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Plant Cryopreservation

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1. Introduction

Two basic approaches to conservation of plant genetic resources are *ex situ* and *in situ* conservation. *Ex situ* conservation includes seed storage, *in vitro* storage, DNA storage, pollen storage, field genebanks and botanical gardens while the *in situ* approach encompasses genetic reserves, on farm and home garden conservation.

Cryopreservation is a part of biotechnology. Biotechnology plays an important role in international plant conservation programs and in preservation of the world's genetic resources (Bajaj, 1995; Benson, 1999). Advances in biotechnology provide new methods for plant genetic resources and evaluation (Paunesca, 2009). Cryopreservation, developed during the last 25 years, is an important and the most valuable method for long-term conservation of biological materials. The main advantages in cryopreservation are simplicity and the applicability to a wide range of genotypes (Engelmann, 2004). This can be achieved using different procedures, such as pre-growth, desiccation, pregrowth-desiccation, vitrification, encapsulation-vitrification and droplet-freezing (Engelmann, 2004). Cryopreservation involves storage of plant material (such as seed, shoot tip, zygotic and somatic embryos and pollen) at ultra-low temperatures in LN (-196°C) or its vapor phase (-150°C). To avoid the genetic alterations that may occur in long tissue cultures storage, cryopreservation has been developed (Martin *et al.*, 1998). At this temperature, cell division, metabolic, and biochemical activities remain suspended and the material can be stored without changes and deterioration for long time. Walters *et al.* (2009) proposed that this assumption, based on extrapolations of temperature-reaction kinetic relationships, is not completely supported by accumulating evidence that dried seeds can deteriorate during cryogenic storage. After 30 years of cryogenic storage, seeds of some species exhibited quantitatively lower viability and vigor. In cryopreservation method, subcultures are not required and somaclonal variation is reduced. Advantages of cryopreservation are that germplasm can be kept for theoretically indefinite time with low costs and little space. Besides its use for the conservation of genetic resources, cryopreservation can also be applied for the safe storage of plant tissues with specific characteristics. Different types of plant cell, tissues and organs can be cryopreserved. Cryopreservation is the most suitable long-term storage method for genetic resources of vegetatively maintained crops (Kaczmarczyk *et al.*, 2008). For vegetatively propagated species, the best organs are shoot apices excised from *in vitro* plants. Shoot apices or meristems cultures are suitable because of virus-free plant production, clonal propagation, improving health status, easier recovery and

less mutation (Scowcroft, 1984). Seed and field collections have been the only proper for the long-term germplasm conservation of woody species, while a large number of forest angiosperms have recalcitrant seeds with a very limited period of conservability. The species, which are mainly vegetatively propagated, require the conservation of huge number of accessions (Panis and Lambardi, 2005). The storage of this huge number needs large areas of land and high running costs. Preservation of plant germplasm is part of any plant breeding program. The most efficient and economical way of germplasm storage is the form of seeds. However, this kind of storage is not always feasible because 1) some seeds deteriorate due to invasion of pathogens and insects, 2) some plants do not produce seeds and they are propagated vegetatively, 3) some seeds are very heterozygous thus, not proper for maintaining true-to-type genotype, 4) seeds remain viable for a limited time, and 5) clonally propagated crops such as fruit, nut, and many root and tuber vegetables cannot be stored as seed (Chang and Reed, 2001; Bekheet *et al.*, 2007). Cryopreservation offers a good method for conservation of the species, especially woody plant germplasm (Panis and Lambardi, 2005). Cryostorage of seeds in LN was initially developed for the conservation of genetic resources of agriculturally important species (Rajasekharan, 2006). The development of simple cryostorage protocols for orthodox seeds has allowed cryopreservation of a large number of species at low cost, significantly reducing seed deterioration in storage (Stanwood, 1987). Only few reports are available on the application of cryopreservation on seeds of wild and endangered species and medicinal plants (Rajasekharan, 2006). New cryobiological studies of plant materials has made cryopreservation a realistic tool for long-term storage, for tropical species, which are not intrinsically tolerant to low temperature and desiccation, has been less extensively investigated (Rajasekharan, 2006). Cryopreservation has been applied to more than 80 plant species (Zhao *et al.*, 2005). Number of species, which can be cryopreserved has rapidly increased over the last several years because of the new techniques and progress of cryopreservation research (Rajasekharan, 2006). The vitrification/one-step freezing and encapsulation dehydration methods have been applied to an increasing number of species (Panis and Lambardi, 2005). A new method, named encapsulation- vitrification is noteworthy (Sakai, 2000). These techniques have produced high levels of post-thaw and minor modifications (Rajasekharan, 2006). In cryopreservation, information recording such as type and size of explants, pretreatment and the correct type and concentration of cryoprotectants, explants water content, cryopreservation method, rate of freezing and thawing, thawing method, recovery medium and incubation conditions is very important (Reed, 2001 ; González-Benito *et al.*, 2004; Bekheet *et al.*, 2007). All germplasm requires safe storage because even exotic germplasm without obvious economic merit may contain genes or alleles that may be needed as new disease, insect, environmental, or crop production problems arise (Westwood, 1989). It is important to record also the recovery percentage after a short conservation period. A major concern is the genetic stability of conserved material.

For many plant species which produce orthodox seeds, i.e. which can be dehydrated extensively and stored dry at low temperature, the emphasis for genetic resource conservation will be on seed/embryo storage. Recalcitrant seeds cannot tolerate desiccation to moisture content that would permit exposure to low temperature. They are often large with considerable quantities of fleshy endosperm. Therefore, recent investigations have identified species displaying an intermediate form of seed/embryo storage. As regards the balance of techniques employed within complementary strategies developed for conserving the genetic resources of these problems species, the emphasis in the case of non-orthodox (intermediate/recalcitrant) forest tree species will be on *in situ* conservation in genetic reserves, while for

species which are propagated vegetatively the emphasis will be on *ex situ* conservation techniques, including field genebank and *in vitro* storage. However it is essential to recognize that owing to various problems and limitations encountered with both genetic reserves and field genebanks, cryopreservation currently offers the only safe and cost effective option for the long-term conservation of genetic resources of these problem species. Significant progress has been made during the past 10 years in the area of plant cryopreservation with the development of various efficient cryopreservation protocols. An important advantage of these new techniques is their operational simplicity, since they will be applied mainly in developing tropical countries where the largest part of genetic resources of problem species is located. Encouraging results in medicinal plants have been published in recent years which present extensive list of plant species whose embryos and or embryonic axes have been successfully cryopreserved (Kartha and Engelmann 1994, Pence 1995, Engelmann *et al* 1995).

In comparison with results obtained with vegetatively propagated species, it is clear that research is still at a very preliminary stage for recalcitrant seeds. The desiccation technique is mainly employed for freezing embryos and embryonic axes, the survival achieved are extremely uneven. And also survival is often limited and regeneration often restricted to callusing or incomplete development of plantlets. In only a limited number of cases, the whole plants have been regenerated from cryopreserved material (Chin and Pritchard 1988, Assy Bah and Engelmann 1992). Seeds and embryos of recalcitrant species also display various characteristics which make their cryopreservation difficult. One of the characteristics of recalcitrant seeds is that there is no arrest in their development, as with orthodox seeds. It is very difficult to select seeds at a precise developmental stage, even though this parameter is often of critical importance to achieve successful cryopreservation. Seeds of many species are too large to be frozen directly and embryos or embryonic axes have to be employed. However, embryos are often very complex tissue composition which display differential sensitivity to desiccation and freezing, the root pole seeming more resistant than the shoot pole (Pence 1995). In some species, embryos are extremely sensitive to desiccation and even minor reduction in their moisture content down to levels much too high to obtain survival after freezing leads to irreparable structural damage. It should be emphasized that selecting embryos at the right developmental stage is of critical importance for the success of any cryopreservation experiment (Engelmann *et al.*, 1995) However, in these cases basic protocols for disinfection, *in vitro* germination of embryos or embryonic axes, plantlet development and possibly limited propagation will have to be established prior to any cryopreservation experiment.

Cryostorage of seed was initially developed for the preservation of genetic resources of agriculturally important species for breeding and selection. The development of comparatively simple cryostorage protocols allowed seeds of over 155 agricultural species (Stanwood, 1985) to be stored at low cost, in an environment without obvious problems of seed ageing, genetic variations and predation common to many conventional seed storage methods. With the regular use of cryostorage system for seeds of agri-crops, the same process is now viewed as having important application for preserving seeds of medicinal plants (Decruse *et al.*, 1999), endangered species (Touchel and Dixon 1994) and other native plant species (Pence 1991, Touchel and Dixon 1993, Decruse and Seeni 2002). For the long-term preservation of species producing recalcitrant seeds, zygotic embryos were used for cryopreservation. Incidentally excised zygotic embryos or embryonic axes were successfully employed for the cryopreservation of coconut (Assy-Bah and Engelman, 1992 a,b, Chin *et al.*,

1989), cocoa (Pence, 1991, Chandel *et al.*, 1995) oil palm (Chabrillange *et al.*, 1997), walnut (de Boucaud *et al.*, 1991), jack fruit (Chandel *et al.*, 1995, Thammasiri, 1999), rubber (Normann, 1986), tea (Chaudury *et al.*, 1991) and neem (Berjack and Dumet, 1996).

National Gene bank for Medicinal and Aromatic Plants at Tropical Botanic Garden and Research Institute (TBGRI) is one among the four (CIMAP, Lucknow, NBPGR, New Delhi and RRL, Jammu) having the mandate of conserving the medicinal and aromatic plants (MAPs) of Peninsular India through biotechnological intervention including collection, *ex situ* conservation and characterization of the precious taxa that are rare, endangered, threatened, endemic, vulnerable or over exploited as the case may be. TBGRI has significantly developed cryopreservation protocol on rare and endangered medicinal plants of India (Decruse *et al.*, 1999, Decruse and Seeni, 2002, Radha *et al.*, 2006). A cryobank was also established which now holds more than 25 accessions of medicinal and aromatic plants (Decruse *et al.*, 1999b, Decruse and Seeni 2002b, Radha *et al.*, 2010).

2. Cryopreservation of excised embryonic axes of *Nothapodytes nimmoniana* (Graham) Mebberly, a vulnerable medicinal tree species of the Western Ghats

Nothapodytes nimmoniana (Graham) Mebberly, of family Icacinaceae is a small vulnerable medicinal tree distributed in India, Sri Lanka, Myanmar, Thailand, Malaysia and China. In India it is distributed in upper ranges of the Western Ghats particularly in the Nilgiris and Palni hills of southern peninsula. The stem and roots are an important source of the anti-tumour quinoline alkaloid camptothecin (Hsiang *et al.*, 1985) and also find applications against retrovirus and human immunodeficiency virus. Consequently natural population of this species in the Western Ghats are severely depleted owing to habitat destruction and over exploitation (Cragg *et al.*, 1993, Ravikumar and Ved, 2000) and hence conservation efforts are undertaken by certain agencies in the region.

Seeds of *N. nimmoniana* are large intermediate type showed 100% germination under controlled conditions. Embryonic axes with cotyledons having moisture content of 55.7% presumed to be intermediate in nature, lose their viability within a short period after maturity. Cryopreservation of zygotic embryos is recognized as an effective tool for the long-term preservation of such plant species those produce recalcitrant/large seeds (Engelmann, 1997).

Desiccation and cryopreservation. The seeds were separated from the fruits (drupe), rinsed in running tap water for one hour to remove the mucilage and washed in commercial detergent (1% Teepol, Godrej, India Ltd., Mumbai) for 10 min. followed by thorough washing in running tap water for 10-20 min. Seeds were then surface decontaminated by immersion in 0.01% (w/v) HgCl₂ for 5-10 min. followed by 3-5 rinses in sterile distilled water. Seed coat was broken and embryos with cotyledons were dissected out free of the endosperm in aseptic condition in the laminar air flow cabinet. Immediately after dissection, batches of 20 embryos each were subjected to dehydration under laminar airflow for 30, 60, 90, 120, 150, 180 and 210 min. period. A sample of 10 embryos was inoculated into MS medium (Murashige and Skoog, 1962) devoid of PGR as fresh control and cultured under 10/14h light/dark periods (30 - 50 $\mu\text{mol m}^{-2} \text{s}^{-2}$) at 25 \pm 2 $^{\circ}\text{C}$ for 8 weeks. After desiccation at 30 min intervals, equally divided samples of 10 embryos were transferred to germination medium and another 10 packed in 2ml cryovial and transferred to LN (at -196 $^{\circ}\text{C}$) After 24h

storage, the vials were retrieved from LN and rewarmed in a water bath at 40°C for 1-2 min. The rewarmed embryos were also transferred to germination medium and cultured under stated conditions for recovery. The whole experiment was repeated three times.

Observations on the germination of embryos were made after 8 weeks and results analyzed statistically in a completely randomized model. Survival rate was assessed as the percentage of embryonic axes that exhibited any kind of growth, including seedling development; shoot growth and root growth.

Moisture content determination. Moisture content (MC) of the embryos was determined by constant temperature oven method (103 °C) for 17h.

The embryonic axes with cotyledons (Fig 1a) freshly dissected from the seeds possessed 55.7% MC and exhibited 86.67% germination and normal growth in MS medium devoid of PGRs within a week of culture. Dehydration under laminar airflow reduced the MC to 43.7% after 30min and 31.3% after 60min. without appreciable reduction in viability so that 76-77% of them germinated (Fig.1). Dehydration for 120min reduced MC to 19.6% and germination to 66.67% and was the optimum dehydration period (Fig.2) to get maximum germination (60%) after LN treatment (Fig.2). Root and shoot emergence was observed after one week of culture (Fig.1b) in 60% of the desiccated (120 min.) and LN treated embryonic axes and well developed seedlings were obtained within 20 days of culture (Fig.1c). Dehydration beyond 120 min. gradually reduced MC and drastically reduced viability. The MC came down to 12.1% after 210 min. when none of the embryos survived. Prolonged dehydration (150-180min) not only reduced survival down to 16.67-10% but also caused abnormal growth with only radicle development in the survived embryos (Fig.1d).

Research in the past two decades has shown that most orthodox seeds remain viable for long periods of storage after attaining appropriate desiccation levels of about 3-5% moisture content (Roberts, 1973). Contrary to this, recalcitrant seeds of several tropical and temperate species are desiccation sensitive, eg. Tea, Cocoa, Citrus, Jack fruit (Chin and Roberts, 1980). There are various options available to improve storage of non-orthodox seeds/embryos. Desiccation is the simplest procedure since it consists of dehydrating explants, and then freezing them rapidly by direct immersion in LN has been applied to embryonic axes extracted from recalcitrant and intermediate seeds (Engelmann, 1997). It should also be noted that selection of embryos at the right developmental stage is of critical importance for the success of any cryopreservation experiment (Engelmann *et al.*, 1995). The conservation efforts of *N. nimmoniana* are hampered mainly due to relatively large and intermediate type of seeds with desiccation sensitivity. The viability of the embryos was not much affected when the embryos were desiccated from 55.7% to 43.7% (i.e. 30 min. desiccation). Significant loss of viability due to further reduction of moisture content shows the intermediate nature of the embryos is in line with the report of Dussert *et al* (1995). Safe moisture content of the embryonic axes as obtained in the present study is 19.6% (60% survival). Damage to plumule rather than radicle occurred due to excessive dehydration of *N. nimmoniana* embryos is as observed earlier in *Auracaria hunstenni* where desiccation damage is reported to be more serious in the plumule (Pritchard and Prendergast, 1986). The exact causes of embryonic death and its relationship with moisture content are not fully understood. Chin *et al* stated that seed death could be due either to the moisture content falling below a critical value or simply a general physiological deterioration with time. If embryonic axes have been desiccated to around 20% moisture content without loss of viability, it is possible that

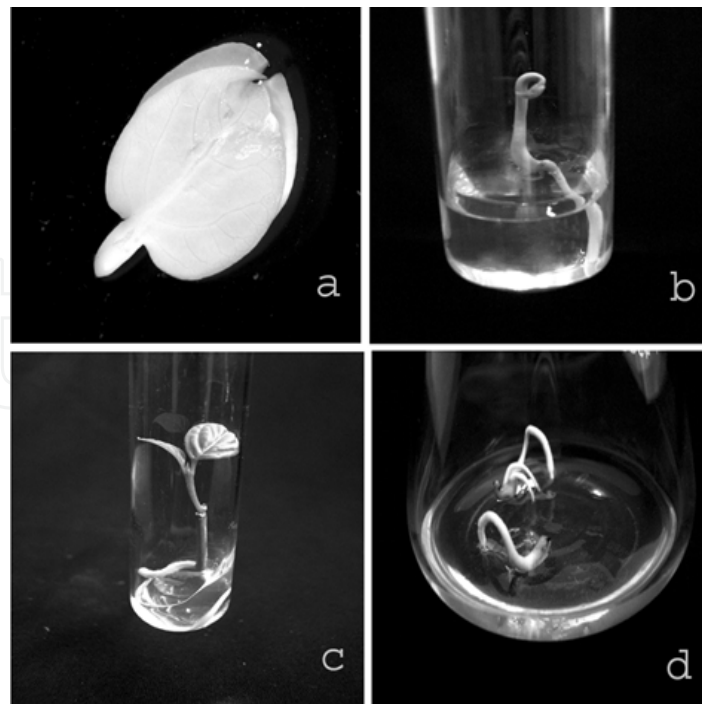


Fig.1 a. Isolated embryonic axes, b. Cryopreserved embryo showing germination after 30 days of culture on MS basal medium, c. Seedling from Cryopreserved embryo after 60 days of culture on MS basal medium and d. radicle development and degeneration of plumule in embryo subjected to desiccation for 180 min.

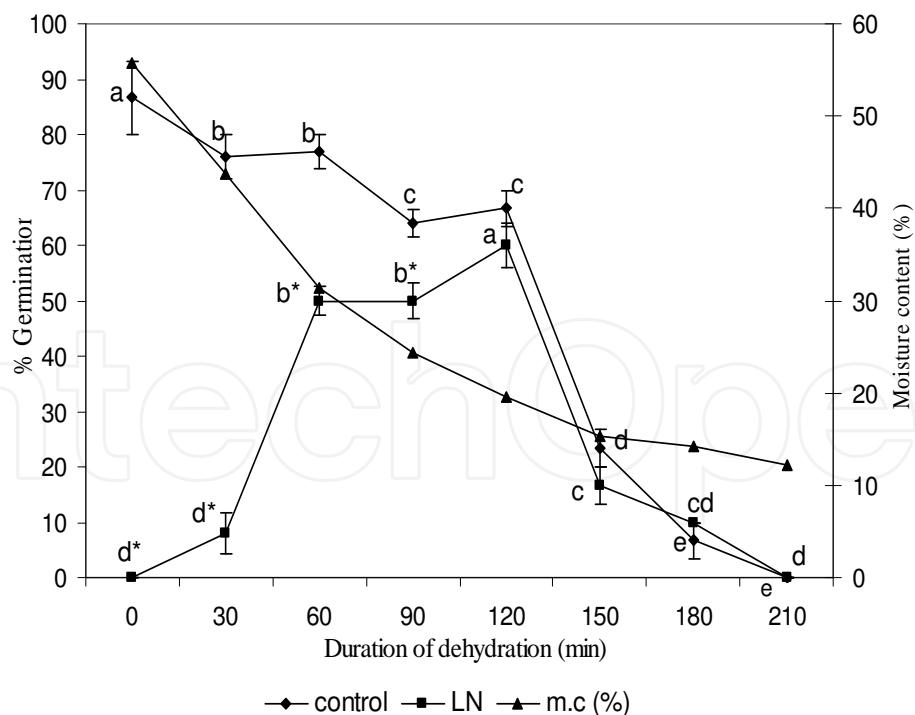


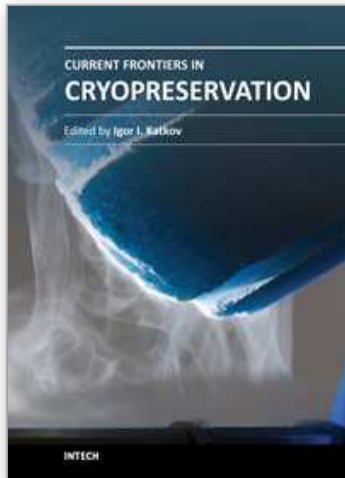
Fig. 2. Effect of cryopreservation on germination of *N. nimmoniana* zygotic embryos. Different letter (s) in a data series shows significant difference at 5% level based on LSD multiple 't' test. *Control and LN treated values differ significantly at 5% level based on Student 't' test. The bars represent SEM.

cooling and storage in LN will be progressed more easily. In most of the reports of successful cryopreservation, excised embryos or embryonic axes have been used for desiccation sensitive species, i.e. zygotic embryos of Citrus (Mumford and Grout, 1979) Oil palm (Grout *et al.*, 1983) Coconut (Chin *et al.*, 1989) Hevea (Normah *et al.*, 1986) where the embryos withstand freezing after being subjected to partial desiccation. The desiccated embryonic axes do not lose viability after rapid cooling and storage at the temperature of LN. At such temperature there should be no change in the tissue either genetic or developmental, over a period of decades (Ashwood *et al.*, 1977). This situation together with the ease to develop independent plants *in vitro* (Satheeshkumar and Seeni, 2000, Ravishankar Rai, 2002) from embryonic axes suggest cryopreservation is an effective technique for the long-term conservation of *N. nimmoniana*, a medicinal tree species producing large intermediate type of seeds.

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Almost a decade has passed since the last textbook on the science of cryobiology, *Life in the Frozen State*, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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