Methods, Tissues and Species Requiring *In Vitro* Conservation

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Introduction

Conservation of plant biodiversity, in the rapidly depleting scenario of natural resources, has gained global importance for ensuring food security and sustainable agriculture. The decline in plant genetic diversity is attributable to many factors important among those being partial degradation of the natural habitat, destabilization of ecosystem, introduction of alien species and anthropogenic pressure. Consequently, there has been a dramatic increase in the number of threatened species. According to a rough estimate, if the pace of destruction continues at this rate, roughly 60,000 plant species may become extinct or threatened by the middle of this century. The situation is more alarming in the tropical and sub-tropical regions which harbor the richest and most important plant genetic resources. The need of the hour is to safeguard these valuable plant germplasm resources to ensure crop production and, meet the growing environmental challenges and climate change.

The most notable and prevalent approach of conserving plant genetic resources is represented by *ex situ* conservation. The highly specialized facilities termed as genebanks offer the convenience of conserving germplasm as seed in specially designed cold modules or, in the case of vegetatively propagated crops and crops with recalcitrant seeds, as living plants in field genebanks or as tissue cultures in *in vitro* gene banks. Owing to limitations of these methods for long-term conservation, cryopreservation at the temperature of liquid nitrogen (LN) (-196 °C) offers the possibility of long-term conservation of vegetative tissues, pollen or embryos. Cryobanking is already being widely practised for human, animal and microbial resources, but less so for plant species.

Ex situ conservation

The most cost-effective *ex situ* approach of conserving plant germplasm is in the form of seeds. However, many crop species fail to produce seeds or if they do, produce few or recalcitrant (desiccation-sensitive) seeds that cannot be conserved using conventional seed-banking procedures. Moreover, in heterozygous species, there is a need to preserve a larger sample to capture maximum genetic variation within a population which is labor-and cost-intensive. Plants maintained under natural conditions succumb to several environmental stress and also changing government policies and anthropogenic pressures. For many species, basic information regarding reproductive biology is still not adequate.

Thus, for aforementioned group of species, available options include maintenance in field gene banks or conservation of dormant vegetative propagules at low temperature (Reed, 2008). These methods have financial and security limitations for long-term maintenance.

In vitro culture offers an important *ex situ* method of clonal crop germplasm conservation facilitating rapid and pathogen-free multiplication, and maintenance of germplasm away from climatic perturbence (Normah and Reed, 2013). Two basic criteria underlying *in vitro* conservation comprise reduced growth for extended duration and genetic integrity of the conserved material.

In vitro conservation methods offer the following advantages: conservation of clonally propagated crops or species with recalcitrant seeds as also of endangered plants, facilitating establishment of *in vitro* genebanks and cryobanks, and exchange of germplasm across international boundaries.

In vitro conservation methods

Past over four decades have witnessed increasing application of in vitro techniques for conservation of plant genetic resources (Engelman 2011, Reed *et al.* 2013). In vitro clonal multiplication is a pre-requisite for in vitro conservation and it harnesses the inherent potential of a cell to produce the whole organism under more or less defined nutrient conditions in an artificial environment.
The foremost aim of an *in vitro* conservation program is to conserve the clonal material under sterile conditions by maintaining the highest possible standards in a given situation (Reed *et al*., 2004). Cultures may be conserved under normal growing conditions (short-term) or under growth restrictive conditions comprising slow growth; (short-to-medium term) and cryopreservation (-196^{0}C; long-term). The choice of a technique whether for medium-or long-term storage, is dictated largely by the genotype and the available infrastructure. The ultimate aim of *in vitro* storage is minimal maintenance for an extended duration and the regeneration of genetically stable, healthy plants with reasonably good multiplication rate (Reed *et al.* 2013).

Normal growth

This involves cultures maintenance under standard culture room conditions with periodic subculture on fresh medium facilitating virtually indefinite storage of cultures under the realms of constant supply of nutrients and avoidance of accidents. This strategy is widely applicable for naturally slow growing culture systems or for built up of stock cultures or for those for which there is no available method. Several crop species such as alliums, banana, black pepper, ginger, sweet potato, taro, turmeric, yams and several medicinal and aromatic plants are being maintained under culture room conditions in the *in vitro* genebank of ICAR-NBPGR, New Delhi. There are, however, limited reports on use of this strategy for germplasm conservation as cultures are prone to losses due to contamination or mislabeling error, somaclonal variation, loss of regenerative capacity with time etc. As cultures are under normal growing conditions, the chances of stress-induced variations are minimal besides there being no requirement for low temperature facility. Cultures are, however, readily available for multiplication and distribution.

Slow growth

The primary aim of this strategy is to reduce the rate of growth of cultures with the sole aim of extending the subculture duration. Several options are available for slowing down the growth of cultures which can be used alone or in combination:
Reduction in temperature and/or light, inclusion of osmotica or growth retardants in the medium, minimal growth media, modification of gaseous environment, volume and type of culture container, culture tube closure, induction of storage organs, desiccation and encapsulation (Chandel & Pandey,1996).

The most successful of the aforementioned methods has been a combination of low temperature with low light intensity or complete darkness. For temperate species, storage temperature in the range of 0-5^{0}C is suitable, whereas for tropical crops, which are often sensitive to low temperatures, 15-22^{0}C is beneficial. Successful conservation has been reported in a large number of crops including *Allium* spp., apple (*Malus domestica)*, banana, cassava, *Gentiana kurroo,* oil palm, *Pyrus communis*, *Rauvolfia serpentina*, strawberry (*Fragaria*× ananassa) etc. (Chandel and Pandey 1992, see Mandal *et al*.2003).
Use of osmotica like mannitol, sorbitol or sucrose, in combination with low temperature has also been effective in limiting growth of cultures in potato (6^{0}C and 4% mannitol) garlic (4^{0}C and 10% sucrose) and sweet potato (5% sorbitol) (see Normah *et al.* 2013 and references therein). Modification of culture medium by reducing mineral content in combination with low temperature has proved beneficial in *Allium* spp.. Use of growth retardants like cycocel (CCC), maleic hydrazide (MH), n-

dimethyl succinamic acid (DSA) and phosphon-D has proved useful in limited cases. Use of growth retardants is generally avoided as they are known to cause genetic instability.

Among other approaches, lowering of oxygen pressure in the culture container also results in growth reduction. This can be achieved either by controlling atmospheric pressure or decreasing the oxygen concentration inside the vessel. Following this technique, plantlets of *Chrysanthemum* and tobacco could be stored for 6 weeks at 1.3% oxygen, whereas, somatic embryos of oil palm were stored for 4 months at 1% oxygen (see Mandal 1995). Another way of reducing oxygen is to cover the cultures with mineral oil, paraffin oil or liquid medium. Though reported successful for calli of carrot, *Catharanthus* and grapes, it needs to prove its worth particularly for organized culture systems. The success with the use of gas permeable culture vessels such as star packs for storage of cultures, offers the possibility of controlling the gaseous atmosphere of entire incubation chamber which may be more practical than controlling the atmosphere of individual culture containers. This technique though not widely used, can be viewed as a promising one.

The type of culture vessel, volume of the medium and the closures of the culture container also affect the survivability of the cultures. Replacement of cotton plugs with polypropylene caps has been beneficial in prolonging subculture duration in species like *Rauvolfia serpentina*, yams, sweet potato, ginger, turmeric, *Allium* species and banana (see Mandal *et al*. 2000, 2003). Induction of *in vitro* storage organs like bulblets in alliums, micro-rhizhomes in ginger and micro-tubers in potato and sweet potato has been conducive for increasing the storage duration up to one year which is several months more than the average sub-culture period in above crop species (Chandel and Pandey, 1992, Mandal *et al*. 2000).

Desiccation as a storage strategy has been successful in conserving somatic embryos of alfalfa and carrot up to one year. Encapsulation of somatic embryos/shoot tips/axillary buds is also a promising strategy for *in vitro* storage of germplasm. Successful examples include *Morus indica, Santalum album, Picea glauca* and *Valeriana wallichii*. The storage period though limited to few months to one year in above species, still needs to be worked out in greater detail for becoming an effective and promising strategy for germplasm conservation.

The aforementioned approaches though hold great promise in specific cases, need to be worked out in greater detail to be applicable to larger number of species. In several cases, combination of treatments may be more beneficial than an individual strategy for *in vitro* conservation. The responsibility rests with the investigator in terms of a cautious approach for safeguarding the genetic stability of the conserved germplasm.

Cryopreservation

A promising tool for long-term conservation, cryopreservation offers the opportunity of storing vegetatively-propagated plant germplasm at the ultra-low temperature of liquid nitrogen preferably at -196^{0}C, for theoretically indefinite period, in a limited space with minimal maintenance (Engelmann 2000, Reed 2008). Avoidance of lethal, intracellular freezing, which occurs during rapid cooling in liquid nitrogen, leads to successful cryopreservation of biological systems. Plant tissues comprise highly hydrated cells which need to be sufficiently dehydrated to avoid intracellular ice formation and consequent cell death. Cryopreservation can be attempted using classical or new cryopreservation techniques. The former involves freeze-induced dehydration and the latter is based on vitrification.

Classical cryopreservation techniques : This controlled cooling cryopreservation technique involves slow cooling down to a defined prefreezing temperature using a programmable freezer and subsequent immersion in liquid nitrogen. It is technically more demanding and cost-intensive owing to the

requirement of a programmable freezer. Controlled cooling procedure involves : pregrowth of samples, cryoprotection, slow cooling (0.1-2.0^{0}C/min) down to -40^{0}C, rapid plunging in liquid nitrogen, storage, rapid thawing and regrowth. The critical step here is cooling. These techniques have been more successful with dormant buds, cell suspension, calli and shoot apices of temperate species (apple, pear, *Rubus* spp., strawberry). Success with tropical germplasm is limited (Engelmann and Dussert 2013).

New cryopreservation techniques : In the new, vitrification-based cryopreservation protocols, there is elimination of most or all freezable water from samples following exposure to highly concentrated cryoprotectant solutions (7-8M) and/or air desiccation. Intracellular ice formation is thus precluded owing to vitrification of intracellular aqueous compartment. This technique is more suitable for shoot tips, embryos or embryonic axes. This technique is operationally simple and economical due to non-requirement of a programmable freezer. It can thus be widely practiced in tropical countries with at least an established tissue culture facility and a constant source of liquid nitrogen supply.

The critical step to achieve survival in these new techniques is dehydration unlike freezing in classical protocols. Seven different procedures are identified in these new techniques as detailed below:

1) Vitrification (apices, cell suspension, somatic embryos of temperate & tropical species) (successful with 60 species)
2) Encapsulation-dehydration (apices, cell suspension, somatic embryos of temperate & tropical species) (successful with 70 species)
3) Encapsulation-vitrification (apices of carnation, lily, wasabi yams, pineapple) (successful with 12 species)
4) Pregrowth (*Musa* apices; 0.5M sucrose for 3 wks)
5) Desiccation (Zygotic embryos and embryonic axes of non orthodox seed species)
6) Pregrowth desiccation (*Asparagus* nodal segments, shoot apices of cassava , oil-palm somatic embryos, Coconut zygotic embryos)
7) Droplet freezing (meristems of *Asparagus* and yams, shoot tips of potato, *Musa*)

Cryoprotectants : A cryoprotectant is a chemical or a mixture of chemicals which protect tissues under conditions of exposure to ultra low temperatures. A variety of compounds (sucrose, glucose, proline, mannitol, glycerol, sorbitol, trehalose, polyethylene glycol, ethylene glycol) may be used to cryoprotect plant tissues. Vitrification solutions contain two or more of the above-mentioned components and applied either at 0^{0}C or at 25^{0}C and at greater concentrations (15-30% (v/v)or (w/v). The additives are generally toxic at these concentrations hence duration of exposure needs to be taken care of.

Genetic stability of *in vitro* conserved germplasm

In vitro slow growth and cryopreservation employ tissue culture as a tool which is prone to the incidence of genetic instability despite taking utmost care with respect to choice of explant for regeneration (meristems/shoot tips), mode of regeneration (direct or adventitious) and usage of growth regulators (type and concentration). It is thus mandatory to verify the genetic stability of in vitro slow-grown and cryopreserved-regrown plant germplasm. According to Engelmann and Dussert (2013), there are no report of changes at the phenotypical, biochemical, cytological or molecular level following cryopreservation.

***In vitro* and cryogenebank at ICAR-NBPGR**

In vitro genebanks are a valuable adjunct to the field genebank besides being a source of explants or stock cultures for a cryogenebank. Significant progress has been made the world over regarding application of *in vitro* slow growth and cryopreservation protocols for conservation of valuable

germplasm of vegetatively propagated crops. Tissue Culture and Cryopreservation Unit (TCCU) (erstwhile NFPTCR) representing the unique multi crop repository at ICAR-NBPGR, New Delhi was established with the funding support from Department of Biotechnology (DBT), Government of India in 1986. Over the years, research work carried out on a range of economically important, vegetatively propagated crop species has led to the conservation of a total of 1,821 accessions *in vitro*, by periodic subculture in the form of 39,000 cultures and/or *in vitro* cryopreserved meristems/shoot tips in the *In Vitro* Active Genebank (IVAG)(Table 1) or *In Vitro* Base Genebank (IVBG) (Table 2), respectively. These accessions comprise horticultural crops like fruit, tuber, bulb, spices, plantation, industrial crops, and medicinal, aromatic and rare/endangered plants. The cultures have been conserved under culture room conditions (25^0C temperature, 16/8 h photoperiod regime) or at low temperature (4-10^0C, in dark). The average subculture period under these conditions variesfrom 2-24 months, depending on the species/genotype and the conservation strategy employed. This represents a unique multi crop repository in the world as various tropical and temperate crops are being conserved under one roof.

Table1. **Status of *in vitro*-conserved germplasm in** IVAG at ICAR-NBPGR

Crop group	Genera (no.)	Species (no.)	Cultures (no.)	Accessions (no.)	Major collections (no. of accessions)
Tropical fruits (banana)	1	14	10,000	**420**	*Musa* spp. (420)
Temperate and minor fruits (apple, apricot, blackberry, blueberry, pear, strawberry)	10	42	8,000	**330**	*Actinidia* spp. (6), *Aegle marmelos* (2), *Artocarpous lakoocha* (1), *Fragaria* x *ananasa* (81), *Malus domestica* (23), *Morus* spp. (61), *Prunus* spp. (5), *Pyrus communis* (68), *Rubus* spp. (62), *Vaccinium* spp. (21)
Tuber crops (sweet potato, taro, yam)	5	14	6,000	**518**	*Alocasia indica* (4), *Colocasia esculenta* (90), *Dioscorea* spp. (154), *Ipomoea batatas* (260), *Xanthosoma sagittifolium* (10)
Bulbous and other crops (garlic, gladiolus)	4	14	3,500	**171**	*Allium* spp. (157), *Dahlia* sp. (6), *Gladiolus* sp. (7)
Medicinal and aromatic plants	23	30	4,000	**155**	*Coleus forskohlii* (14), *Plumbago zeylanica* (19), *Rauvolfia serpentina* (13), *Tylophora indica* (10), *Valeriana wallichii* (16)
Spices and industrial crops (ginger, turmeric, pepper, cardamom, vanilla, hops, jojoba)	8	20	6,500	**227**	*Curcuma* spp. (110), *Elettaria cardamomum* (5), *Humulus lupulus* (8) *Piper* spp. (7), *Simmondsia chinensis* (12), *Vanila planifolia* (4), *Zingiber* spp. (80)
TOTAL	**52**	**134**	**39,000**	**1,821**	

Conclusion

In vitro conservation and cryopreservation techniques provide alternative and complimentary conservation strategies for maintenance and sustainable utilization of plant genetic diversity. In vitro slow growth techniques including cryopreservation have been attempted in a fairly large number of crop species yet future challenges concern optimizing cryopreservation protocols not only for species with recalcitrant seeds but also for species which have irregular reproductive cycles or are prone to losses in their natural environment. Conservation of plant genetic diversity will greatly benefit from the timely integration of *in vitro* and cryoconservation technologies with traditional conservation practices. Several cryobanks for vegetatively propagated plant species have already been established yet, their application is limited to only a few laboratories. New vitrification-based techniques though operationally convenient need large scale experimentation in tropical countries which house majority of important, problem species. Future research should be aimed at developing simplified protocols, applicable across genotypes of a particular crop germplasm. It is expected that coming years would witness increased understanding of mechanisms involved in desiccation tolerance with cryopreservation being put to routine application in large number of tropical species.

Table 2. Status of *in vitro*-conserved germplasm in IVBG at ICAR-NBPGR

Crop/Species	No. of accessions	Technique(s)*	Explant (s)#
Allium sativum	68	V, DV	ST
A. chinense	3	V, DV	ST
A. hookeri	1	V, DV	ST
A. tuberosum	3	V, DV	ST
A. ramosum	1	V, DV	ST
Dioscorea bulbifera	2	V	ST
D. deltoidea	5	V	ST
Musa spp.	65	DV, V	SM, ECS
M. acuminata	5	DV	SM
M. balbisiana	5	DV	SM, ZE
M. textilis	1	DV	SM
Rubus hybrid	6	ED	ST
Vaccinium ovatum	5	ED	ST
TOTAL	**170**		

*DV= droplet vitrification; V=Vitrification; ED=Encapsulation-dehydration; EV= Encapsulation-vitrifcation

ST=shoot tip; SM=shoot meristem; ZE=Zygotic embryo;

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