Implementing Cryotechniques for Plant Germplasm: Storing Seeds, Embryonic Axes, Pollen and Dormant Buds

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Abstract

National Cryogenebank at National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India developed, over a span of 26 years, species-specific cryoprotocols using seeds, embryos, embryonic axes and pollen of difficult-to-store species belonging to non-orthodox seed species and dormant buds of temperate fruits and nuts. Cryopreservation protocols were standardized primarily for tropical species of horticultural, plantation, agroforestry and industrial importance. Desiccation-freezing, vitrification and encapsulation-dehydration and classical techniques of step-wise slow freezing were applied to range of germplasm resulting in base collection of several indigenous species representing wide genetic diversity. More than 6000 accessions of difficult-to-store non-orthodox seed species, 3200 accessions of orthodox seeds, 387 accessions of buds and 404 accessions of pollen have so far been cryostored. The intermediate and recalcitrant seeds and dormant buds were investigated for their morphological and physiological parameters especially for desiccation and freezing tolerance. For more than 250 accessions of 48 species tested, original viability of explants was found to be retained for various periods, maximum being 25 years of cryostorage. Degree to which low temperature prolonged life span depends on how seeds are handled before cryostorage. High survival and healthy plantlets have been obtained from species with intermediate seed storage behaviour and dormant buds of *Morus* species. Improvement in recovery and regrowth was achieved by manipulating maturity stage of explant, duration and rate of desiccation and freezing and also by improving recovery media. Efforts are continued to conserve sizable variability of additional indigenous and exotic species.

INTRODUCTION

Germplasm conservation at National Cryogenebank, National Bureau of Plant Genetic Resources (NBPGR) has been prioritised based on the national and institutional needs. The Cryogenebank conserves diverse germplasm in the form of various explants from both sexually and asexually propagated species for effective management of species producing intermediate and recalcitrant seeds having sizable indigenous diversity. Threatened and endangered plant species with critically small population size, wild and weedy relatives of crop plants, registered germplasm, exotics, medicinal and aromatic plants, core collections and released cultivars are the other priorities for cryobanking. Cryopreserved explants include seeds, embryos, embryonic axes, pollen grains, dormant buds, meristems and shoot apices. Various techniques have been experimented and recovery of explants optimised before a base collection is established for long-term banking.

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MATERIALS AND METHODS

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 quickly processed for storage within a week of extraction. For storing them briefly, seeds were treated with fungicides and stored at temperatures between 15 to 20°C. Seed morphology was studied to decide the most appropriate explant (whole seed/embryo/embryonic axes) for conservation.

Whole Seeds and Embryonic Axes Cryostorage

1. 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 determined using moist filter paper/peat moss/perlite or in rolled paper. The embryonic axes, controls and after treatments, were cultured in vitro.

2. Air Desiccation-Freezing. Orthodox 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.

3. Freezing. Cryopreservation procedures and standards were refined on a species-specific basis as new reports on post-storage behaviour and cryoprotocols became available. 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, 1995a,b; Chaudhury and Malik, 2006; Malik and Chaudhury, 2010; Malik et al., 2011, 2012).

4. Vitrification. For vitrification experiments, procedure described in Malik et al. (2012) was followed. Embryonic axes were precultured followed by PVS2 vitrification. Cryovials were thawed after at least 24 h at 38°C for 5 min and cultured suitably.

5. Encapsulation Dehydration. Following the procedure described in Malik et al. (2012) encapsulated embryonic axes were pre-cultured (in 0.3, 0.5 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.

6. Containers for Storage. Small seeds and EA were packed in 2 ml polypropylene cryovials. Larger seeds and excised buds were packed in cryovials of capacity 5 or 50 ml and largest seeds and bud twigs in polyolefin tubing. The National Cryogenebank may hold a quarter million samples (1 ml capacity cryovials), and presently about 42,000 containers of different capacities are held.

7. Rewarming and Assessment of Recovery. The cryopreserved seeds, embryos and embryonic axes were later transferred to large capacity (1000 L 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 after 48 h of storage followed by testing in years 6 to 15 as per retrieval and experimental schedule. Rewarming 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 three months in culture. In few of the cases healthy plantlets were transferred to pots.

Pollen Cryostorage

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 for 2-5 h for achieving moisture levels between 7-10% before packing in cryovials and stored at -196°C. After 24 h 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 Bud Cryostorage

Twigs of mulberry, almond and walnut were harvested at regular intervals, during peak winters (December to February) from field genebanks at Srinagar and Hosur and airlifted to cryolab at New Delhi. Buds were processed as per the procedure detailed in Rao et al. (2007, 2009). The in vitro cultured buds were incubated under complete darkness at 25±2°C for seven days before transferring under dim light for 5-7 days. Cultures were finally transferred to normal light conditions of 35 µE m^{-2} s^{-1} with a 16 h photoperiod.

For vitrification, pre-cultured buds were treated with PVS2 for periods of 20, 40, 60, 90 and 120 min at room temperature before LN exposure. After 24 h storage the vials were re-warmed at 38°C for 5 min. PVS2 was replaced with unloading solution and buds were cultured on suitable MS medium. Encapsulation was done for the buds pre-cultured for 48 h in MS basal media with 0.7% agar. Beads were prepared as described for embryonic axes and dehydrated in MS medium with sucrose for 17-24 h. Beads were desiccated for 4-5 h when the MC% of the beads fell to between 20-25% and were rapidly cooled and held in LN for 24 h. Vials were rewarmed slowly and beads cultured. After rewarming, the buds were rehydrated in sterile moist moss grass for 2-4 hours at room temperature in mulberry and up to six days at 5°C for almond and walnut before culturing in vitro or grafting.

For long-term large scale cryobanking of germplasm as a base collection, a minimum of 60 explants (axes and buds) of each accession were packed as 15 explants per cryovial, creating a minimum of four replicates. For orthodox seeds up to 2000 seeds of each accession were cryostored distributed in 8-12 vials depending upon the seed size.

RESULTS AND DISCUSSION

Cryobanking of Orthodox Seeds

Initiation of cryobanking of prioritized orthodox seed species as a pilot project in 1986 at NBPGR led to a full-fledged cryogenebank conserving 3,213 accessions (Anonymous, 2013) (Table 1, Fig. 1). Several tree species and wild species producing orthodox seeds have been cryostored to obviate need for their regeneration. Table 2 depicts the retesting data generated in representative 30 genera. Original viability values were ensured as they were tested for a maximum of up to 25 years of cryostorage.

The dried seeds of orthodox species can be stored for long periods at -196°C, leading to successful banking. It is, however, essential to retest the seed viability from cryobanks after regular intervals to study any differences in seed longevity among species (Priestley et al., 1985). Although cryogenic storage (at temperatures between -160 to -196°C) is assumed to provide nearly infinite longevity to cells and tissues, that actual shelf life is still not available for several plant species. There are reports of variability in the extent of deterioration among species and accessions within a species (Walters et al., 2004). Very few crop species have been tested for longevity at cryogenic temperatures or the data generated has not been under the best of storage conditions as required for cryobanking necessitating additional studies. In our cryolab data for more species is being generated.

Cryobanking of Non-Orthodox Seeds

Non-orthodox seeds/fruits were abundantly collected from the moist ecosystems of tropics and subtropics from several Indian states. Seed storage behaviour and the developmental stage when the seeds exhibited maximum tolerance to desiccation were ascertained as the prerequisites to actual storage (Chaudhury and Malik, 2004). Based on this, more than 30 species of indigenous tropical and temperate species were categorized

(Malik et al., 2003). Recalcitrant seeds at various developmental stages showed varying degrees of tolerance to desiccation (Chandel et al., 1995). This also varied among species e.g. immature/partially mature embryos of jackfruit and litchi were more adaptable to manipulation than mature embryos/embryonic axes. Rapid and careful handling, vitrification, encapsulation and use of EA were found effective for the cryopreservation of 27 *Citrus* spp. Vitrification and encapsulation were attempted in embryonic axes of *Artocarpus heterophyllus*, *Litchi chinensis*, *Poncirus trifoliata* and *Citrus* species (Chaudhury and Malik, 2000; Malik and Chaudhury, 2006) and in other labs in mango (Huang et al., 2000) and jackfruit (Thammasiri, 1999) with varying success.

The air desiccation-freezing of EA and desiccation of whole seeds over silica gel followed by fast freezing was found best. Using these techniques, success was achieved at NBPGR for EA in almond (Chaudhury and Chandel, 1995b), tea (Chaudhury et al., 1991) and whole seeds of neem, intermediate seed species (Chaudhury and Chandel, 1991), black pepper (Chaudhury and Chandel, 1994), cardamom (Chaudhury and Chandel, 1995a) and Jatropha and Pongamia (Chaudhury and Malik, 2006). The base collection of more than 12 indigenous species representing wide genetic diversity of fruits, nuts, agroforestry and forestry species, plantation crops, spices and industrial crop species was cryoconserved at NBPGR's National Cryogenebank (Fig. 2). A total of 6,032 accessions of difficult-to-store non-orthodox seed species are cryostored (Table 1). Basic cryobiological investigations in highly recalcitrant seed species of jackfruit, litchi, cocoa and *Garcinia* species (Malik et al., 2005a,b) were done to elucidate the biochemical, biophysical and ultrastructural basis of desiccation and freezing sensitivity. Cryoconserved samples were randomly tested for up to 10 years and accessions retained the original viability.

The suitability of a protocol for cryopreservation can be assessed only in terms of good recovery growth of cryopreserved explants. Growth analysis is a common method where signs of regrowth are considered as survival and the development of whole plantlet is considered to be the recovery growth. The selection of optimal culture media and culture conditions played an important role in achieving recovery growth from cryopreserved explants, especially during the first weeks of culture. In vitro recovery methods after cryostorage were standardised for tea, almond, citrus species (more than 20 spp.), neem, walnut, etc. The explants are placed in optimal conditions to trigger rapid and direct growth. Tea EA were recovered on modified Nakamura medium with 0.1% yeast extract. The axes of *Citrus* sp., neem, walnut, apricot, pilu and almond could be recovered on medium defined by Chin et al. (1988). The embryos of jatropha were cultured on MS medium with 1 mg L^{-1} benzylaminopurine. In *Salvadora* sp. in vitro recovery of excised embryonic axes from cryostored seeds was essential as the cotyledons and endocarp were found to impede the growth of viable embryonic axes. Rehydration of desiccated explants was also required to avoid any imbibition injuries.

Cryobanking of Pollen

In vitro germination and Fluorochromatic Reaction (FCR) tests of cryostored pollen was used for testing pollen viability in different species. In vitro germination provided 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 and wild species of more than 380 accessions was successfully cryostored (Table 1).

Cryobanking of Dormant Buds

Cryostorage of mulberry dormant buds 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. This was the first report of successful cryopreservation of mulberry dormant buds, not subjected to any freezing temperatures in field conditions, belonging to species *M. indica*, *M. latifolia*, *M. serrata*, *M. laevigata*, *M. australis*, *M. bombycis*, *M. alba*, *M. sinensis*, *M multicaulis* and *M. rotundiloba* (Rao et al., 2007).

Regrowth of cryopreserved mulberry germplasm accessions after 1-3 years of storage indicated no survival loss and 33-40% of the accessions showed viability above 40% and up to a maximum of 100%. Encapsulation of buds, with 7-8 scales removed, was attempted in *M. indica* with survival on 0.3 M sucrose of 40%, 0.5 M sucrose of 52% and 74% for buds desiccated with 0.75 M sucrose.

Winter dormant buds of walnut, almond and apricot were tested for the 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 two out of eight accessions with 40-50% survival in vitro using step-wise freezing. For the encapsulation method, survival in vitro of 30 and 20% was observed for buds (with 7-8 scales removed) dehydrated with 0.3 and 0.75 M sucrose, respectively. In vitrification of buds (with 7-8 scales removed), survival in vitro of 23 and 10% was apparent for PVS2 treatments of 40 and 60 min, respectively. In almond 8-10% survival was observed using encapsulation and vitrification. Experiments are continued for improving the regrowth.

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Tables

Table 1. Status of germplasm at National Cryogenebank at NBPGR.

Categories	Accessions (no.)
Recalcitrant & Intermediate	
Fruits & Nuts	2888
Spices & Condiments	151
Plantation Crops	22
Agroforestry, Industrial & Medicinal crops	2971
Total	6032
Orthodox	3213
Dormant buds	387
Pollen samples	381
Total	10013

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

Crops	Genera	Species	No. of accessions	Viability (%)	Years of cryostorage
Cereals	3	8	46	90-100	8-18
Millets	4	6	29	85-100	9-22
Pseudo-cereals	1	2	17	70-100	9-24
Legumes	5	8	51	85-100	12-23
Oil seeds & fibres	6	10	54	85-100	9-24
Vegetables	7	9	46	60-100	7-25
Medicinal, narcotics and dye plants	4	5	7	80-100	3-23
Total	30	48	250		

Figures

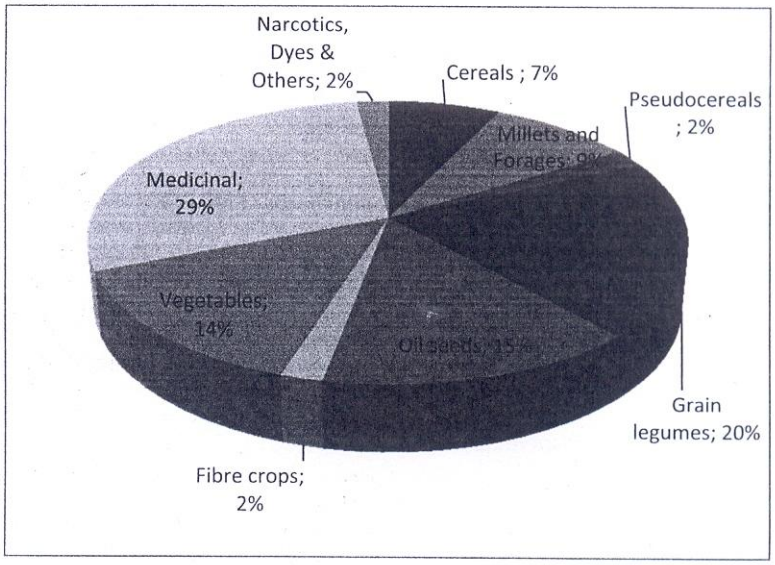

Fig. 1. Major crop groups of orthodox seed species in Cryogenebank at NBPGR. No. of crop species conserved: 505.

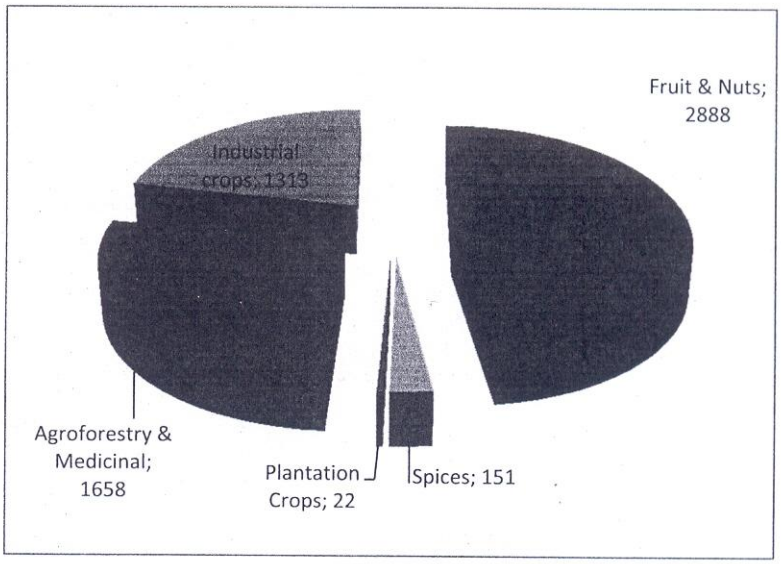

Fig. 2. Major crop groups of non-orthodox seed species in Cryogenebank at NBPGR.