CROP IMPROVEMENT

Long-term Conservation of Horticultural Species-Current Status and Potential Uses

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Introduction

National Cryogenebank at National Bureau of Plant Genetic Resources (NBPGR) New Delhi has been conserving diverse germplasm in the form of various explants like seeds, embryos, embryonic axes, pollen grains, dormant buds, meristems and shoot apices from both sexually and asexually propagated species. Species producing non-orthodox (intermediate and recalcitrant) seeds having sizable indigenous diversity which mainly belong to horticultural species are the first priority. Threatened and endangered plant species, wild and weedy relatives of crop plants, medicinal and aromatic plants, core collections and released varieties are the other priorities. Various techniques have been experimented and recovery of explants optimised after which the base before a base collection is established for long-term banking.

Methodology of Crystorage

Transport and handling

Whole fruits of non-orthodox seed species were transported to the laboratory by courier in moisture-retaining bags. Seeds were extracted on the day of experimentation and within a week processed for storage. For short term storage seeds were treated with fungicides and stored at temperatures between 15 to 20 ^{o}C.

Whole seeds and embryonic axes cryostorage

Moisture content and viability determination: Moisture content (MC) of seeds and embryonic axes (EA) was determined gravimetrically using low constant temperature oven method (ISTA, 1985; Malik and Chaudhury, 2010) and germination was assessed using moist filter paper/ peat moss/ perlite or in rolled paper. The embryonic axes, controls and after treatments, were cultured *in vitro*.

Air desiccation-freezing: Seeds were desiccated to between 5-7% MC over silica in desiccators. EA of non-orthodox seed species were desiccated to between 11-

18% MC in laminar air-flow depending on the desiccation sensitivity. While handling almond and walnut, the nuts were cracked to collect the seeds (kernels). Germplasm accessions which showed satisfactory germination were further processed for cryostorage.

Freezing: The processing followed for seeds and embryonic axes of *Citrus* spp., *Prunus armeniaca*, *P. amygdalus*, *Jatropha curcas*, *Pongamia pinnata*, *Juglans regia* and *Azadirachta indica* was as reported earlier (Chaudhury and Chandel, 1995 a&b; Chaudhury and Malik, 2006; Malik and Chaudhury, 2010; Malik *et al*., 2010).

Vitrification: For vitrification experiments, procedure described in Malik *et al*. (2012) was followed. Embryonic axes were precultured followed by PVS_2 vitrification. Cryovials were thawed after at least 24h at 38 °C for 5 min. and cultured suitably.

Encapsulation-Dehydration: Following the procedure described in Malik *et al*. (2012) encapsulated embryonic axes were pre-cultured (in 0.3 M, 0.5 M, or 0.75 M sucrose solutions). Beads were dehydrated for 6 h before LN fast freezing. The cryovials were thawed and axes cultured in vitro.

Containers for storage: Polypropylene cryovials of 2 ml capacity were used for packing small seeds and EA. Cryovials of capacity 5 or 50 ml were packed with larger seeds and excised buds and largest seeds and bud twigs in polyolefin tubings.

Thawing and Assessment of Recovery

The frozen seeds, embryos and embryonic axes were later transferred to large capacity (1000 litres capacity) cryotanks MVE model XLC 1830 at -160 to -180 °C temperature in the vapour phase of liquid nitrogen. For long-term storage 10 cryovials each with 8-10 embryos and 10-15 axes for each accession were maintained for facilitating retrieval of part material at regular intervals for periodic viability testing. Recovery growth and survival was recorded on the first testing after 48h of storage followed by years (2 to 24 years). Thawing was done in a water bath at +37 °C for 5 min. and explants regenerated by the method used before cryopreservation. Data was recorded after about 3 months in culture. Efforts were made to transfer healthy plantlets to pots.

Cryostorage

Pollen

The mango and litchi pollen were collected using cyclohexane method as detailed in Tandon *et al*. (2007) and Chaudhury *et al.* (2010). Pollen samples for rest of the species were collected dry from field and desiccated suitably before packing in cryovials and storing at –196 °C. After 24 hrs the cryovials were shifted to the vapor phase of LN. At regular intervals samples were thawed by keeping for 30 min. at room temperature prior to a viability test reported earlier.

Dormant buds

For mulberry, almond, walnut, cherry, salix and apricot twigs containing dormant winter buds were harvested during peak winters (December to February) from field genebanks

at Srinagar, Shimla and Hosur and airlifted to cryolab at New Delhi. Buds were processed as per procedure of 2-step freezing detailed in Rao *et al*. (2007; 2009).

For vitrification, pre-cultured buds were treated with PVS_2 for periods ranging from 20 to 120 min at 25-30 °C. After 24 h storage the vials were re-warmed in 38 °C for 5 min and PVS_2 replaced with unloading solution before culturing. Encapsulation was done for pre-cultured buds and dehydrated in MS medium supplemented with sucrose for 17-24 h. Beads were desiccated for 4-5 h to achieve MC% between 20-25% and fast frozen for 24 h. After thawing, the buds before culturing *in vitro* or grafting were rehydrated in sterile moist moss grass for 2-4 h at room temperature in mulberry and upto 6 days at 5 °C for others.

Cryobanking

Orthodox Seeds

Initiation of cryobanking of prioritized orthodox seeds as a pilot project in 1986 has led to a fullfledged cryogenebank presently conserving 10, 230 accs (Anonymous, 2014). Vegetables and medicinal seed species belonging to orthodox category have been cryoconserved (Table 1). Several tree species and wild species producing orthodox seeds have been cryostored to obviate need for their regeneration as required when stored at -20 °C Seed genebank. Table 2 depicts the retesting data generated for seeds of vegetables, medicinal narcotics and dyes for several years. Original viability values has been ensured as tested maximum upto 20 years of cryostorage.

Using the cryoprotocols developed, the collection of more than 438 accessions of 23 genera representing wide genetic diversity in vegetables, including varieties and wild species, has been cryoconserved at NBPGR's National Cryogenebank in the form of seeds. In addition seeds of legume vegetables comprising 34 accessions have so far been cryostored. Pollen of *Cucumis sativus*, wild species of *C. hardwikii* and of *Abelmoschus* namely *A callei*, *A manihot*, *A pungens* etc. have been

Table 1: Status of horticultural germplasm at National Cryobank, NBPGR, New Delhi

Category	Accessions (No.)
Non-orthodox (Recalcitrant & Intermediate)	
Fruits & Nuts	2972
Spices & Condiments	151
Plantation Crops	22
Agro-forestry, Industrial & medicinal crops	2971
Total	**6116**
Orthodox	
Vegetables	438
Medicinal crops	946
Dormant buds (mulberry)	387
Pollen grains (various horticultural crops-mango, vigna, etc.)	466
Total	8353

cryostored for use by breeders in crossing programmes (unpublished). The dried seeds of orthodox species have been reported to be survive LN exposure (Stanwood and Roos, 1979; Salomao, 2002) stored for long periods at -196 ^{O}C leading to successful banking. It is, however, essential to retest the seed viability after regular intervals from cryobank to study any differences in seed longevity between species. There are reports of variability in the extent of deterioration among species and accessions within a species (Walters *et al.*, 2004). In our cryolab data for more and more species is being generated.

Non-orthodox Seeds

For non-orthodox seeds seed storage behaviour and the developmental stage with maximum tolerance to desiccation was ascertained and chosen (Chaudhury and Malik, 2000) and 30 species of indigenous tropical and temperate species has been categorized (Malik *et al.*, 2010) (Table 3). Recalcitrant seeds at various developmental stages showed varying degree of tolerance to desiccation. This also varied among species, *e.g.* immature/partially mature embryos of jackfruit and litchi were been found to be more adaptable to manipulation than mature embryos/embryonic axes (Chaudhury and Malik, 2004). Rapid and careful handling, vitrification, encapsulation and use of EA were found effective in cryopreservation for 27 different *Citrus* spp. Vitrification and encapsulation were attempted in embryonic axes of *Artocarpus heterophyllus*, *Litchi chinensis*, *Poncirus trifoliata* and *Citrus* species (Chaudhury and Malik, 2000) and in other labs in mango (Huang, 2000) and jackfruit (Thammasiri, 1999) with varying success.

The air desiccation-freezing of EA and seeds using fast freezing was found optimum in almond (Chaudhury and Chandel, 1995b), tea (Chaudhury *et al.,* 1991) and whole seeds of intermediate seed species of black pepper (Chaudhury and Chandel, 1994), cardamom (Chaudhury and Chandel, 1995a), apricot (Malik and Chaudhury, 2010a).

Extensive work on cryobanking of tropical underutilised fruit species (Malik *et al*., 2011) have been attempted. Genetic diversity of highly nutritious and rare underutilized fruits commonly used as vegetables in several parts of India such as *Capparis decidua* (*Ker*), *Carissa* species (*Karonda*), *Cordia* species (*Lasora*), *Emblica offcinalis* (*Aonla*) and *Moringa oleifera* (drumstick) have been conserved in the Cryogenebank at NBPGR (Malik *et al*., 2011). Detailed experiments have been conducted at NBPGR on more than 20 indigenous *Citrus* spp. and seed and EA have been successfully cryobanked (Malik *et al*., 2010b). Explants subjected to air-desiccation freezing, vitrification and encapsulation showed success to different levels (Table 4) (Malik and Chaudhury, 2006, Malik *et al*., 2012). The base collection of more than 12 indigenous species of fruits, nuts, agroforestry, plantation crops and spices has been cryoconserved (Table 5). In all a total of 6,116 accessions of difficult-to-store horticultural non-orthodox seed species have so far been cryostored (Table 1).

Table 2: Retesting of orthodox horticultural seed spp. cryobanked for various periods at NBPGR

Crop	Genera	Species	No. of Acc.	Viability (%)	Years of cryostorage
Vegetables	7	9	46	60-100	7-25
Medicinal, narcotics, & dyes plants	4	5	7	80-100	3-23
Total	11	14	53		

Table 3: Seed storage behaviour and storage life of seeds in fruit species investigated at NBPGR, New Delhi

Species	Storage in months till 50% *via* at RT, unless defined as days	Max survival (%) to LN exposure	Seed storage behaviour
Artocarpus heterophyllus	20 (days)	30	Recalcitrant
Aegle marmelos	24	85	Intermediate
Annona squamosa	6	90	Orthodox
Buchanania lanzan	5	75	Intermediate
Capparis decidua	6	80	Intermediate
Carissa congesta	3	90	Intermediate
Cordia myxa	6	90-100	Intermediate
Grewia asiatica	12	80-90	Intermediate
Garcinia cambogia	10 (days)	0	Recalcitrant
G. indica	10 (days)	0	Recalcitrant
G. xanthochymus	10 (days)	0	Recalcitrant
Litchi chinensis	3 (days)	15-20	Recalcitrant
Madhuca indica	10 (days)	0	Recalcitrant
Manilkara hexandra	4	65-75	Intermediate
Mimusops elengi	10	70-85	Intermediate
Phyllanthus emblica	10	100	Orthodox
Pithocelobium dulce	14	100	Intermediate
Poncirus trifoliata	10 (days)	75-80	Recalcitrant
Salvadora oleoides	10 (days)	40-85	Intermediate
Tamarindus indicus	18	100	Orthodox
Zizyphus mauritiana	24	80-85	Orthodox

Cryoconserved samples have been randomly tested upto 10 years and retention of original viability observed. *In vitro* recovery methods with direct growth after cryostorage have been standardised for tea, almond, citrus species (more than 20 species), (Malik *et al*., 2012), walnut, etc. In *Salvadora* sp. *in vitro* recovery of embryonic axes excised from cryostored seeds has been found essential as the cotyledons and endocarp were found to impede the growth of viable embryonic axes. There are several other non-orthodox seeded species which needs investigation for their long-term conservation (Table 6).

Pollen

In vitro germination and Fluorochromatic Reaction (FCR) test of cryostored pollen was used for testing pollen viability in different species. *In vitro* germination provided

Table 4: Seed storage behaviour and successful cryopreservation of *Citrus* species undertaken at Cryogenebank, NBPGR, New Delhi

Species	Seed storage behaviour	Explant stored	Method of cryostorage	Recovery (%)	Total accession(s)
C. reticulata	Intermediate	SeedEA	SDFFDFF, ED, VT	93.375,80,70	53
C. sinensis	Intermediate	SeedEA	SDFFDFF, ED, VT	7090,80,90	75
C. aurantifolia	Intermediate	SeedEA	SDFFDFF, VT	5557,67	51
C. limon Intermediate	Seed	SDFF	88	55	
C. medica	Intermediate	SeedEA	SDFFDFF, VT	8085,77	32
C. grandis	Intermediate	SeedEA	SDFFDFF, ED, VT	8595,100,90	60
C. paradisi	Intermediate	SeedEA	SDFFDFF, ED, VT	8875,100	18
C. jambhiri	Recalcitrant	SeedEA	SDFFDFF, ED, VT	68.796,100,90	62
C. karna Recalcitrant	SeedEA	SDFFDFF, ED, VT	60.573,70,70	19	
C. latipes	Intermediate	SeedEA	SDFFDFF, ED, VT	6064, 45,77	2
C. macroptera	Intermediate	SeedEA	SDFFDFF, ED, VT	7087,62,92	7
C. indica Intermediate	SeedEA	SDFFDFF, ED, VT	8690,87,90	27	
C. aurantium	Intermediate	Seed	SDFF	90	10
C. limetta	Intermediate	Seed	SDFF	80	6
C. limettioides	Intermediate	Seed	SDFF	80	5
C. limonia	Intermediate	Seed	SDFF	85.6	26
C. pseudolimon	Intermediate	Seed	SDFF	74	10
C. ambelycarpa	Intermediate	Seed	SDFF	90	1
C.maderaspantna	Intermediate	Seed	SDFF	50	1
C. madurensis	Intermediate	Seed	SDFF	52	4

(Contd).

Species	Seed storage behaviour	Explant stored	Method of cryostorage	Recovery (%)	Total accession(s)
C. myritifoila	Intermediate	Seed	SDFF	60	2
C. pectinifera	Intermediate	Seed	SDFF	56.6	5
C. reshni Intermediate	Seed	SDFF	87.5	10	
C. regulosa	Intermediate	Seed	SDFF	71	2
C. samperflorens	Intermediate	Seed	SDFF	70	2
C. taiwanica	Intermediate	Seed	SDFF	90	3
C. tangerina	Intermediate	Seed	SDFF	90	3
Citrus spp.	Intermediate	Seed	SDFF	74.8	115
Poncirus trifoliata hybrid	Intermediate	SeedEA	SDFFDFF	88.370	5

EA = Embryonic axes; SDFF = Silica drying followed by fast freezing; DFF = Desiccation in sterile conditions followed by fast freezing; ED = Encapsulation-Dehydration; VT = Vitrification

Table 5: The germplasm of horticultural non-orthodox seed species conserved as base collection in National Cryogenebank at NBPGR

Species	Explant conserved	Accss (no)	Type of variability
Citrus spp.	Embryos & EA	<600	Mainly from NE Indian regions
P. armeniaca	Seeds & EA	<400	Collections from Himalayan regions
Prunus amygdalus	EA	<200	Belonging to bitter, sweet, exotic, released varieties and local types
Juglans regia	EA	<140	Diverse variability from Himalayan regions
Manilkara hexandra	Seeds & EA	<60	Variability from Western and Central India
Salvadora oleoides andS. persica	Seeds	<40	Variability from dry areas of Western India

a reliable simple method. Fertilizing ability was also conducted in mango pollen and fruit set quantified (Chaudhury *et al*., 2010). Pollen of mainly recalcitrant seeded species of horticultural crops and wild species of more than 466 accessions have been successfully cryostored (Table 1). Viability has been tested after cryostorage for utilization in breeding programmes (Table 7).

Dormant Buds

Cryostorage of mulberry species like *Morus indica*, *M. latifolia*, *M. serrata*, *M. laevigata*, *M. australis*, *M. bombycis*, *M. alba*, *M. sinensis*, *M multicaulis* and *M. rotundiloba* from sub-tropical conditions of less cold-hardy types was made for the first time and success in survival after exposure to LN was achieved for 329 accessions (Rao *et al*., 2007). Regeneration of cryopreserved mulberry germplasm accessions after 1-3 years of storage indicated no survival loss and genetic instability and 33-40% of the accessions showed viability above 40% and upto maximum of 100% (Choudhary *et al*., 2013). Encapsulation was attempted in *M. indica* with survival of 40%, 52% and 74% for buds desiccated with 0.3 M, 0.5 M and 0.75 M sucrose, respectively.

Winter dormant buds of walnut, almond and apricot were tested for degree of cold hardiness during peak winters using physiological markers. Following cryostorage the effect of rate of thawing, rehydration for different durations and dark incubation for several days was studied. In *Juglans regia* success in cryostorage was evident in 2 out of 8 accessions with 40-50% survival *in vitro* using step-wise freezing. In

Table 6: Horticultural species reported to exhibit non-orthodox seed storage behaviour which need efforts for long-term conservation

Common name	Botanical name
Arecanut	*Areca catechu*
Banana (wild)	*Musa* spp.
Black pepper	*Piper nigrum*
Cardamom	*Elettaria cardamomum*
Citrus	*Citrus* spp.
Cocoa	*Theobroma cacao*
Coffee	*Coffea arabica*
Coconut	*Cocos nucifera*
Hazel nut	*Corylus avellana*
Jamun	*Syzygium cuminii*
Longan	*Euphoria longan*
Mango	*Mangifera indica*
Papaya	*Carica papaya*
Pecan nut	*Carya illinoensis*
Rambutan	*Nephelium lappaceum*
Sapota	*Achras zapota*
Walnut	*Juglans regia*

Table 7: Two different viability tests for fresh and cryostored pollen of different horticultural species at NBPGR, New Delhi

Species	Cultivars/varieties	Viability percentage			
		IVG test (Values in parenthesis are years of cryostorage)		FCR test (Values in parenthesis are years of cryostorage)	
		BC	AC	BC	AC
Elaeis guineensis		52	49 (**8**)	60	54.0 (**8**)
Mangifera indica	Amrapali	-	-	85.5	87.0 (**4**)
	Bombay Green	-	-	55.0	56.8 (**4**)
	Neelum	-	-	75.0	70.6 (**4**)
	Bangalora	-	-	71.7	73.2 (**4**)
	Kishan Bhog	-	-	82.5	80.0 (**4**)
Litchi chinensis	CHES-6	28	31.5 (4)	69.9	72.2 (4)
	China	25	25.5 (4)	48.7	46.5 (4)
	Kasba	30.5	27.7 (4)	58.7	60.0 (4)
Abelmoschus esculentus	-	75.6	45.5 (1)	82.3	51.7 (1)
Cucumis sativus		30	25 (6 month)	-	-

IVG = In vitro germination; FCR = Fluorochromatic reaction; BC = Before cryostorage; AC = After cryostorage; Values in parenthesis denote year of cryostorage; mon = Months of storage

encapsulation method survival *in vitro* of 30% and 20% was observed for beads dehydrated with 0.3 M and 0.75 M sucrose. In vitrification, success, *in vitro* of 23% and 10% was apparent for PVS2 treatments of 40 and 60 min, respectively. In almond survival was observed using encapsulation and vitrification (Choudhary *et al*., 2014).

In vitro cryopreservation

In vitro cultures comprising shoot tips, meristems, somatic embryos and cells contain high amounts of cellular water and are, thus, highly sensitive to freezing injury. Some defined regions of the meristematic zone, due to relatively small size, low vacuolation and high nucleo-cytoplasmic ratio, make them withstand desiccation and freezing to a high extent. Vitrification has been successful with shoot tips/meristems of garlic, yams, cassava and *Solanum* spp., while encapsulation-dehydration has been reported successful for cryopreservation of potato, grapes, pear, sweet potato and yams. Droplet freezing has been applied widely with success in potato (Mix-Wagner *et al*., 2003). Successful cryopreservation of shoot apices, shoot tips etc has been achieved in several species at NBPGR (Table 8).

In vitro cultures of tuber crops mainly of sweet potato, yams and taro totalling 619 accs have been conserved in the *in vitro* Repository for last more than 20 years. Various cultivated and wild species of *Allium* are being maintained as *in vitro* cultures and cryopreservation to achieve short-medium and long term conservation and more than 171 accessions are presently being maintained in the *in vitro* repository (Anon, 2014). *Musa* species are yet another group being experimented for *in vitro* cryobanking with good success (Agrawal *et al*., 2011). Efforts are pursued to conserve

Table 8: Development of cryoprotocols for vegetatively propagated vegetables biodiversity at NBPGR, New Delhi

Species	Explant	Methodology	Reference
Ipomoea batatas	Somatic embryo & embryonic callus		Ahuja *et al*. (2001) Dixit *et al*. (2003)
Dioscorea deltoidea	Shoot tip	Encapsulation-dehydration Vitrification	Chaudhury *et al*. (2001) Anonymous, (2010)
Dioscorea rotundata	Shoot tip	Encapsulation-dehydration	Mandal, 2000
Dioscorea alata	Shoot tip	Encapsulation-dehydration and vitrification	Mandal and Dixit (2000)
Dioscorea bulbifera	Somatic embryo & embryogenic callus	Encapsulation-dehydration Vitrification	Mandal *et al*. (1999) Mukherjee *et al*. (2009)
Dioscorea floribunda	Shoot tip, somatic embryo & embry-ogenic callus	Encapsulation-dehydration	Mandal and Ahuja-Ghosh (2007)
Allium spp.	Shoot tip, Shoot bases	Encapsulation-dehydration, Vitrification	Unpublished data (Pandey on *A. sativum* 2010)

sizable variability of additional species and to cryostore these accessions as meristems and shoot tips. This will complement the conservation in the field genebank where they are difficult to maintain due to various biotic and abiotic stresses.

Potential Uses

Seed Conservation Needs and Advantages

There is vast diversity of tropical fruits in Asia comprising more than 500 species of edible tropical fruits. Conservation efforts for these have been hastened due to their importance and threat perception. However, efforts are slow as they generally possess non-orthodox nature emphasising *in situ* and *ex situ* conservation in field genebanks, *in vitro* conservation and cryopreservation. Conservation of germplasm in the form of seeds for underutilised fruits that are predominantly cross pollinated, only ensures genepool conservation. As most of the species are wild or semi-wild and propagated through seeds in nature, seed storage behaviour needs investigation before storage. The presence of high degree of polyembryony in many *Citrus* species provides the opportunity to conserve the original genotype as seed despite high level of heterozygosity.

Non-orthodox Seed Storage Behaviour

These difficult-to-store non-orthodox (intermediate and recalcitrant) seeds are subjected to high humidity during seed development, maturation and at harvest. Due to these conditions seeds do not undergo maturation drying as a final, pre-shedding development phase. Short-, medium- and long-term seed storage can only be recommended when seed storage behaviour and developmental stage, when the seeds exhibit tolerance to desiccation, is known (Chaudhury and Malik, 2004). The highly recalcitrant (e.g. mango, litchi, jackfruit, *mahua*, *jamun*, mangosteen etc.), moderately recalcitrant/ intermediate (papaya, several *Citrus* species, black pepper, banana etc.) and weakly recalcitrant (some *Citrus* species, *bael*, custard apple etc.) species need careful investigations (Normah *et al*., 2013).

Explants and Techniques

Longevity of intermediate seeds ranges from few months to years while recalcitrant seeds from weeks to months. Intermediate and recalcitrant seeds are desiccation intolerant and lose viability after being dried below a critical limit usually between 12-30% moisture (Chaudhury *et al.,* 1990; Chandel *et al.,* 1995). The germplasm of all highly recalcitrant and few intermediate species cannot be stored as whole seeds, because of large size. Hence, techniques for conserving excised embryos and embryonic axes (EA) as an alternative method have been devised. Protocols for handling, dehydration method and explants used have been developed in important species. Rapid and careful handling, vitrification, encapsulation and use of embryonic axis have been found effective in cryopreservation for tea, black pepper, cardamom, almond, citrus, trifoliate orange and other tropical fruit species. Embryos and EA, being deep seated were not found invariably to carry infections and hence only whole seeds are sterilized. The simplest cryotechnique used has been the excision of embryonic axes aseptically, desiccation to around 11-16%

moisture level using air drying in laminar air flow and desiccation of whole seeds over silica gel and exposing both the explants to LN by direct plunging.

Cryobanking at NBPGR

Infrastructure

With proven wide applicability of cryotechniques in plant sciences, diverse germplasm of national and international importance has been cryoconserved at National Cryogenebank at NBPGR. Over a span of 26 years cryoprotocols have been developed, in most cases on species-specific basis, using explants like seeds, embryos, embryonic axes, pollen, dormant buds and vegetative meristems/shoot tips of these difficult-to-store species. In view of large storage capacity of National Cryogenebank at NBPGR to hold quarter million samples (1 ml capacity cryovials), presently it holds about 42,000 containers (cryovials of 2, 5, 50 ml capacity, and polyolefin tubings of 2 different diameters) storing more than 10,000 accessions belonging to more than 740 plant species. Using the cryoprotocols developed, the base collection of more than 12 indigenous species representing wide genetic diversity of fruits, nuts, agroforestry, plantation crops and spices has been cryoconserved at NBPGR's National Cryogenebank. Basic cryobiological investigations, a strong component of this programme, to elucidate the biochemical, biophysical and ultrastructural basis of desiccation and freezing sensitivity is being pursued. Cryoconserved samples have been randomly tested upto 25 years. The aim had been to retrieve accessions, 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. Accessions have shown retention of original viability values.

Recovery and Regeneration

Cryobanking of diverse germplasm of 400 species in the form of embryos, embryonic axes, dormant buds etc necessitated the *in vitro* regeneration into healthy plants before and after storage. After cryostorage for various periods, the *in vitro* recovery methods mainly using MS medium with various hormone combinations have been standardised for tea, almond, citrus species (more than 20 spp.), neem, walnut, etc. the explants are required to be placed in optimal conditions to trigger rapid and direct growth. The axes of *Citrus* sp., walnut, apricot, *pilu* and almond could be recovered by culturing on medium defined by Chin *et al.* (1988) containing Murashige and Skoog's macro and microelements, 0.17 g l^{-1} NaH_2PO_4, vitamins, iron, 1 mg l^{-1} each of benzyl aminopurine and naphthalene acetic acid and 2 g l^{-1} activated charcoal. Axes that formed a well-defined root and shoot were considered viable. In temperate species like walnut and apricot, this method obviates the need to subject the seed materials to pre-chilling for several weeks. The cultures are usually placed in the dark or under reduced light for several days to reduce deteriorative photo-oxidation phenomena. After few days or weeks the cultures are brought back to standard culture conditions. In addition, before culturing, rehydration of desiccated explants by different methods is resorted to avoid any imbibition injuries. Modifications, even when minor, in the *in vitro* culture conditions have led to improvement in the recovery rate for several species.

Alternative methods for highly recalcitrant species not amenable to cryostorage

Rapid regeneration of plantlets *via* adventitious bud differentiation in three *Garcinia* species and *Calophyllum* sp. has been attempted for *in vitro* establishment and multiplication of selected superior clones for obtaining true-to-type plants. Attempts for cryobanking of meristems from these *in vitro* plantlets are underway. In addition, rare and endangered species like *C. indica, C. macroptera* and *C. megaloxycarpa* are being attempted for cryobanking using meristems of *in vitro* raised plantlets.

Cost of cryobanking

Based on liquid nitrogen and other associated expenditures, the approximate cost of cryobanking are estimated to be:

- Cost per cryovial (1 ml capacity) - ₹ 6/- per year (App. 10 cents)
- Cost per accession contained in 8 cryovials - ₹ 48/- per year (App. 0.8 US $)

Other costs which would be needed to be added are:

- Long term costs based on retesting schedules, working personnel, etc.
- Replenishment of LN Cryotanks after their long life of approx 40-70 years

As per our experience and literature from other International Institutes, cryobanking is the cheapest method of conservation when compared to Seed genebanks, *In vitro* Repository and Field genebank.

Important world cryobanks and HRD

In India cryobanking is being carried out at IIHR, Bengaluru, CPCRI, Kasaragod, JNTBGRI, Palode, Thiruvanathapuram, IISR, Kozhikode, and CPRI, Shimla. World Cryogenebanks holding important horticultural species in sizeable numbers are i) National Center for Genetic Resources Preservation (NCGRP), Fort Collins, Colorado, USA ii) Association Foret Cellulose (AFOCEL), France iii) National Institute of Agrobiological Resources (NIAR), Japan iv) Institute of Plant Genetics and Crop Plant Research (IPK), Germany. National Cryogenebank is aiming to develop strong linkages with Field genebanks of horticultural Institutes and NBPGR Regional Stations to prioritise conservation. Tissue Culture and Cryopreservation Unit at NBPGR has already been designated as "**Centre of Excellence"** for imparting International training in the field of *in vitro* and cryopreservation where so far more than 100 trainees have been trained from more than 15 countries.

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