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Cryopreservation Studies on Plant Germplasm

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Annual and biennial seed crops belonging to the orthodox crop species are generally amenable for preservation in seed 'Gene Banks' at low (+10°C) and ultra low (−20°C) temperatures. Until now it has proved to be the best means of germplasm storage all over the world. For several vegetatively propagated plant species, in-vitro *conservation techniques now offer new possibilities. Pollen preservation is yet another important method by which part of genome can be preserved, although it plays only a complementary role. However, recent researches in cryopreservation indicate that seeds, meristems, shoot tips, buds and both zygotic and somatic embryos as well as embryonic axes can be preserved and the trends indicate that perhaps these can be preserved for indefinite period without loss of viability or deterioration in qualiiy and genetic integrity. Cryopreservation work undertaken presently at National Facility Plant Tissue Culture Repository, (NFPTCR) using seeds, pollen, and* in-vitro *cultures has made significant progress and the information has been briefly presented in this paper.*

Several strategies for germplasm conservation have been employed by national and international organisations. These can be categorised under *in-situ* and *ex-situ* conservation. The former involves continuance of wild species in their natural habitats or biosphere reserves (genetic reserves) where process of evolution can continue, while *ex-situ* conservation involves maintenance of germplasm in the field by growing frequently for rejuvenation of seeds (Field Gene Banks), or holding germplasm in Seed Gene Banks, gardens, plantation, orchards or in green houses. Besides, *in-vitro* techniques developed recently offer potential for storage of meristem, shoot tips, buds and embryos under controlled physical and nutritional regime using minimal media, low temperature or through growth retardant over short, medium or long term periods. Cryopreservation techniques offer yet another strategy for the preservation of germplasm. However, practical considerations are important for adopting one or some of these methods for genetic conservation. Researches indicate that in *in-vitro* storage and cryopreservation provide useful adjuncts in the wake of specific problems associated with some plant species (Morel, 1975; D'Amato, 1975; Henshaw, 1975; Withers and Williams, 1985). Pollen preservation can help in crop breeding, in the development of haploids (inbreds) as well as dihaploids (genetically homozygous). Although it plays only a complementary role in the long term conservation strategy (Towill, 1985; Ganeshan, 1986a), yet it helps in conservation of germplasm.

Cryopreservation techniques provide alternative strategy for the conservation of seeds, pollen, embryos and *in-vitro* cultures at ultra low cryogenic temperature (Liquid nitrogen,—196°C). However, recalcitrant seeds are desiccation sensitive and are easily killed on dehydration below a critical value between 12-35 percent moisture content. Recalcitrant seeds are produced by some aquatic species, most of tropical fruits and many economically important species like coconut, oilpalm, jackfruit, mango, litchi, walnut, rubber and nutmeg. Partially desiccation tolerant species include tea, coffee, citrus and several others. According to Chin and Roberts (1980), although efforts have been regularly made for the storage of such species, however, none of the methods ensure long-term storability beyond four years without any loss of viability. Currently germplasm conservation work is being carried out intensively in several laboratories of the world (Plucknett *et al.*, 1987). India has also established its first National Plant Tissue Culture Repository and NBPGR already possesses a well established network (Paroda, 1988). The cryopreservation work being currently persued at the National Plant Tissue Culture Repository, NBPGR, is being presented here.

MATERIALS AND METHODS

In order to devise freeze preservation experiments, random samples drawn from seed lots of different orthodox species were used for present studies. The seed lots were dehydrated to 5-8 per cent moisture. The 100 grain weight (1000 grain weight in case of *Nicotiana tabacum*) was determined in different species/accessions. For long-term cryopreservation work (base collection), a minimum of 10,000 seeds were stored for cross pollinated crops (e.g. pearl millet) while about 5,000 seeds were stored for self pollinated crops. For determination of moisture content in orthodox seed species, ISTA rules (1985) were followed. Low constant temperature oven method was routinely followed for recalcitrant species. Seed lots were subjected to viability test and actual germination was used as a criterion of viability. In each case, 50 seeds in 2 replicates were germinated on top of filter paper discs. All desiccation sensitive and partially desiccation sensitive species were germinated in open trays or in polythene bags in mixture of 1 : 1 garden soil and sand, and were maintained at room temperature (25-32°C). Seedling vigour was tested for pearl millet, onion, *Brassica* and *Plantago*, etc., following the method suggested by Myhill and Konzak (1967). Shoot/root length was noted after a suitable germination period for different species.

(I) Cryopreservation Technique For Seeds

For conservation experiments, seeds were distributed in small capacity cryovials (1/1.8 ml) or in goblets (250 ml) made of polypropylene and were kept at different temperatures; (1) ambient, (2) —20°C and (3) —150° to —180°C (vapour phase of liquid nitrogen). The seeds of cocoa and pepper were simultaneously stored in closed petriplates subimbibed in different concentrations of abscicic acid (10^{-3}, 10^{-4} and 10^{-5} M) with a filter paper laid underneath and maintained at 5°C).

(II) Freeze Preservation Technique For Pollen

For pollen storage the material comprised of primitive landraces of maize (*Zea mays*) from Sikkim and North-eastern region of India as well as introduced germplasm of related genera and species, *Coix lacryma jobi*, *Chionachne*, *Zea parviglumis*, *Z. mexicana*, and *Z. diploperennis*. Pollen was collected in the early morning hours (between 8.30 a.m. and 11.00 a.m.) from actively dehiscing tassels. The *in-vitro* germination of pollen grains on semisolid medium and *in-vitro* pollination of silks (Mishra, 1984) was used as a criterion of viability. Water content was determined by drying to a constant weight at 80-90°C temperature and the percentage of water content of the pollen was determined. For freeze preservation of pollen, fresh samples of each species immediately after collection were dried over dry silica maintained in laboratory at ambient temperature for 3 hours before actually storing in cryovials. The loss of water was determined at the end of desiccation period by determining the decrease in weight. Stored seeds and pollen samples were thawed rapidly by immersing the storage vial in waterbath maintained at 40°C. Viability was tested following the same method as used before storage of these samples.

(III) *In-vitro* Freeze Preservation Technique

In order to set the *in-vitro* freeze preservation experiments, tobacco (*Nicotiana tabacum*) was taken up as a model system for meristem/shoot tip preservation. Meristem with 2-3 leaf primordia was excised aseptically from seedlings grown on MS basal medium supplemented with DMSO (5 per cent) for 48 hours. Cultures were further maintained at 5°C for 24 h. Meristems were transferred to 5 ml of chilled cryoprotectant free MS basal medium. 5 ml of 10 per cent PEG/DMSO was added dropwise to it over a period of 30 min. to a final concentration of 5 per cent and maintained further for 20 min. Meristem alongwith cryoptotectant solution were then stored aseptically in 1.8 ml cryotubes and frozen rapidly. After 24 hours cryotubes were thawed rapidly using water bath (40°C). One set of meristems was recultured on MS basal medium directly whereas the other set was washed repeatedly in sterile water before culturing. Controls were run using cryoprotectant alone without involving any freezing.

RESULTS AND DISCUSSION

Cryopreservation of orthodox seeds

All orthodox seed species under study possessed high viability (> 80 per cent) as indicated by the actual germination test at the time of storage (Table 1). Seedlots indicated a range of moisture content of 3.5 to 7.8 per cent. Seed samples were successfully stored for variable length of times as evident by the data available for well over two years (experiments still continuing). Pearl millet and onion seeds were tested periodically and all the samples were found to retain the initial viability, germination percentage and seedling vigour. Cryopreservation attempts on orthodox seeds have been undertaken only at a few selected laboratory, such as National Seed Storage Laboratory (NSSL), Fort Collins, Colorado, USA (Stanwood and Bass, 1981). At NSSL, seveal hundred accessions belonging to the orthodox

TABLE 1. VIABILITY, MOISTURE CONTENT AND STORABILITY OF DIFFERENT PLANT SPECIES CRYOPRESERVED IN LIQUID NITROGEN

Plant species	Initial Viability (%)	Moisture content (%)	Period of storage (days)
ORTHODOX SEED SP.			
Pearl millet			
Pennisetum typhoides var. BJ 104	80	6.7	900
MH 179	94	4.3	900
Onion			
Allium cepa var. Pusa Ratnar	96	7.8	933
Ratnar selection	96	7.69	933
Nicotiana			
Nicotiana tabacum var. NPN-190	98	4.53	520
PL- 5	97.8	4.5	520
Anand-119	100	4.5	520
Anand- 2	100	4.5	520
S- 20	98	4.4	520
Brassica			
Brassica juncea var Pusa Bold	93	4.4	507
Varuna	90	4.06	507
PR- 45	95	3.85	507
RLM-198	87	5.5	507
B. napus var. ISN-129	95	5.7	507
ISN- 11	90	5.19	507
706	91	3.53	507
BO- 54	90	3.99	507
ISN-106	91	4.0	507
B. carinata 1	90	4.95	507
Isabgol			
Plantago ovata var. Gujarat Isabgol-1	95	5.66	514
Sunflower			
Helianthus annuus var. Morden	96	6.4	518
Peredovik	94	6.5	518
Surya	95	6.63	518
*RECALCITRANT SEED SP.***			—
Theobroma cacao	95	41.3	—
Artocarpus heterophyllus	100	53.2	—
Piper nigrum	0*	31.9	—
Myristica fragrans	0	32.9	—
Camellia sinensis	100	30.0	—
Aegle marmelos	100	3.86	90

*10 per cent germination was noted in seeds plated on semi-solid medium (B_5 medium with 0.7 per cent agar) in test tubes maintained at 23°C constant under light.

**Success not achieved, Experiments continued.

species (desiccation tolerant) have been cryoreserved for well over 8 years. It is considered that at the temperature of —196°C, most or all metabolic and cell division activities cease. The decline in viability at cryogenic temperature (—196°C) is argued to be well over several decades and it perhaps happens due to low kinetic energy of molecules and negligible diffusion characteristics (Mazur, 1976). Background irradiation during the storage period is thought to be the major potential source for damage to cells. Although cryopreservation had not been well studied in many systems, the conclusion is still that virtually no change should occur over a long period of time (Ashwood-Smith and Grant, 1977). Thus the potential of liquid nitrogen storage is considerable. It also provides better quality of seeds and improved maintenance of plant genetic resources much sought for crop improvement.

Cryopreservation of pollen

Pollen of maize landraces and allied genera/species used for freeze preservation studies showed very high moisture content (51 per cent). The pollen were desiccated to 12 per cent moisture level before storage without much loss of viability. Sufficient numbers and amount of pollen samples were stored for each genera/species and testing of viability was also undertaken after one year of storage in liquid nitrogen. *In-vitro* pollinations on maize cob silks gave a better estimate of viability as compared to *in-vitro* germination.

Reports on successful cryopreservation of pollen in liquid nitrogen have already appeared in the literature. Pollen of *Allium cepa, Carica papaya, Citrus limon, Vitis vinifera, Solanum melongena* and *Lycopersicon esculentum* were reported to be successfully cryopreserved for one or more years using freeze-preservation techniques (Ganeshan, 1986a, 1986b). Pollen banks have been established by various research organisations for the purpose of basic research in pollen biology, plant breeding and crop production programme. Yet the concept of pollen preservation for conservation of plant genetic resources is rather recent. Cryopreservation of pollen has several advantage, as it can facilitate wide hybridization between plants that are separated physiologically, geographically and also due to seasonal barriers. It would also help to conserve genetic diversity. Pollen can be used for the production of haploids to develop inbred lines rapidly as well as dihaploids, which can be of tremendous significance in plant breeding programme and genetical studies (Chandel *et al.*, 1988; Chandel and Pandey, 1990).

Cryopreservation of desiccation sensitive species

Majority of the recalcitrant seed species possess very high moisture (30-53 per cent). Also the literature on the seed germination of recalcitrant species is very scanty and this provided difficulty as no guidelines were available for standard germination procedures to be followed. Laboratory experiments were thus conducted for the standardization of germination for various desiccation sensitive/partially desiccation sensitive species presently being studied at NFPTCR.

Pepper seeds were extracted from berries on arrival of freshly harvested consignment. These showed very low germination only when plated on agar medium. Out of the berries received from CPCRI, Kasargoad, some showed drying and some already died before reaching the destination perhaps due to dehydration. Rest of the seed species showed very high germination.

Recalcitrant seeds were used for experimentation within few days after the freshly harvested lot became available. Storage of cocoa, jackfruit and pepper seeds wrapped in charcoal powder did not prove beneficial because of frequent fungal infection. Cacoa and pepper seeds were thus extracted from pods and berries, respectively just before cryopreservation experiments. Seeds of jackfruit were stored as such in polythene bags at 8-10°C.

In our preliminary studies, the seeds of several recalcitrant/partially desiccation sensitive species were not found amenable to LN_2 storage except bael (*Aegle marmelos*) seeds which behaved like orthodox seeds in the storage characteristics. Desiccation experiments conducted on jackfruit showed that reduction in moisture content by 10 per cent does not cause any damage, while it proved highly deterimental in case of cocoa.

Use of cryoprotectant in our experiments for seed storage in liquid nitrogen did not prove beneficial. The failure of LN_2 storage cannot be attributed to any single factor as the damage might have occurred at any stage during desiccation, cryoprotectant treatment, freezing, thawing or post-thaw treatment. It is evident that large size of recalcitrant seeds (oil palm, cocoa, jackfruit, mango, litchi, etc) itself may cause problem for efficient ingress of cryoprotectant into the seeds and also in precise control of freezing and thawing. The inter and intra-cellular ice crystal formation and changes in osmotic regulatory mechanism related to membrane composition and activity can result in nucleation leading to ice formation. This might cause death of embryos. Similarly, in sub-imbibed storage of pepper and cocoa seeds, fungal infection proved to be a constant menace which once set in a lot, could perhaps spoil the entire seed lot.

The review of the literature suggested that cryogenic storage so far has been restricted only to few plant species. Successful reports exist for *Anthurium* (Stanwood, 1986), coffee (Yilmaz, Pers Commu) and rubber (Normah and Chin, 1989).

Preservation of excised embryos have been reported from young coconut (Chin *et al.*, 1989) and *Veitchia* and *Howea* palm embryos (Chin *et al.*, 1988). Previously some of the tree species such as *Citrus* were considered to be recalcitrant, but recent researches have shown that lime and lemon belong to the orthodox group (Mumford and Grout, 1979).

Ultrastructural changes were observed in *Hevea* seeds stored at 10°C, 22°C and 27°C. Membrane degeneration appeared to be the most common feature of deterioration. At all storage temperatures, the plasmalemma was observed to be increasingly folded, disintegrated or withdrawn from the cell wall. The dissolution of the tonoplast was also widely observed (Normah and Chin, 1989). The reports on the cryopreservation of young coconut embryos have appeared recently (Chin

et al., 1988) where excised coconut embryos survived the cryopreservation treatment but only with callus formation from parts of embryos. In later experiments, success was achieved in cryopreservation of young coconut embryos with formation of organised growth after fifteen months of culture (Chin *et al.*, 1989). Further, it was highlighted that basic studies on the identification of causes of desication sensitivity in recalcitrant seeds and their intolerance to low temperatures of 10 to 15°C should also be given high priority. These factors are important since any long-term storage of recalcitrant seeds requires preventive measures for seed deterioration and viability loss. According to the above authors, alternatives to imbibed seed storage, such as partial drying or lowering moisture contents to just above a critical level in conjunction with fungicide treatments also need specific attention.

In-vitro freeze preservation

Meristem and shoot tip cultures initiated on tobacco (*Nicotiana tobacum*) on MS basal medium were observed periodically for growth and development. Meristem pretreated with 5% PEG (without LN_2 exposure) kept as control showed good growth, whereas in all other treatments, meristem slowly turned yellowish white and showed no further growth. It was found that PEG (5 per cent) was not toxic to meristem cultures while DMSO (5 per cent) was cytotoxic. Expriments were continued to bring improvement and refinement in the methods and protocols for successful storage.

Researches on cryopreservation of plant species were systematized only in the last decade, although the first success in the cryopreservation of a cell suspension was reported by Quatrano (1968) in flax at —50°C while another report was by Nag and Street (1973) who regenerated somatic embryos from a cell culture of carrot (*Daucus carota*) frozen at —196°C. Since then, there has been increase in number of species shown to be amenable to being exposed to low temperature with good percentage of recovery and survival. Cryopreservation process for *in-vitro* cultures can be divided into six stages; pregrowth, cryoprotection, freezing, storage, thawing, estimation of viability and cryoinjury (Wilkins and Dodds, 1983).

Cryopreservation researches have made considerable success in case of preservation of cells, suspension and protoplast cultures in plants. (Bajaj and Reinert, 1977; Withers 1980; Kartha, 1985). Cassava meristems have been also cryopreserved at CIAT, Cali, Columbia (Kartha *et al.*, 1982). Successful freeze preservation of meristems of strawberry (Kartha *et al.*, 1980) and apple (Caswell *et al.*, 1986) have been reported.

Cryogenic storage of semen, ova and fertilized eggs have been obtained in animal system. There are infact reports of cryogenic storage of complete human organs and also bodies (Mazur, 1970; Meryman, 1966; Mericka, *et al.*, 1988; Grischenko *et al.*, 1988). However, researches on basic aspects of cryobiology are required to obtain indepth knowledge of cryopreservation, particularly cryobiological aspects including physical and biochemical aspects of membrane integrity, osmotic regulatory mechanism, nucleation, cryoinjury, etc. Thus future research

efforts would be directed towards the solving of such problems so that effective and reliable methods of freeze preservation could be developed.

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