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Cryopreservation of Spices Genetic Resources

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1. Introduction

Plant genetic resources - constituting genotypes or populations of cultivars (landraces, advance/improved cultivars), genetic stocks, wild and weedy species, which are maintained in the form of plants, seeds, tissues, etc. - hold key to food security and sustainable agricultural development (Iwananga, 1994). They are non-renewable and are among the most essential of the world's natural resources. Due to deforestation, spread of superior varieties and selection pressure, genetic variability is gradually getting eroded. This demands priority action to conserve germplasm be it at species, gene pool or ecosystem level, for posterity (Frankel, 1975).

Whilst ecologists focused on *in situ* conservation might argue that *ex situ* conserved germplasm cannot offer the advantages afforded by selection and adaptation as a result of environmental pressures, there is no denying that if species are under threat—or worse, near extinction—then *ex situ* conservation of even limited germplasm is preferable to extinction. The opportunities offered by conservation biotechnology should not be missed or restricted by lack of interconnectivity between traditional and contemporary conservation practitioners.

2. Spices and germplasm conservation

Spices and herbs are aromatic plants—fresh or dried plant parts like foliage, young shoots, roots, bark, buds, seeds, berries and other fruits of which are mainly used to flavour our culinary preparations, confectionary. They are also major ingredients in indigenous medicine and perfumery. Spices and herbs are grown throughout the world—different plant species in different regions. Peninsular India is a rich repository of spices and over 100 species of spices and herbs are grown. The other major spice growing countries are Brazil, China, Guatemala, Indonesia, Madagascar, Nigeria, West Indies, Malaysia, Sri Lanka, Spain, Turkey, Mediterranean region and the Central America. Black pepper, cardamom, ginger, turmeric, vanilla, capsicum, cinnamon, clove, nutmeg, tamarind, coriander, cumin, fennel, fenugreek, dill, caraway, anise and herbs like saffron, lavender, thyme, oregano, celery, anise, sage and basil are important as spices. India being the native home of many spices, their conservation and characterization are one of the priority programmes. Deforestation, habitat degradation and overexploitation caused considerable loss of diversity in spices.

In many spices, conventional seed storage can satisfy most of the conservation requirements. But in crops with recalcitrant seeds and those having conservation needs cannot be satisfied by seed storage, have to be stored *in vitro*. Most field gene banks are prone to high labour cost, vulnerable to hazards like natural disasters, pests and pathogens attack (especially viruses and systemic pathogens), to which they are continuously exposed and require large areas of space. This supports *in vitro* and cryo conservation. In addition, other resources like continuous supply of standard stock cultures for experiments to examine physiological and biochemical processes, cell and callus lines developed for *in vitro* synthesis of valuable secondary products, flavours and other important compounds will benefit strongly from *in vitro* cultures. Most of the spice crops are either vegetatively propagated or have recalcitrant seeds. The spices germplasm is mostly conserved in field gene banks. Most of the spices are plagued by destructive and epidemic diseases caused by viruses, bacteria and fungi. This makes germplasm conservation in field gene bank risky. Thus *in vitro* and cryo storage system becomes important in the overall strategy of conserving genepool. Each technology should be chosen on the basis of utility, security and complementarily to other components of the strategy. A balance needs to be struck between seed, field gene bank, *in vitro* and cryo conservation of propagules, tissues, pollen, cell lines and DNA storage for overall objective of conserving gene pool.

3. Methodologies

3.1 Micropropagation

Plant regeneration and successful cloning of genetically stable plantlets in tissue culture is an important pre-requisite in any conservation effort of recalcitrant species. These techniques form the base for establishing tissue cultures and developing *in vitro* and cryo conservation technology for conservation. Simultaneously these tissue-cultured plants should be evaluated for their morphological and genetic stability in culture. The *in vitro* storage experiments, as much as possible, use growth regulators free media to reduce the rate of multiplication which in turn will reduce the extent of variation.

Micropropagation (culture initiation, multiplication, plant regeneration and *in vitro* rooting) form the cycle of events that form the backbone of cryopreservation studies. For initial culture establishment earlier protocols developed by Nirmal Babu *et al.*, 1997 can be used.

Murashige and Skoog (1962), Woody Plant (McCown and Amos, 1979) and Schenk and Hildebrandt (1972) media can be used depending upon the crop for micropropagation Table 1. The miniaturized *in vitro* grown shoots can be used for cryopreservation.

Micropropagation protocols for stable cloning of elite genotypes of spice crops were standardised. Protocols were available for black pepper and its related species cardamom, ginger, turmeric and related genera, large cardamom, kasturi turmeric, mango ginger, *Kaempferia galanga*, *K. rotunda*, *Alpinia* spp, large. Cardamom, vanilla and related species, cinnamon, camphor, cassia seed and herbal spices like lavender, celery, thyme, mint, anise, savory, spearmint and oregano (Nirmal Babu *et al.*, 1997, 2005, Mino 2002). These techniques form the base for establishing tissue cultures and developing *in vitro* technology for conservation. The basal media used are MS (Murshige and Skoog, 1962) for crops like cardamom, ginger, turmeric, kasturi turmeric, mango ginger, large cardamom, *Kaempferia*, *Vanilla* spp. seed and herbal spices and WPM-Woody Plant Medium (Mc Cown and Amos,

1979) for black pepper and its related species, cinnamon, camphor and cassia. Simultaneously these tissue-cultured plants are being evaluated for their morphological and genetic stability in culture (Luckose *et al*, 1993, Chandrappa *et al*, 1997, Nirmal babu *et al* 2003, Madhusoodanan *et al* 2005). Though micropropagation protocols were standardized using growth regulators, all the *in vitro* storage experiments were carried out using growth regulators free media to reduce the rate of multiplication which in turn will reduce the extent of variation.

Composition	Molecular formula	Concentration (mg ^l ⁻¹) MS	Concentration (mg ^l ⁻¹) WPM	Concentration (mg ^l ⁻¹) SH
Macronutrients				
Ammonium nitrate	NH ₄ NO ₃	1650.00	400.00	-
Ammonium phosphate	NH ₄ H ₂ PO ₄	-	-	300.00
Potassium nitrate	KNO ₃	1900.00	-	2500.00
Calcium chloride	CaCl ₂ .2H ₂ O	440.00	-	-
Calcium chloride	CaCl ₂	-	72.50	151.00
Calcium nitrate	Ca(NO ₃) ₂ .4H ₂ O	-	386.00	-
Potassium di hydrogen orthophosphate	KH ₂ PO ₄	170.00	170.00	-
Potassium sulfate	K ₂ SO ₄	-	990.00	-
Magnesium sulphate	MgSO ₄ .7H ₂ O	370.00	180.70	195.40
Micronutrients				
Sodium EDTA	Na ₂ EDTA	37.30	37.30	20.00
Ferrous sulphate	FeSO ₄ .7H ₂ O	27.80	27.800	15.00
Boric acid	H ₃ BO ₃	6.20	6.20	5.00
Manganese sulphate	MnSO ₄ .4H ₂ O	22.30	22.30	10.00
Potassium iodide	KI	0.83	-	1.00
Zinc sulphate	ZnSO ₄ .7H ₂ O	8.60	8.60	1.00
Sodium molybdate	Na ₂ MoO ₄ .2H ₂ O	0.25	0.25	0.10
Copper sulphate	CuSO ₄ .5H ₂ O	0.025	0.25	0.20
Cobalt chloride	CoCl ₂ .6H ₂ O	0.025	-	0.10
Vitamins				
Myo-inositol	C ₆ H ₁₂ O ₆	100.00	100.00	1000
Thiamine HCl	C ₁₂ H ₁₇ CIN ₄ OS.HCl	0.10	0.50	100
Nicotinic acid	C ₆ H ₅ NO ₂	0.50	0.025	1.00
Pyridoxine HCl	C ₆ H ₁₁ NO ₃ .HCl	0.50	0.025	1.00
Amino acid				
Glycine	C ₂ H ₅ NO ₂	2.00	1	-

*Murashige and Skoog, 1962, McCown and Amos, 1979, Schenk and Hildebrandt 1972

Table 1. Composition of MS*, WPM* and SH* basal media

Protocols are available for micropropagation and multiplication of many endangered species like *Piper hapnium*, *P. silent vallyensis*, *P.schmidtii*, *P. wightii*, *P. barberi*, *Vaniilla aphylla*, *V. pilifera*, *V. walkyrie*, *V. wightiana*, *K. rotunda* and *Alpinia galanga* are available (Peter *et al* 2002, Mino 2002, Nirmal Babu *et al* 1999, 2005).

Bertaccini *et al* (2004), Du *et al* (2004) reported micropropagation and establishment of mite-brone virus-free garlic.

3.2 Callus and cell culture systems

Quatrano (1968) and Nag and Street (1973) reported the first successful experiments on cryopreservation of plant cells. Since then a large number of cell suspension and calli cultures have been successfully cryopreserved (Engelmann *et al* 1994). In general, callus cultures are more difficult to cryopreserve than cell suspensions, because of the relative volume of the callus, its slow growth rate and the cellular heterogeneity (Withers 1987). One successful cryopreservation procedure that is applicable to all different cell suspensions or calli cultures has not been developed yet. Research focuses on optimizing the factors on which successful cryopreservation of plant organs cells suspensions and calli depends, such as: (i) starting material, (ii) pretreatment, (iii) cryopreservation procedure, and (iv) post-thaw treatment.

Plant cells cultured *in vitro* produce wide range of primary and secondary metabolites of economic value. Production of phytochemicals from plant cell cultures has been presently used for pharmaceutical products. Production of flavour components and secondary metabolites *in vitro* using immobilised cells is an ideal system for spices crops. Production of saffron and capsaicin was reported using such system (Ravishankar *et al.*, 1988; 1993, Johnson *et al.*, 1996; Venkataraman and Ravishankar 1997). Johnson *et al* (1996) reported biotransformation of ferulic acid vanillamine to capsaicin and vanillin in immobilised cell cultures of *Capsicum frutescens*. Reports on the *in vitro* synthesis of crocin, picrocrocin and safranal from saffron stigma (Himeno and Sano, 1995) and colour components from cells derived from pistils (Hori *et al*, 1988) are available for further scaling up. Callus and cell cultures were established in nutmeg, clove, camphor, ginger, lavender, mint, thyme, celery etc. Cell immobilization techniques have been standardized in ginger, sage, anise and lavender (Ilahi and Jabeen, 1992; Ravindran *et al*, 1996; Sajina *et al*, 1997).

Studies on conservation of cell lines is yet to become popular in spices. Suspensions of embryogenic cell lines of fennel, conserved at 4 °C for up to 12 weeks produced normal plants upon transfer to normal laboratory conditions (Umetsu *et al*, 1995).

3.3 Somatic embryogenesis and plant regeneration

In black pepper primary embryogenic cultures can be established as per the method described by Nair and Dutta Gupta (2003). Culture the surface sterilized seeds on agar gelled full-strength, PGR-free SH (Schenk and Hildebrandt, 1972) medium containing 3.0% (W/V) sucrose under darkness. Primary somatic embryos (PEs) derived from micropylar tissues of germinating seeds after 90 days could be utilized for inducing secondary somatic embryogenic cultures.

Primary somatic embryo clumps having pre-globular to torpedo shaped embryos (5–6 visible embryos per seed) were carefully detached and inoculated on half strength PGR-free SH medium containing 1.5 % sucrose and gelled with 0.8% agar (Bacteriological grade, Himedia). The pH of the medium was adjusted to 5.9 prior to autoclaving. Cultures were maintained at darkness at a temperature of 25±2°C. The culture conditions remained the same for all further experiments unless otherwise specified. While inoculating, the PEs were uniformly spread on the surface of the medium. Secondary embryogenic cultures were further maintained by subculturing on SH medium containing 1.5% sucrose at intervals of 20 d. The proliferating SEs were spread periodically on the surface of the medium, to facilitate proliferation.

3.4 Pollen storage

Pollen storage can be considerable value supplementing the germplasm conservation strategy by facilitating hybridisation between plants with different time of flowering and to transport pollen across the globe for various crop improvement programmes in addition to developing haploid or homozygous lines. No significant work was done in India, except a few initial reports.

The technique of pollen storage is comparable with that of seed storage, since pollen can be dried (less than 5% moisture content on a dry weight basis) and stored below 0°C. There are limited reports on the survival and fertilizing capacity of cryopreserved pollen more than five years old. Pollen might represent an interesting alternative for the long-term conservation of problematic species (IPGRI, 1996). However, pollen has a relatively short life compared with seeds (although this varies significantly among species) and viability testing can be time-consuming and uneconomical. Other disadvantages of pollen storage are the small amount produced by many species, the lack of transmission of organelle genomes via pollen, the loss of sex-linked genes in dioecious species and the general inability to regenerate into plants. Pollen, therefore, has been used to a limited extent in germplasm conservation (Hoekstra, 1995). An advantage is that pests and diseases are rarely transferred by pollen (excepting some virus diseases). This allows safe movement and exchange of germplasm as pollen.

3.5 Cryo preservation

For long-term conservation of the problem species, cryopreservation is the only method currently available. Dramatic progress has been made in recent years in the development of new cryopreservation techniques and cryopreservation protocols have been established for over 100 different plant species.

Cryopreservation is an attractive option for long-term storage. Liquid nitrogen (-196°C) is routinely used for cryogenic storage, since it is relatively cheap and safe, requires little maintenance and is widely available. Below -120°C the rate of chemical or biophysical reactions is too slow to cause biological deterioration (Kantha 1985). Only in the long term might there be a small risk of ionising radiation causing genetic changes in materials stored at cryogenic temperatures (Grout 1995).

An array of plant material could be considered for cryopreservation as dictated by the actual needs *vis-a-vis* preservation. These include meristems, cell, callus and protoplast cultures, somatic and zygotic embryos, anthers, pollen or microspores and whole seeds (Withers, 1985; Kantha, 1985).

Plant germplasm stored in liquid nitrogen (-196°C) does not undergo cellular divisions. In addition, metabolic and most physical processes are stopped at this temperature. As such, plants can be stored for very long time periods and both the problem of genetic instability and the risk of losing accessions due to contamination or human error during subculturing are overcome. Most cryopreservation endeavours deal with recalcitrant seeds, *in vitro* tissues from vegetatively propagated crops, species with a particular gene combination (elite genotypes) and dedifferentiated plant cell cultures. Care must be taken to avoid ice crystallisation during the freezing process, which otherwise would cause physical damage

to the tissues. The existing cryogenic strategies rely on air-drying, freeze dehydration, osmotic dehydration, addition of penetrating cryoprotective substances and adaptive metabolism (hardening), encapsulation, vitrification or combinations of these processes.

Cryopreservation methods have been developed for more than 80 different plant species in various forms like cell suspensions, calluses, apices, somatic and zygotic embryos (Kantha and Engelmann, 1994; Engelmann, 1997, 2000, Engelmann *et al* 1994, 1995). However, their routine utilisation is still restricted almost exclusively to the conservation of cell lines in research laboratories

For small volumes, long-term storage is practicable through storage of cultures in cryopreservation at ultra-low temperature, usually by using liquid nitrogen (-196°C). At this temperature all cellular divisions and metabolic processes are virtually halted and consequently, plant material can be indefinitely stored without alteration or modification.

The normal approach of tissue culture is to find a medium and set of conditions that favour the most rapid rate of growth with a subculture interval of 20 – 30 days. For cryopreservation storage biological materials are stored in liquid nitrogen for long term with out subculturing. Cryopreservation, i.e., the storage of biological material at ultra low temperature usually that of liquid nitrogen (-196°C) can be achieved by different techniques like direct freezing, encapsulation- dehydration, encapsulation- vitrification and vitrification.

3.5.1 Encapsulation - Dehydration

A simplified methodology for vitrification is given below (Yamuna 2007).

The *in vitro* plants already established were used as mother plants for source of explants. This in turn facilitates the reduction in size of the plantlets and smaller somatic embryos which made them suitable for cryopreservation.

1. Suspend *in vitro* grown shoots/ somatic embryos in MS basal medium supplemented with 4% (w/v) Na alginate, 2M Glycerol and 0.4 M sucrose.
2. Drop the mixture containing microshoots, with a sterile pipette into 0.1M CaCl₂ solution containing 2M Glycerol and 0.4M sucrose and left for 20 min to form beads about 4 mm in diameter, each bead containing at least one shoot.
3. Preculture the encapsulated shoots – stepwise - on MS medium enriched with different concentration of 0.3, 0.5, 0.75 and 1.0M for four days with one day on each.
4. Place the precultured beads on sterile filter paper in Petridishes (diameter 90mm) and dehydrated by air drying on a flow bench (at room temperature and humidity) for periods of 0-10 h to determine the optimal dehydration time.
5. Measure the water content of the beads was by weighing them prior and after drying in an oven at 80°C for 48h.
6. Transfer the dehydrated beads into a 2 ml cryovial (ten beads per tube) and directly immerse in liquid nitrogen for 24h.

3.5.2 Vitrification

A simplified methodology for vitrification is given below (Yamuna 2007).

1. Shoots (1-2mm)/ somatic embryos were excised and cultivated on MS medium supplemented with 0.3 M sucrose for 24h at 25°C.

2. The treated explants were then cultured on MS medium supplemented with sucrose at 0.75 M for 1 day in the same conditions.
3. After pretreatments explants were transferred to a cryovial with 1.8 ml of loading solution (2 M Glycerol + 0.4 M sucrose) and kept for 15 min.
4. Different incubation periods in PVS2 (40-100 minutes) were tested for osmoprotected explants
5. Cryovials containing 8-10 explants were directly immersed in liquid nitrogen and kept for 24 h.

3.5.3 Encapsulation – Vitrification

A simplified methodology for encapsulation - vitrification is given below (Yamuna 2007).

1. Suspend pre-cultured shoots (1-2mm)/ somatic embryos with 2-3 apical domes on 0.3M sucrose for 16h in MS basal medium supplemented with 4% sodium alginate and 0.3 M sucrose.
2. Dispense the mixture including shoots, were with a sterile pipette into MS medium supplemented with 0.1M CaCl₂ and 0.4 to 1.0M sucrose, with or without 2M Glycerol gently shaken (20 rpm) on a rotary shaker for 1h at 25°C.
3. The encapsulated and osmo-protected shoots were dehydrated with 20 ml PVS2 in a 100 ml Erlenmeyer flask at 25°C and plunged into LN and held for at least 24 h at -196°C.

3.6 Thawing and recovery of conserved materials

After LN storage, cryovials warm rapidly in a 40 °C water bath for 2-3 minutes. The solution was drained from the cryovials and replace twice at 10 min intervals with 1 ml 1.2 M sucrose solution in the case of encapsulation- vitrification and vitrification methods. The composition of recovery medium was MS/WPM/SH basal medium supplemented with 2.22 – 4.44 µM and BA, 2.69- 5.37 µM NAA.

In the Encapsulation - dehydration, Encapsulation - vitification and vitrification procedures, surviving shoots can be identified by greening of explants following 2 weeks of post culture. Regrowth can be defined as the shoots that regenerated to shoots in 6 weeks of postculture. Elongated shoots can be used for micropropagation and rooting and subculture was done every 4 weeks. For rooting well grown shoots can be transferred to solid MS medium used for multiplication.

3.7 Genetic stability of conserved materials

An important prerequisite for any conservation technique is that the regenerants produced from the conserved material should be true-to-type. There are ample evidences to indicate that under certain culture conditions the materials undergo genetic changes (somaclonal variations) and as a consequence lose their integrity and uniformity. This would be highly undesirable in spices varieties where the purpose is not only to conserve a genotype but also retain its specific quality traits. Thus testing for the genetic stability of *in vitro* conserved materials is of utmost importance. Besides morphology, cytology and isozyme profiling sophisticated biochemical and DNA-based techniques have enabled more critical analysis of the genetic stability of *in vitro* materials.

RAPD, ISSR and SSR analysis can be done to evaluate genetic fidelity of the cryopreserved lines of Spices. DNA isolation can be done as per CTAB method (Ausubel *et al.*, 1995 or Sambrook *et al.* 1989). RAPD and ISSR, SSR profiles were developed as per the method suggested by Williams *et al.*, (1990), Nirmal babu *et al.*, (2003, 2007) and Ravindran *et al.*, (2004).

Morphological characters coupled with RAPD profiles using 24 operon primers have indicated genetic fidelity among randomly selected micropropagated plants of Subhakara and Aimpriyan, indicating that micropropagation protocol can be used for commercial cloning of black pepper (Nirmal Babu *et al.*, 2003). Genetic uniformity of micropropagated *Piper longum* using RAPD profiling was reported by Ajith (1997) and Parani *et al.* (1997) for conservation.

Peter *et al* (2001) and Ravindran *et al* (2004) reported that the conserved materials of all the species conserved by them showed normal rate of multiplication when transferred to multiplication medium after storage. The normal sized plantlets when transferred to soil established with over 80% success. They developed into normal plants without any deformities and were morphologically similar to mother plants. RAPD profiling of these conserved plants also showed their genetic uniformity.

Ravindran *et al* (2004), Yamuna *et al* (2007) and Yamuna (2007) reported genetic uniformity was observed in cryo preserved and recovered plants of cardamom, ginger, black pepper and endangered species of *Piper*, *P. barberi* based on RAPD and ISSR profiling.

4. Status of cryo conservation in spices

Reports on cryopreservation of spices are meager and limited. The present status of cryo preservation in major spices is given Table 2. The number of accessions conserved in cryo genebank at the National Bureau of Plant Genetic Resources (NBPGR), New Delhi are given in Table 3.

Application	Technique	Reference
Black pepper (<i>Piper nigrum</i>) and related species	Meristem culture	Philip <i>et al.</i> , 1992
Disease eradication propagation	Shoot Culture Leaf/root	Broome and Zimmerman, 1978
Cryopreservation	Seeds	Chaudhury and Chandel, 1994
Cryopreservation	synseeds	Ravindran <i>et al.</i> , 2004; Nirmal Babu <i>et al.</i> , 2007; Yamuna 2007
Cryopreservation	Seed	Decruse and Seeni, 2003
Slow growth storage and cryopreservation	Plantlets and shoot tips	Ravindran <i>et al.</i> , 2004; Nirmal Babu <i>et al.</i> , 2007; Yamuna 2007
<i>Allium</i> Spp		
Disease eradication	Meristem culture and themotherapy	Conci and Nome, 1991
Cryopreservation	Shoot culture,	Keller 1991

Application	Technique	Reference
	microbullbets	
Cryopreservation	Shoot tips	Niwata 1995
Cardamom (<i>Elettaria cardamomum</i> Maton)		
Disease eradication	Meristem culture	Nadagauda <i>et al.</i> 1983
Cryopreservation	Seeds	Chaudhury and Chandel, 1995
Slow growth storage and cryopreservation	Plantlets and shoot tips	Ravindran <i>et al.</i> , 2004; Nirmal Babu <i>et al.</i> , 2007; Yamuna 2007
Zingiber spp.		
Disease eradication	Shoot cultures, shoot buds	Balachandran <i>et al.</i> ,1990
Propagation	Somatic embryo regeneration	Hosoki and Sagawa ,1977, Nirmal Babu, 1997
Cryopreservation	Synseeds	Sharma <i>et al.</i> , 1994
Slow growth storage and cryopreservation	Plantlets and shoot tips	Ravindran <i>et al.</i> , 2004; Nirmal Babu <i>et al.</i> , 2007; Yamuna <i>et al</i> 2007 ; Yamuna 2007
Curcuma spp		
Slow growth storage and cryopreservation	Plantlets and shoot tips	Ravindran <i>et al.</i> , 2004 ; Nirmal Babu <i>et al.</i> , 2007
Vanilla spp.		
Disease eradication	Apical meristem	Cereveta and Madrigal, 1981
Cryo preservation	Synthetic seeds	Ravindran <i>et al.</i> , 2004
Pollen Cryo preservation	Pollen	Minoo, 2002; Minoo <i>et al</i> 2011
Slow growth storage and cryopreservation	Plantlets and shoot tips	Ravindran <i>et al.</i> , 2004; Nirmal Babu <i>et al.</i> , 2007, Minoo and Babu 2009
Herbal spices		
Slow growth storage	<i>In vitro</i> plantlets	Nirmal Babu <i>et al.</i> 1996
Capsicum		
Cryopreservation	Seed	Peter <i>et al</i> 2002 ; Ravindran <i>et al</i> 2004
Cryopreservation	Pollen	Alexander <i>et al.</i> , 1991
Cryopreservation	Pollen	Rajasekharan and Ganeshan, 2003
Fennel (<i>Foeniculum vulgare</i>)		
Cold storage	Embryogenic	Umetsu <i>et al.</i> , 1995

Application	Technique	Reference
	suspension cells	
Coriander (<i>Coriandrum sativum</i>)		
Cryopreservation	somatic embryos	Elena <i>et al.</i> , (2010)
Mint (<i>Mentha spp.</i>)		
Cryopreservation	Somatic embryos	Leigh and Remi 2003
Ocimum spp		
Slow growth	Encapsulated beads	Mandal <i>et al</i> (2000)
<i>Syzygium francissi</i>		
	Shoot tips	Shatnawi <i>et al</i> (2004).
<i>Armoracia rusticana</i>		
Cryopreservation	Hairy root cultures	Phunchindawan <i>et al</i>
Crocus spp.		
	Encapsulated calluses	Chand <i>et al</i> (2000); Baghdadi <i>et al.</i> , (2010)

*Ashmore, 1997, 2002 and Nirmal Babu *et al*, 1999, 2007, Yamuna 2007; Yamuna *et al* 2007

Table 2. Present status of information on cryo conservation of spices

Species	No.of accessions
Maintained as in vitro cultures	
Spices and industrial crops	380 accessions (7 genera, 27 species)
Medicinal and Aromatic plants	169 accessions (21 genera, 28 species)
Maintained in cryo bank	
Spices and Condiments	148 accessions
Medicinal and Aromatic plants	5 accessions
Total	702

Source: Annual Report NBPGR 2010-11

Table 3. Present status of Spices in *in vitro* and Cryo genebank at NBPGR

4.1 Black pepper and related species

Cryopreservation of black pepper (*Piper nigrum* L.) seeds in liquid nitrogen (LN₂) was reported by Choudhary and Chandel, (1994), and Choudhury and Malik (2004). Pepper seeds are recalcitrant and the seed viability decreases with reduction in moisture content. Seeds desiccated to 12% & 6% moisture contents were successfully cryopreserved in liquid nitrogen at -196°C, with a survival rate of 45% & 10.5% respectively (Chaudhury and Chandel 1994).

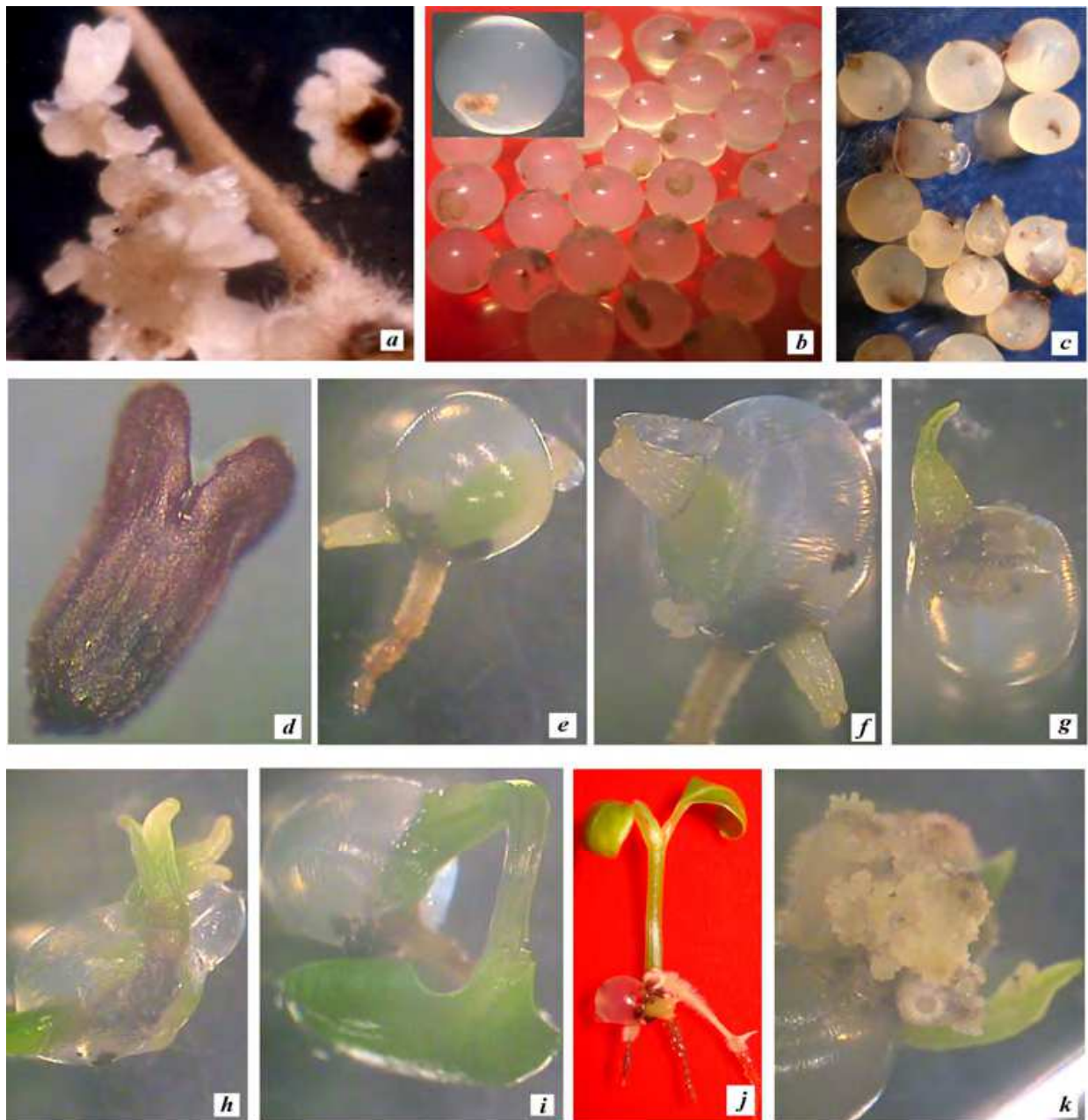


Fig. 1. Cryopreservation of black pepper somatic embryos by encapsulation dehydration. a) Somatic embryos used for cryopreservation, b) Somatic embryos encapsulated in Na-alginate, c) Encapsulated and dehydrated somatic embryos, d) Viable somatic embryo stained in red colour after cryopreservation, e), f), g), h) & i) Various stages of development of somatic embryos to plantlet after cryopreservation, j) Fully developed plantlet from a somatic embryo cryopreserved by encapsulation dehydration, k) A cluster of somatic embryos at different stages of development, originated from an embryogenic line after cryopreservation

Yamuna (2007) reported the effect of encapsulation-dehydration and vitrification methods on survival of cryo preserved somatic embryos in black pepper. In encapsulation dehydration treatment, the best survival rates (62 %) of somatic embryos was obtained after freezing, by preculturing in 0.7 M sucrose (direct) for 1 day, followed by dehydration in the

laminar air flow for 6 h which resulted in 21 % moisture content. In the vitrification procedure, the somatic embryos were precultured for 3 days on SH basal medium containing 0.3 M sucrose and subjected to vitrification treatment for 60 minutes at 25°C resulted in 71 % survival after cryopreservation. The study concluded that the embryogenic lines of *Piper nigrum* cultivar karimunda can be successfully cryopreserved following an encapsulation dehydration/desiccation procedure (62 % success). This success rate can be enhanced to 71 % using a vitrification/one step freezing in liquid nitrogen (Fig. 1). This was mainly because of the nature of somatic embryos which is more suitable to cryopreservation compared to shoot buds. The genetic stability of the conserved somatic embryos was proved by RAPD and ISSR profiling. Cryopreservation of encapsulated shoot buds of endangered *Piper barberi* was reported by Peter *et al* (2001) and Ravindran *et al* (2004).

Encapsulated shoot tips of *Piper barberi* were cryopreserved with 60% success using vitrification technique. In encapsulation vitrification the encapsulated shoot tips were precultured on MS medium, supplemented with 0.3 M, 0.5 M and 0.7 M sucrose (pH 5.8) for three days followed by dehydration with PVS2 solution (100%) at 0°C for 3 hours. After dehydration the beads (10 encapsulated shoot tips in 0.8 ml PVS2 solution per 1.5 ml cryotube) were frozen rapidly by direct immersion in to liquid nitrogen (-196 °C) and kept for one hour (Peter *et al* 2001 and Ravindran *et al* 2004). Yamuna 2007 also reported that studies on cryopreservation of endangered *P.barberii* shoot tips revealed that, the encapsulation- vitrification procedure produced higher survival (70 %) of cryopreserved shoot tips (Fig. 2) compared to encapsulation - dehydration which gave 40 % survival. Genetic fidelity studies showed that the regenerated plants were similar to the controls. Thus encapsulation - vitrification as a simple and efficient method for long term preservation of *P.barberii* propagules.

4.2 Cardamom and related species

Choudhary and Chandel (1995) attempted cryo-conservation of cardamom (*Elettaria cardamomum* Maton.) seed. They tried to conserve seeds at ultra-low temperature by suspending seeds in cryovials in vapor phase of liquid nitrogen (-150°C) by slow freezing and also by direct immersion in liquid nitrogen (-196°C) by fast freezing. The result showed that seeds possessing 7.7-14.3% moisture content could be successfully cryo-preserved with 80% germination when tested after one-year storage in vapor phase of liquid nitrogen (at -150°C).

Shoot tips(1.0-2.0mm) from *in vitro* grown plantlets of cardamom were subjected to progressive increase of sucrose concentrations (0.1, 0.3, 0.5, 0.7, 0.9, and 1.0) for two days each under the same cultural conditions as the parent plantlets. These shoot tips were transferred to 1.8ml cryotube containing ice cold PVS2 solution (30%(v/v) glycerol + 15% (v/v) ethylene glycol + 15% (v/v) DMSO in culture medium with 0.4 M sucrose, pH (5.8)) at 0°C for 3 hours. After 3 hours equilibration at 0°C, the shoot tips were directly immersed into liquid nitrogen for 1 hour. Vials were thawed in 40°C water for 1 minute. The cryoprotectant was removed and the shoot tips were washed 2-3 times in 1.2M sucrose solution. About 70% Shoot tips were recovered on MS medium supplemented with BAP and NAA. But the encapsulation vitrification method gave only 60% success (Ravindran *et al* 2004).

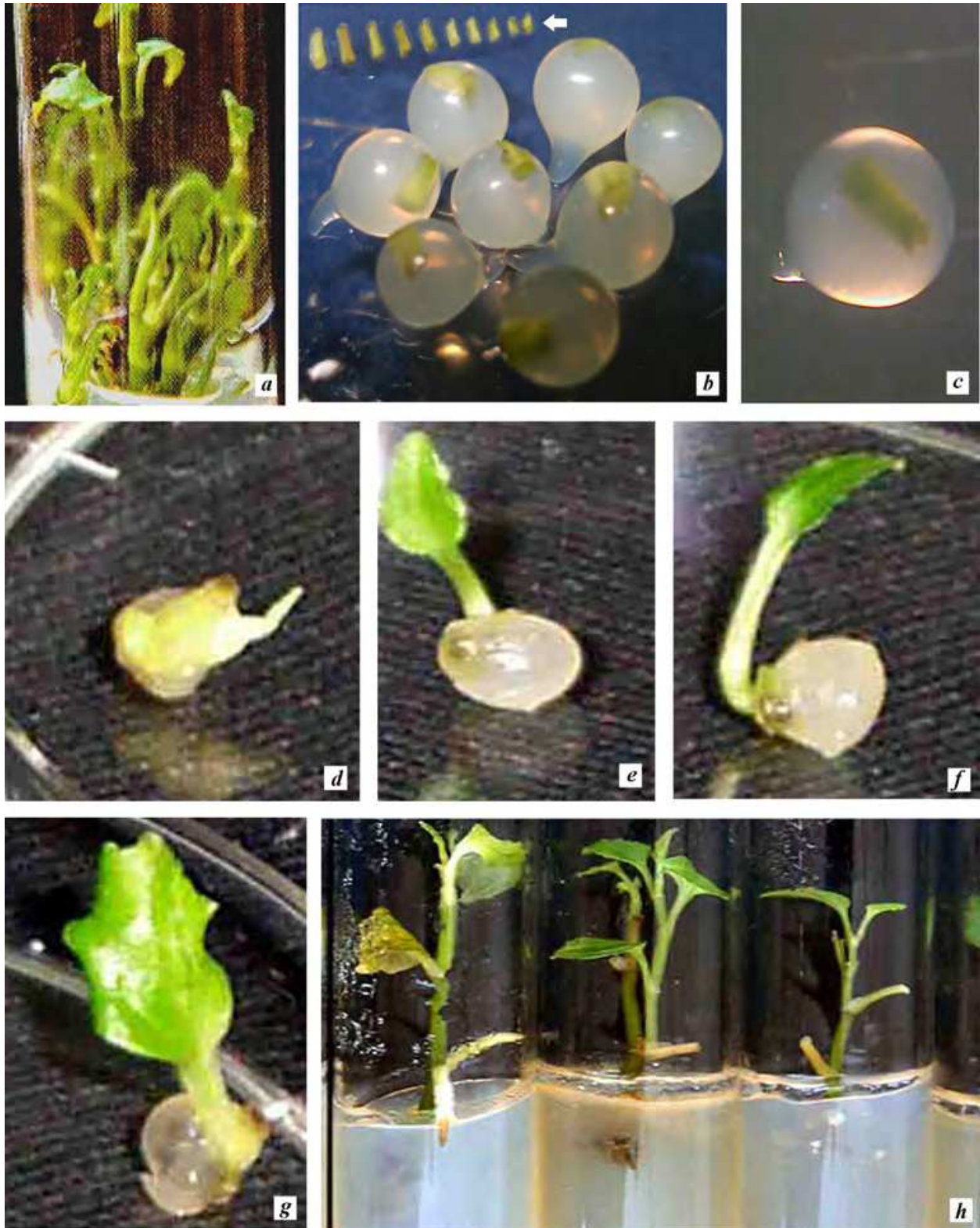


Fig. 2. Cryopreservation of *Piper barberi* by encapsulation vitrification. a) *In vitro* culture of *P. barberi*, b) & c) Shoot tips encapsulated in Na-alginate, arrow indicates shoot tip used as explants, d), e), f) & g) Various stages of development of cryopreserved shoot tips after post culturing, h) Regenerated plantlets after 3 months of post culturing

Yamuna (2007) tested the effect of encapsulation - dehydration, encapsulation vitrification and vitrification methods on cryopreservation of cardamom. In the vitrification treatment, to enhance tolerance to vitrification solution (PVS2), a two step sucrose preculture with 0.3 M and 0.75 M sucrose for one day each and an osmo protection step with a loading solution (LS) of 2 M glycerol and 0.4 M sucrose were performed prior to PVS2 treatment. The shoots

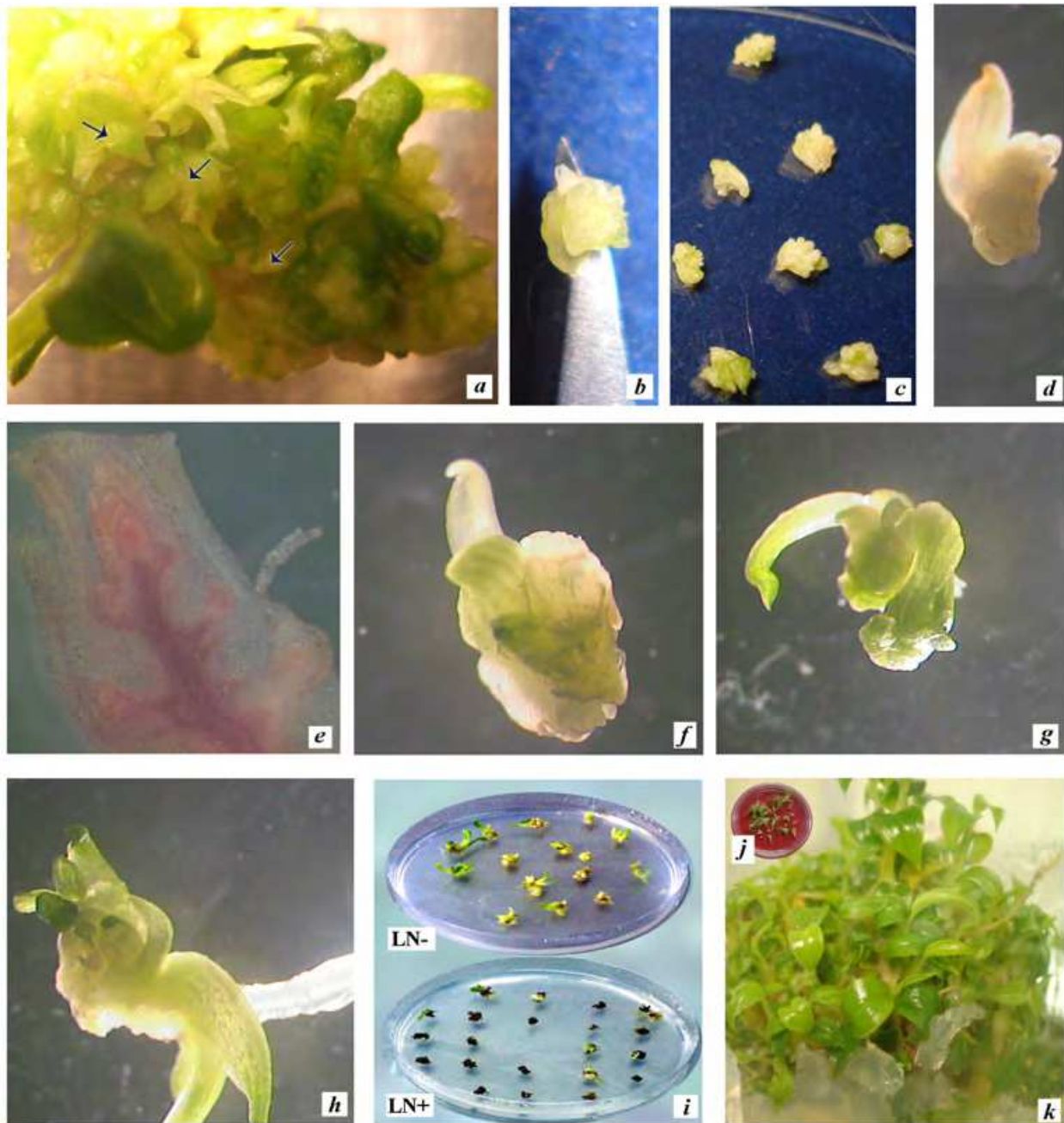


Fig. 3. Plant regeneration from cryopreserved miniature shoots of cardamom by vitrification. a) Cardamom culture with miniature shoots, b) & c) Excised meristematic clumps used for cryopreservation, d) Explant turned brown after cryopreservation, e) Viable tissues stained in TTC after cryopreservation, f), g), h), & i) Shoot development after 10, 14 and 25 days of post culturing, j) regenerating shoot buds in a petridish, k) Development of multiple shoots after 4 months of post culturing

dehydrated with PVS2 for 60 min retained a high level of shoot formation (70 %). The vitrification procedure resulted in higher regrowth (70 %) (Fig.3) when compared to encapsulation vitrification (62 %) and encapsulation dehydration (60 %). In all the three cryopreservation procedures tested, shoots grew after cryopreservation without intermediary callus formation. The genetic stability of cryopreserved cardamom shoots were confirmed using ISSR and RAPD profiling.

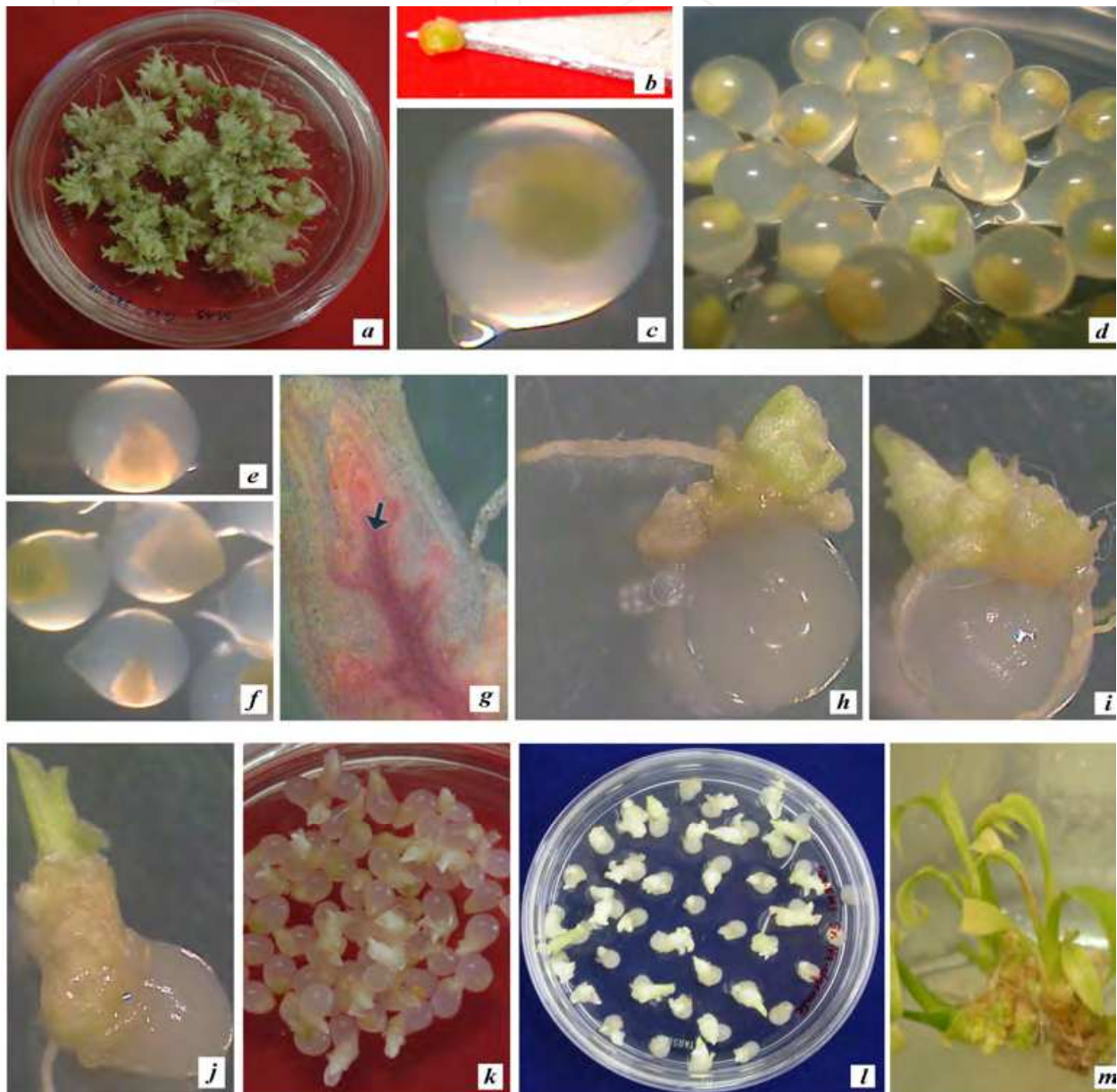


Fig. 4. Plant regeneration from cryopreserved shoot buds of ginger by encapsulation vitrification. a) *In vitro* culture, b) A typically excised shoot bud used for cryopreservation, c) & d) Shoot buds encapsulated in Na-alginate, e) & f) Shoot buds turned brown after thawing, g) Viable apical dome stained in red colour after liquid nitrogen storage (TTC staining), h) Regenerating shoot bud 20 days after post culturing, i) & j) Elongated shoot with no intermediary callus formation, k) & l) Regenerating shoot buds in petriplates, m) Plantlets regenerating from cryopreserved shoot bud

4.3 Ginger, turmeric and related species

Cryopreservation of Ginger (*Zingiber officinale* Rosc) and turmeric (*Curcuma longa* L.) shoot tips was successfully done with 80% of recovery using vitrification method. But the rate of recovery was only 40% when encapsulated shoot tips were dehydrated in progressive increase of sucrose concentration together with 4- 8 hrs. of desiccation (Peter *et al* 2001 and Ravindran *et al* 2004).

Efficient cryopreservation techniques were developed for *in vitro* grown shoots of ginger based on encapsulation dehydration, encapsulation vitrification and vitrification procedures (Yamuna *et al* 2007 and Yamuna 2007. The vitrification procedure resulted in higher re-growth (80 %) when compared to encapsulation vitrification (66 %) and encapsulation dehydration (41 %). The genetically stability of shoot apices was confirmed by molecular profiling. The RAPD and ISSR assays performed suggested that no genetic aberrations originated in ginger plants during culture and cryopreservation (Fig. 4).

4.4 Vanilla and related species

Technology for cryopreservation of vanilla germplasm - using encapsulation and vitrification methods - were available. Encapsulated *in vitro* grown shoot tips of vanilla could be cryo preserved with 70% success when pretreated with progressive increase of sucrose concentration (0.1M-1.0M) for one day each and dehydrated for 8 hrs (Peter *et al* 2001; Mino 2002 and Ravindran *et al* 2004) (Fig. 5).

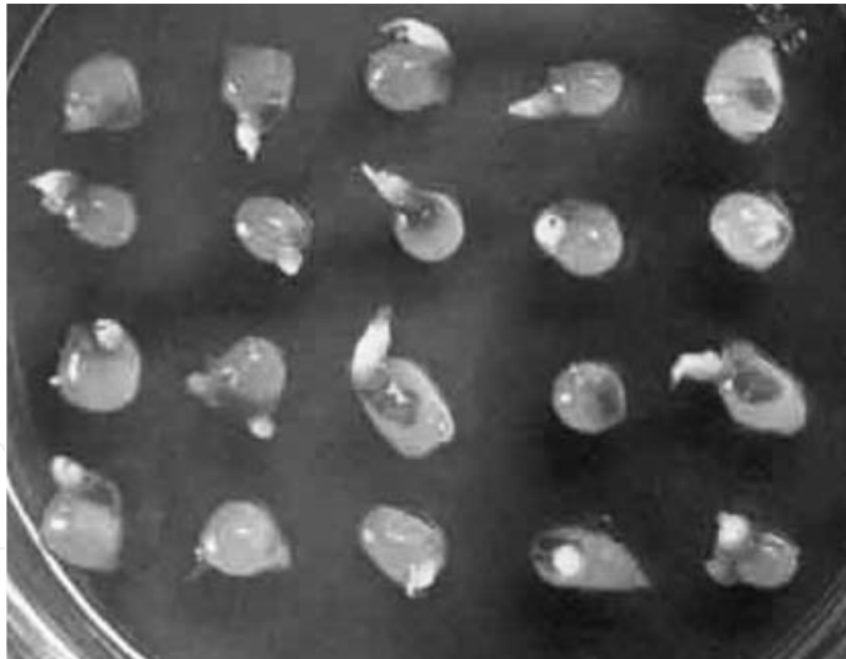


Fig. 5. Germination of cryopreserved encapsulated shoot tips protocorms of vanilla

Ginzalez-Arno, *et al.*, (2009) attempted to cryo-preserve *V. planifolia* Andr. using *in vitro* fragmented explants (IFEs) and the apices derived from them. Cryopreservation of apices from *in vitro* grown plants was achieved using the droplet vitrification protocol. Maximum survival (30%) and further regeneration (10%) of new shoots were obtained for apices derived from clusters of *in vitro* plantlets produced from microcuttings through a three-step droplet vitrification protocol: 1-d preculture of apices on solid MS medium with 0.3 M

sucrose; loading with a 0.4 M sucrose + 2 M glycerol solution for 20–30 min; and exposure to plant vitrification solution PVS3 for 30 min at room temperature.

Minoo (2002) reported cryopreservation of vanilla pollen for conservation (Fig. 6) of haploid genome as well as assisted pollination between species that flower at different seasons and successful fertilisation using cryopreserved pollen (Minoo, 2002, Minoo *et al* 2011). Pollen from two asynchronously flowering species of *Vanilla viz.*, cultivated *V. planifolia* and its wild relative *V. aphylla*, were cryopreserved after desiccation to 12 % moisture content, pretreated with cryoprotectant Dimethyl sulphoxide (5%) and cryopreserved -196°C in Liquid Nitrogen. This cryopreserved pollen was latter thawed and tested for their viability both *in vitro* and *in vivo*. A germination percentage of 82.1% and 75.4% in *V. planifolia* and *V. aphylla* pollen respectively were observed indicating their viability (Fig.6). This cryopreserved pollen of *V. planifolia* was used successfully to pollinate *V. aphylla* flowers resulting in fruit set (Fig.7). The seeds thus obtaines were sussfully cultured to develop hybrid plantlets. This system is of great importance and can be used for conserving the haploid gene pool of *Vanilla* in cryobanks and their subsequent utility in crop improvement (Fig. 6 and 7)



Fig. 6. Germination of cryopreserved Vanilla pollen



Fig. 7. Fruit set after pollination with cryopreserved pollen

4.5 Capsicum

Plants could be successfully regenerated (Fig 8) from cryopreserved seeds of capsicum (Peter *et al* 2001 and Ravindran *et al* 2004). Alexander *et al* (1991) and Rajasekharan and Ganeshan. (2003) reported freeze preservation of capsicum pollen (*Capsicum annuum*) in liquid nitrogen (-196°C) for 42 months.



Fig. 8. Successful germination of cryopreserved seeds of capsicum

4.6 Seed herbal and other spices

Elena *et al.*, (2010) successfully cryopreserved coriander (*Coriandrum sativum* L.) somatic embryos using sucrose pre-culture and air desiccation procedure utilized embryo clumps (ECs). The regrowth after cryopreservation and average number of new embryos developed from cryopreserved ECs were retained at the level of the untreated control (98% and 13 embryos per clump, respectively). Both normal and abnormal plants were produced from control and cryopreserved cultures, indicating that appearance of abnormalities was not related to cryopreservation. The regenerants with normal phenotype showed the same peaks of relative DNA content regardless of cryopreservation. The results suggest that simple desiccation method is effective for cryopreservation of coriander somatic embryos with subsequent regeneration. Plants could be regenerated from cryopreserved seeds of Anise. (Peter *et al* 2001).

Successful Cryopreservation of seeds, meristems, somatic or zygotic embryos were reported in *Allium* Spp (Niwata, 1995, Hyung *et al* 2003, Haeng *et al* 2003, 2004, 2005, Jung *et al* 2005, Gayle *et al* 2004). Preliminary success was reported in cryo preservation of Mint (Leigh and Remi 2003).

Most of the reports are confined to a few genotypes and hence the techniques standardized needs to be extended to more genotypes before adopting them for routine conservation. Reports of cryoconservation of spices like *Ocimum*, *Lavendula*, *Salvia* are available from National Bureau of Plant Genetic Resources (NBPGR), New Delhi.

Mandal *et al* (2000) reported propagation and conservation of four pharmaceutically important herbs, *Ocimum americanum* L. syn. *O. canum* Sims. (hoary basil); *O. basilicum* L. (swett basil); *O. gratissimum* L. (shrubby basil); and *O. sanctum* L. (sacred basil) using synthetic seed technology. Synthetic seeds were produced by encapsulating axillary vegetative buds harvested from garden-grown plants of these four *Ocimum* species in calcium alginate gel. The gel contained Murashige and Skoog (MS) nutrients and 1.1-4.4 μM benzyladenine (BA). Shoots emerged from the encapsulated buds on all six planting media tested. However, the highest frequency shoot emergence and maximum number of shoots per bud were recorded on media containing BA. Of the six planting media tested, both shoot and root emergence from the encapsulated buds in a single step was recorded on growth regulator-free MS medium as well as on vermi-compost moistened with halfstrength MS medium. Rooted shoots were retrieved from the encapsulated buds of *O. americanum*, *O. basilicum*, and *O. sanctum* on these two media, whereas shoots of *O. gratissimum* failed to root. The encapsulated buds could be stored for 60 d at 4°C. Plants retrieved from the encapsulated buds were hardened off and established in soil.

An efficient procedure for the *in vitro* propagation and cryogenic conservation of *Syzygium francissi* was developed by Shatnawi *et al* (2004). Shoot tips excised from *in vitro*-grown plants were successfully cryostored at -196°C by the encapsulation-dehydration method. A preculture of formed beads on MS medium containing 0.75 M sucrose for 1 d, followed by 6 h dehydration (20% moisture content) led to the highest survival rate after cryostorage for 1h. This method is a promising technique for *in vitro* propagation and cryopreservation of shoot tips from *in vitro*-grown plantlets of *S. francissi* germplasm.

Hairy root cultures of *A Armoracia rusticana* Gaertn. Mey. et Scherb. (horseradish) were successfully cryopreserved by two cryogenic procedures (Phunchindawan *et al.*, 1997). Encapsulated shoot primordia were precultured on solidified Murashige-Skoog medium supplemented with 0.5M sucrose for 1 day and then dehydrated with a highly concentrated vitrification solution (PVS2) for 4 h at 0°C prior to a plunge into liquid nitrogen. The survival rate of encapsulated vitrified primordia amounted to 69%. In a revised encapsulation-dehydration technique, the encapsulated shoot primordia were precultured with a mixture of 0.5M sucrose and 1M or 1.5M glycerol for 1 day to induce dehydration tolerance and then subjected to air-drying prior to a plunge into liquid nitrogen. The survival rate of encapsulated dried primordia was more than 90%, and the revived primordia produced shoots within 2 weeks after plating. A long-term preservation of shoot primordia was also achieved by the technique. Thus, this revised encapsulation-dehydration technique appears promising as a routine method for the cryopreservation of shoot primordia of hairy roots

The effect of sucrose concentration and dehydration period on survival and regrowth of encapsulated calluses were also studied in 2 species of *Crocus* (Chand *et al* 2000). Highest survival (83.3; 88.9%) and regrowth (77.6; 83.3%) rates were obtained when encapsulated unfrozen calluses of *Crocus hyemalis* and *C. moabiticus* precultured with 0.1 M sucrose for two days without further air dehydration. After cryopreservation, the highest survival (55.6; 61.1%) and regrowth (16.7; 27.8%) rates were achieved when calluses of *C. hyemalis* and *C. moabiticus* were pretreated with 0.5 M sucrose for two days after two hours of dehydration. Viability of crocus decreased with increased sucrose concentration and dehydration period. Dehydration of encapsulated calluses of *C. hyemalis* and *C. moabiticus* with silica gel for one hour prior to freezing resulted in maximum rates of survival (77.8; 83.3%) and re-growth

(33.3; 72.1%). However, further studies should be initiated to improve regrowth of surviving embryogenic calluses and to study genetic stability after cryopreservation.

5. DNA bank

Concurrent with the advancements in gene cloning and transfer has been the development of technology for the removal and analysis of DNA. DNAs from the nucleus, mitochondrion and chloroplast are now routinely extracted and immobilized onto nitrocellulose sheets where the DNA can be probed with numerous cloned genes. In addition, the rapid development of polymerase chain reaction (PCR) now means that one can routinely amplify specific oligonucleotides or genes from the entire mixture of genomic DNA. These advances, coupled with the prospect of the loss of significant plant genetic resources throughout the world, have led to the establishment of DNA bank for the storage of genomic DNA.

The conserved DNA will have numerous uses viz, molecular phylogenetics and systematics of extinct taxa, production of previously characterized secondary compounds in transgenic cell cultures, production of transgenic plants using genes from gene families, *in vitro* expression and study of enzyme structure and function and genomic probes for research laboratories.

The vast resources of dried specimens in the world's herbaria may hold considerable DNA that would be suitable for PCR. It seems likely that the integrity of DNA would decrease with the age of specimens. Because there are many types of herbarium storage environments, preservation and collections, there is a need for systematic investigations of the effect of modes of preparation, collection and storage on the integrity of DNA in the world's major holdings.

The advantage of storing DNA is that it is efficient and simple and overcomes many physical limitations and constraints that characterize other forms of storage (Adams 1988, 1990, 1997, Adams and Adams 1991, Adams *et al* 1994). The disadvantage lies in problems with subsequent gene isolation, cloning and transfer but, most importantly, it does not allow the regeneration of live organisms (Maxted *et al.*, 1997). DNA banking is yet to catch up in spices. DNA samples of over 600 genotypes of spices is stored in the DNA bank of Indian Institute of Spices Research (IISR), Calicut.

6. Future focus

In contrast to the prevailing attitude among conservation biologists, globally there is considerable interest among cryobiologists in the use of *in vitro*, cold and ultra-cold technology for germplasm conservation. The procedures for plant material are given in-depth coverage by Reed *et al.* (2004) who stress equally the ecological and plant/germplasm health aspects preceding and following storage. Panis and Lambardi (2006) discussed the evolution of technologies for plant material, covering cell suspensions and callus cultures of herbaceous species, pollen, shoot meristems, woody species, as well as seed and embryonic axes. The *ex situ* gene bank at Gatersleben in Germany houses 986 potato accessions are cryopreserved and trials on other species are performed (Börner 2006). The National Bureau of Plant Genetic Resources (NBPGR), New Delhi has over 702 accession of various spices, medicinal and aromatic crops in its cryo gene bank (Table. 3). Keller *et al.* (2008) make the point that cryopreservation affords the best of conditions for the long-term maintenance of

plant material, particularly for vegetatively propagated species. Cryopreservation is the only viable method available for long-term preservation of the both plant and animal origin species. As an ultimate aim of cryoconservation is the reintroduction of preserved material into the field, it is appropriate at this point to consider the concept of restoration a little more closely. In terms of ultimate ecosystem restoration, the possibilities raised by *in vitro* conservation, including cryoconservation, do not mean that species selection should merely take random advantage of what germplasm has or can be conserved as there are many genetic, physiological and phenotypic considerations to be taken into account (Kramer and Havens 2009).

The establishment and maintenance of biological resource centers (BRCs) or germplasm conservatories requires careful attention to implementation of reliable preservation technologies and appropriate quality control to ensure that recovered cultures and other biological materials perform in the same way as the originally isolated culture or material. There are many types of BRC that vary both in the kinds of material they hold and in the purposes for which the materials are provided. All BRCs are expected to provide materials and information of an appropriate quality for their application and work to standards relevant to those applications. There are important industrial, biomedical, and conservation issues that can only be addressed through effective and efficient operation of BRCs in the long term. This requires a high degree of expertise in the maintenance and management of collections of biological materials at ultra-low temperatures, or as freeze dried material, to secure their long-term integrity and relevance for future research, development, and conservation. The application of cryogenic preservation in biotechnology and medicine has recently been a topic of interest. The use of cryogenic preservation in this area has given new horizon to this field of applications.

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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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