPGR cryopreservation has been attempted with *Micrococcus luteus*, *Agrobacterium tumefaciens*, *Acetobacter pasteurianus*, *A. baumannii*, *Escherichia coli*, *Enterobacter cloacae*, *Lactobacillus acidophilus*, *Klebsiella pneumonia*, *Cellulomonas flavigena* and *Brevundimonas diminuta* selected from genepool of NBAIM. Pure cultures of the bacteria inoculated in different glycerol concentrations were cooled slowly and cryobanked at -196$^{o}$C. The viability of the bacteria was checked at regular time intervals and found satisfactory.

## Expanding Applications to Seeds of Orthodox and Rare and Endangered Plant Species, Transgenic Plant Materials, Mycorrhizal Fungi and Bryophytes

Cryopreservation has proven effective for futuristic programmes. For terrestrial native orchids which are dependent on mycorrhizal associations with fungi for survival in field, conservation of both is essential. Long-term *ex situ* storage of orchid seed and fungal symbionts for the conservation of endangered orchids have increasingly being attempted using cryopreservation (Batty *et al*., 2001; Sommerville and Offord, 2014). Pritchard and Nadarajan (2008) and Cruz-Cruz *et al.* (2013) have strongly suggested cryopreservation to be appropriate for integrating as an extra insurance policy for storing all orthodox seeds with sub-sample of each to be cryostored, in addition to the samples stored under classical gene bank conditions of -20°C. There are several examples to illustrate the application of cryopreservation techniques to preserve rare and endangered species for various higher plants, including orchids, bryophytes and ferns.

Reports exists for successful cryopreservation of transgenic materials containing different target genes in *Oryza sativa, Triticum aestivum*, *Carica papaya*, *Citrus sinensis*, *Pyrus pyrifolia*, *Betula pendula*, *Papaver somniferum* (Cho *et al*., 2007; Van Eck and Keen, 2009; Wang *et al*., 2012). Expression of foreign genes remained unaffected and the productive ability of cryopreserved cells containing recombinant proteins was similar to that in non-cryopreserved cultures.

## Looking Ahead for Expanding Applications of Cryopreservation

The future cryobanking activities need a fresh look based on experiences gained by laboratories that are adopting large scale banking. Regular brainstorming with International and national experts at ICAR-NBPGR in recent times contributed to refine and fine tune the concepts, practical approaches to match them with the expectations from the current researches. Laboratories are striving to put in correct perspectives the role of cryopreservation to handle the challenges faced by biodiversity conservation at national and international levels.

The issues needing focus are :

- Role of conventional storage and trends in *ex situ* conservation using cryopreservation techniques;
- Networking approaches – nationally and internationally;
- Best practice, knowledge transfer and infrastructure;
- Need to initiate banking even before techniques are perfected for high survival;
- Competitive basic research to maximize storage success:
    a. Systems biology research to identify suitable markers;
    b. Identifying the model species for basic studies on desiccation tolerance and for freezing tolerance.
- Promoting cryobiology work in the national and international arena through trainings.

## Exploring Wide Applications

Basic research on conservation are challenging as additional data from seed banks emerge and several crops especially wild species and crop wild relatives are left behind in our conservation efforts or show less than expected longevity under -20°C storage. Desiccation sensitive plant materials exist in about 47% of plants of tropical moist forest needing cryobanking. Recalcitrant seeded species, the main targets for cryobanking, may be close to one quarter of the World's higher plants (Walters *et al*., 2013) and hence pose challenge to conservationists in this regard.

### *Aquaculture Cryopreservation*

Sperm cryopreservation, mainly of commercially important fish such as salmonids, carps, sturgeons, catfish, herring, cod and sea bass, has been achieved for several fish species with 10% to 85% post thaw fertility where lesser results are achieved with fresh water species in comparison to marine species. Advances in research on cryopreservation of fish oocytes and embryos are very limited and success is still not much encouraging and needs expansion.

### *Animal Species Cryopreservation Especially Related to Endangered and Threatened Species*

There is increasing interest in maintaining genetic diversity for future conservation of wild endangered animal species. The preservation of valuable breeds using assisted reproductive technologies is limited and is assisted by cryopreservation of biological materials especially gametes from such species. Several animal species classified as threatened, vulnerable and endangered are often difficult to breed both in captivity and under natural conditions and cryobanking assists in restoration of species in large numbers.

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