STUDIES ON CRYOPRESERVATION OF SPICES GENETIC RESOURCES

Thesis submitted to

University of Calicut

for the award of **Doctor of Philosophy** in Biotechnology

By

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UNIVERSITY OF CALICUT

Calicut, Kerala, India

2007

CERTIFICATE

I hereby certify that the thesis entitled 'Studies on Cryopreservation of Spices Genetic Resources' submitted by Yamuna George for the award of the degree of Doctor of Philosophy in Biotechnology of the University of Calicut contains the results of bonafide research work done by her during 2003-2007 at Indian Institute of Spices Research, Calicut, Kerala under my supervision and guidance. No part of this work has been submitted to any other university for the award of any other degree or diploma. All sources of help received by her during the course of this investigation have been duly acknowledged. Certified that she has also passed the required qualifying examination.

Place: Calicut
Date: 07.11.2007

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Declaration

I hereby declare that this thesis entitled 'Studies on Cryopreservation of Spices Genetic Resources' submitted by me for the award of the degree of Doctor of philosophy in Biotechnology of the University of Calicut, contains the results of bonafide research work done by me at Indian Institute of Spices Research, Calicut, under the guidance of Dr. K. Nirmal Babu (Principal Scientist, Indian Institute of Spices Research). This thesis or part of it has not been submitted to any other university for the award of any other degree or diploma. All sources of help received by me during the course of this work have been duly acknowledged.

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a constant source of inspiration and support all through my life. Last but not least, to my husband and son for all their love and support.

Finally I would like to extend my truthful gratitude and sincere gratefulness to the Almighty God, who enabled me to accomplish this effort successfully.

Yamuna George

List of Abbreviations Used

Abbreviation	
ANOVA	Analysis of Variance
BA	Benzyl adenine
CTAB	Cetyl Trimethyl Ammonium Bromide
DNTP’s	
EDTA	Ethylene diamine tetra acetic acid
g / l	Gram per litre
IBA	Indole 3 butyric acid
Kb	Kilobase
LSD	Least significant difference
M	Molar
mg/ l	Milligram per litre
ml	milliliter
mm	Millimetre
mM	Millimole
min	Minutes
NAA	α - naphthalene acetic acid
PCR	Polymerase chain reaction
RAPD	Random Amplified Polymorphic DNA
SD	Standard deviation
SE	Standard Error
TAE	Tris Acetic acid EDTA
Taq	Thermophile aquaticus
TE	Tris EDTA
UV	Ultra violet
V	Volume
W/v	Weight/ volume
2,4-D	2,4 - dichlorophenoxy acetic acid
µM	Micromole
µl	Microlitre
µg	Microgram
d	Days
SEs	Somatic embryos
PEs	Primary embryos
MS	Murashige & Skoog 1962
WPM	Woody Plant Medium
SH	Schenk & Hildebrandt
Na alginate	Sodium alginate
s	Seconds
PVS2	Plant vitrification solution 2
LN	Liquid nitrogen
min	Minutes
TTC	Triphenyl tetrazolium chloride
En De	Encapsulation dehydration
En Vi	Encapsulation vitrification
RAPD	Random Amplified Polymorphic
ISSR	DNA
DMSO	Inter simple sequence repeats
MC	Dimethyl sulfoxide
LS	Moisture content
	Loading solution

Synopsis

Reg. Order No. CDC/B2/2320/2001 dated 18.02.2003
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Studies on Cryopreservation of Spices Genetic Resources

By Yamuna George

India is blessed not only with rich biological diversity but also with the associated indigenous knowledge. The country is one of the twelve mega diversity centres and harbors 3 of the 28 global hotspots (Chauhan, 1996). It is estimated that up to 100,000 plants, representing more than one third of all the world's plant species, are currently threatened or face extinction (BGCI, 2005). Preservation of the plant biodiversity is essential for plant improvement and provides various compounds to the pharmaceutical, food and crop protection industries. India is considered as the land of spices, over 53 major spices are grown. Spices like black pepper, chilli, ginger, turmeric, cardamom, fennel, fenugreek, coriander and cumin form the economic backbone of large number of people in India.

Cardamom, *Elettaria cardamomum* Maton, considered the "Queen of Spices", is a large, herbaceous, rhizomatous perennial, belonging to the family *Zingiberaceae.* Cardamom is native to the moist evergreen forests of the Western Ghats of Southern India and is propagated both through seeds and clonally through

suckers. Being a cross-pollinated, seeds are heterozygous. Ginger, *Zingiber officinale* Rosc. also belongs to the family *Zingiberaceae* is not known to occur in the truly wild state but is under cultivation since ancient times. Ginger is believed to have originated in Southeast Asia and is propagated only through vegetative means. India is the largest producer of ginger with rich cultivar diversity (Lawrence, 1984). India, is also the center of origin and diversity for black pepper (*Piper nigrum* L, family *Piperaceae*) considered the 'king of spices,' one of the most widely used spice in the world. Pepper is predominantly propagated using stem cuttings as seeds are heterozygous and seed progenies are not true to type. *Piper barberi*, a closely related species, is very rare and is reported to be almost extinct (Subramanyam and Henry, 1970).

Conservation of germplasm in seed genebanks by storage of desiccated seeds at low temperature is the most efficient, economical and preferred. But this method is not applicable to crops that do not produce seed (e.g., ginger) or with recalcitrant seed (cardamom & black pepper) as well as to plant species that are propagated vegetatively to preserve the unique genomic constitution of cultivars. Conservation in clonal field repositories is the cheapest alternative. But the field collections are exposed to risks of pests, diseases and adverse weather conditions in addition to being labour-intensive. These risks can be mitigated using *in*

vitro conservation strategy which is also labour-intensive. Somaclonal variation is another factor to be considered and at best *in vitro* conservation can be used only for medium term conservation. Hence, cryopreservation or freeze-preservation at ultra-low temperature (-196°C, i.e., the temperature of liquid nitrogen) is a sound additive to conventional *ex situ* approaches for the long-term conservation of base collections of plant genetic resources, since under these conditions, biochemical and most physical processes are completely arrested and the plant material can be stored for unlimited periods (Withers & Engelmann, 1997).

Efficient technologies for cryopreservation of germplasm in cardamom, ginger and black pepper are not yet optimized. Only one report each are available on cryo conservation of black pepper and cardamom, using seeds (Chaudhury & Chandel, 1994; 1995). Heterozygous nature of seeds in these crops makes these conservation techniques not suitable for germplasm conservation. In the present study cryopreservation were standardized using shoot buds/ somatic embryos for optimizing the true to type long term conservation of cardamom, ginger & black pepper germplasm. The genetic integrity of cryopreserved plants was confirmed using molecular markers - RAPD and ISSR.

Cryopreservation of cardamom

Aseptic shoot buds from *in vitro* cultures of cardamom were cryopreserved by three different techniques based on encapsulation dehydration, encapsulation vitrification and vitrification.

In encapsulation dehydration procedure, shoots were first encapsulated in 4 % sodium alginate and to improve survival after cryopreservation, 0.3 M – 1.0 M sucrose preculture and desiccation in the laminar air flow chamber for 10 h were added. The maximum survival (60 %) was detected when the water content was at 20 % with 8 hrs of dehydration in the laminar air flow chamber.

In encapsulation vitrification treatment, after preculturing with 0.3 M sucrose for 16 h, dehydration tolerance to PVS2 was induced by treating the shoots with 2 M glycerol and 0.6 M sucrose. Encapsulated and osmoprotected cardamom shoots were dehydrated with PVS2 for 2 h at 25^0C and plunged into LN and held for at least 24 h at -196^0C. The cryopreserved shoot was then thawed at 40^0C and cultured on MS + BA (1mg/l) + NAA (0.5mg/l) for post thaw recovery. The encapsulation vitrification treatment resulted in 62 % survival of cryopreserved cardamom shoots.

In the vitrification experiment, 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 dehydrated with PVS2 for 60 min retained a high level of shoot formation (70 %). The vitrification procedure resulted in higher regrowth (70 %) 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.

Thus vitrification procedure was the best for cryopreservation of cardamom shoot buds with 70 % success. This is 20 % higher than earlier reported success of seed conservation. Moreover using clonal material ensures genetic fidelity of conserved material.

Cryopreservation of ginger

An efficient cryopreservation technique for *in vitro* grown shoots of ginger *(Zingiber officinale* Rosc) was developed based on encapsulation dehydration, encapsulation vitrification and vitrification procedures. In the encapsulation dehydration method, ginger shoots consisting of the apical dome with 3-4 leaf primordia were encapsulated in 4 % sodium alginate and subsequently precultured in liquid MS medium containing 0.1, 0.3, 0.5, 0.7, 0.9

and 1.0 M sucrose for 24 h each and were dehydrated in laminar flow cabinet (at room temperature and humidity) for periods of 0-10 h before plunging to liquid nitrogen. The best results in terms of cryopreserved ginger shoot recovery (41 %) were obtained by preculturing in progressive increase of sucrose together with 6 h of dehydration in the laminar air flow chamber.

In encapsulation vitrification treatment, ginger shoot buds were encapsulated in 4 % sodium alginate and to enhance the osmotolerance of ginger shoots in the vitrification solution, the shoot buds were preincubated with 0.1 M sucrose for 24 h after encapsulation and precultured with 0.3 M sucrose for 16 h at room temperature. The precultured and preincubated shoots were then treated with 2 M glycerol and 1.6 M sucrose for 3 h at 25^{0}C before being dehydrated with PVS2 for 40 min at 25ºC. Here, the survival rate of ginger shoots (66 %) was higher than with the encapsulated and dehydrated method.

In the vitrification procedure shoots were: precultured in liquid Murashige-Skoog medium containing 0.3 M sucrose for 3 days; cryoprotected with a mixture of 5% DMSO and 5% glycerol for 20 min at room temperature; osmoprotected with a mixture of 2 M glycerol and 0.4 M sucrose for 20 min at 25ºC; before being dehydrated with a highly concentrated vitrification solution (PVS2) for 40 min at 25ºC. The dehydrated shoots were transferred to 2 ml

cryotubes, suspended in 1 ml PVS2 and plunged directly into liquid nitrogen. In all the three cryopreservation procedures tested, shoots grew from cryopreserved shoot tips without intermediary callus formation. The vitrification procedure resulted in higher regrowth (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. In conclusion, these results show that the three step vitrification procedure can be successfully applied for cryopreservation and recovery of genetically stable ginger shoots and thus for the conservation of ginger germplasm.

Thus vitrification procedure was the best for cryopreservation of ginger shoot buds with 80 % success. This is more than 30 % higher than earlier reported success of ginger shoot buds using encapsulation dehydration method. Moreover using clonal material ensures genetic fidelity of conserved material.

Cryopreservation of black pepper

In black pepper the published studies in this area have used seeds for cryopreservation (Chaudhury and Chandel, 1995) which are highly heterogenous and considered to be recalcitrant.

Cryopreservation of somatic embryos could be useful for reliable long term maintenance of genetic resources and this also reduce somaclonal variation (Cyr, 2000). The present study describes the successful cryopreservation of somatic embryos of black pepper through encapsulation dehydration and vitrification procedures.

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 vitrfication treatment for 60 minutes at 25^0C resulted in 71 % survival after cryopreservation.

In conclusion, this study shows that the embryogenic lines of *Piper nigrum* cultivar karimunda can be successfully cyopreserved following an encapsulation desiccation procedure (62 % success). This success rate can be enhanced to 71 % using a vitrification/one step freezing in liquid nitrogen. 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 *Piper barberi*

Developing *in vitro* and cryopreservation protocol will help in conserving this endangered species. Procedures for micropropagation and *in vitro* conservation were already available (Nirmal Babu *et al*., 1992). In the present study cryopreservation technologies were developed for conservation of *P.barberi* lines

In the encapsulation dehydration procedure, apical and axillary shoot meristems with 2-3 leaf primordia of *P.barberi* (about 0.8 - 1.5 mm in size) were encapsulated in 4 % (w/v) sodium alginate with 2 M glycerol and 0.4 M sucrose and were subjected to direct and stepwise preculture with sucrose concentrations ranging from 0.25 – 1.25 M. The highest survival rate 40 % was obtained when shoot tips were encapsulated in 4 % (w/v) sodium alginate with 2 M glycerol and 0.4 M sucrose and were subjected to stepwise preculture with sucrose concentrations ranging from 0.25 – 0.75 M (the final concentration 0.75 M) at 20 % of water content at 6 h of dehydration.

In the encapsulation vitrification method, survival of cryopreserved *P.barberi* shoot tips increased with increasing sucrose concentrations and the highest survival rate (70 %) was obtained when encapsulated shoot tips were precultured on basic medium using stepwise increasing sucrose concentrations to reach the final concentration 0.75 M which were then were treated with a

loading solution of 2 M glycerol and 0.6 M sucrose for 60 min, followed by dehydration with plant vitrification solution 2 (PVS2) for 3 h at 25^0C prior to direct immersion in liquid nitrogen for 48 h.

In conclusion with the cryopreservation of *P.barberi* shoot tips, the encapsulation vitrification procedure produced higher survival (70 %) of cryopreserved shoot tips compared to encapsulation dehydration which gave 40 % survival as calculated based on the total number of shoot tips treated. Genetic fidelity studies showed that the regenerated plants were similar to the controls. Results here indicate encapsulation vitrification as a simple and efficient method for long term preservation of *P.barberi* plants.

Thus in the present study cryopreservation protocols developed based on encapsulation dehydration, encapsulation vitrification and vitrification procedures, for the long term storage of spice germplasm like cardamom, ginger, black pepper and *P.barberi* are simple and efficient. In all the three cryopreservation procedures tested, plants grew from cryopreserved explants without intermediary callus formation. The genetic stability of cryopreserved plants were confirmed using ISSR and RAPD profiling and the present study of evaluating different primers (RAPD and ISSR) for amplification of genomic DNA in these spices

can also form a basis for the use of these primers for other genetic studies like DNA fingerprinting.

REFERENCES

BGCI (2005). In: *http://www.bgci.org/*

Chauhan K P S 1996 Legal requirements for fulfilling obligations under the convention on biological diversity in India. Indian J. Plant Genetic Resources. 9(2): 183–191.

Chaudhury R & Chandel K P S 1994 Germination studies and cryopreservation of seeds of black pepper (*Piper nigrum* L.) – a recalcitrant species. CryoLetters. 15 : 145 – 150.

Chaudhury R & Chandel K P S 1995 Studies on germination and cryopreservation of cardamom (*Elettaria cardamomum* Maton.) seeds. Seed Sci. & Technol. 23 : 235 –240.

Cyr D R 2000 Cryopreservation: roles in clonal propagation and germplasm conservation of conifers, in Engelmann F & Takagi H (eds) Cryopreservation of tropical plant germplasm. Current Research Progress and Application, JIRCAS, IPGRI, Rome, Italy, pp.261-268.

Lawrence B.M 1984 Major tropical spices: Ginger (Zingiber officinale Rose.). Perfumer & Flav. 9 : 1-40.

Nirmal Babu K, Nair R R, Johnson George K & Ravindran P N 1992 *Piper barberi* Gamble – a redescription of the species with a note on the karyotype.J.Spicea and Aromatic Crops 1(1):88-93.

Subramanyam K & Henry A N 1970 Rare or little known plants from South India. Bull. Bot. Surv. India. 12(1-4):1-5.

India is blessed not only with rich biological diversity but also with the associated indigenous knowledge. The country is one of the twelve mega diversity centres and harbors 3 of the 28 global hotspots (Chauhan, 1996). It is estimated that up to 100,000 plants, representing more than one third of all the world's plant species, are currently threatened or face extinction (BGCI, 2005). Preservation of the plant biodiversity is essential for plant improvement and provides various compounds to the pharmaceutical, food and crop protection industries. Plant genetic resources which include land races, primitive cultivars, advanced/improved varieties and wild relatives of crop plants hold the key to food security and sustainable agricultural development (Iwananga, 1994).

Conservation of germplasm in seed genebanks by storage of desiccated seeds at low temperature is the most efficient, economical and preferred. But this method is not applicable to crops that do not produce seed (e.g., ginger) or with recalcitrant seed (cardamom & black pepper) as well as to plant species that are propagated vegetatively to preserve the unique genomic constitution of cultivars. Conservation in clonal field repositories is the cheapest alternative. But the field collections are exposed to risks of pests, diseases and adverse weather conditions in addition to being labour-intensive. These risks can be mitigated using *in*

vitro conservation strategy which is also labour-intensive. Somaclonal variation is another factor to be considered and at best *in vitro* conservation can be used only for medium term conservation. Hence, cryopreservation or freeze-preservation at ultra-low temperature (-196°C, i.e., the temperature of liquid nitrogen) is a sound additive to conventional *ex situ* approaches for the long-term conservation of base collections of plant genetic resources, since under these conditions, biochemical and most physical processes are completely arrested and the plant material can be stored for unlimited periods (Withers & Engelmann, 1998).

Spices Genetic Resources

India is considered as the land of spices, over 53 major spices are grown. Spices like black pepper, chilli, ginger, turmeric, cardamom, fennel, fenugreek, coriander and cumin form the economic backbone of large number of people in India.

Cardamom, *Elettaria cardamomum* Maton, considered the "Queen of Spices", is a large, herbaceous, rhizomatous perennial, belonging to the family *Zingiberaceae.* Cardamom is native to the moist evergreen forests of the Western Ghats of Southern India and is propagated both through seeds and clonally through suckers. Being a cross-pollinated, seeds are heterozygous. Serious diseases of viral and bacterial origin, such as katte, Nilgiri necrosis and Azhukal disease threaten crop cultivation as well as field

germplasm repositories (Venugopal, 2001). Cardamom is also infested by various insects and nematode pests, among which thrips (*Sciothrips cardamomi* Ramk.) shoot and capsule borer (*Conogethes punctiferalis* Guen.) root grub (*Basilepta fulvicorne* Jacoby) and root knot nematode (*Meloidogyne incognita* Kofoed et White) is important (Premkumar and Madhusoodanan, 1995, Ramana and Eapen, 1992). Hence development of alternate strategies for conservation is important.

Ginger, *Zingiber officinale* Rosc. also belongs to the family *Zingiberaceae* is not known to occur in the truly wild state but is under cultivation since ancient times. Ginger is believed to have originated in Southeast Asia and is propagated only through vegetative means. India is the largest producer of ginger with rich cultivar diversity (Lawrence, 1984). It is cultivated from time immemorial in India and China (Ravindran *et al*.,2005). Over 800 accessions of ginger germplasm are available at National Conservatory for Ginger (Ravindran *et al*.,2005) at Indian Institute of Spices Research (IISR). The major constraints involved in the conservation of the germplasm of ginger are the two soil-borne diseases- rhizome rot caused by *Pythium* spp.(*P. aphanidermatum, P. myriotylum and P. vexans*) and the bacterial wilt caused by *Ralstonia solanacearum* (*Pseudomonas solanacearum*). Added to this, infection by leaf fleck virus also poses serious problems for

conservation. These diseases are extremely difficult to control under field conditions. *In vitro* conservation of ginger germplsm is safe and complementary. Conservation of ginger germplasm under *in vitro* conditions by slow growth was standardized at IISR (Geetha, 2002). At IISR, over 100 unique accessions of ginger are conserved *in vitro* as medium-term storage of germplasm (Geetha, 2002; Nirmal Babu *et al*., 2005; Ravindran *et al*., 2005). The possibility of storage at relatively high ambient temperatures (24-29ºC) by subjecting the ginger and related taxa to stress factors was explored (Dekker *et al*., 1991). *In vitro* conservation of ginger requires subculturing at 8-12-months intervals (Geetha, 2002) and can ensure only medium-term storage and cryopreservation is more appropriate for long-term conservation of ginger germplasm.

India, is also the center of origin and diversity for black pepper (*Piper nigrum* L, family *Piperaceae*) considered the 'king of spices,' one of the most widely used spice in the world. Pepper is predominantly propagated using stem cuttings as seeds are heterozygous and seed progenies are not true to type. *Piper barberi*, a closely related species, is very rare and is reported to be almost extinct (Subramanyam and Henry, 1970). The Western Ghats is very high in endemic species, unfortunately it is also one of the most ecologically threatened areas due to large scale encroachments and human settlements that have taken place

during the past hundred years. Indian Institute of Spices Research holds the world's largest collection of pepper germplasm, which is at present conserved in clonal field repositories, where they are threatened by serious diseases.

Efficient technologies for cryopreservation of germplasm in cardamom, ginger and black pepper are not yet optimized. Only one report each are available on cryo conservation of black pepper and cardamom, using seeds (Chaudhury & Chandel, 1994; 1995). Heterozygous nature of seeds in these crops makes these conservation techniques not suitable for germplasm conservation.

For long-term conservation of the germplasm cryopreservation is the only current method without subculture conferring genetic stability with minimum space and maintenance requirements (Engelmann, 1997). Storage of clonal materials in liquid nitrogen (LN) as a base collection is the goal of many genebanks (Reed *et al*, 2000), but for efficiency and reliability each species requires the development of an appropriate cryopreservation protocol. It is important that cryopreservation is not seen as a full replacement for conventional ex situ approaches (Withers and Engelmann, 1997). It serves the field gene bank as an additional tool to improve the conservation of germplasm and can also be used for efficient pest and pathogen free germplasm exchange.

In the case of black pepper and cardamom, the few published studies in this area have used seeds (Chaudhury and Chandel, 1994; 1995). As per literature available, the most relevant strategy for long-term conservation of vegetatively propagated species, cryopreservation of shoot tips (a genetically stable plant material), has not been investigated for the above crops.

The present study aims at developing cryopreservation strategies for safe long term conservation of base germplasm in important spice crops such as black pepper, cardamom and ginger. In addition this study also attempts to the genetic fidelity of cryopreserved material by molecular markers.

Biological resources are not only essential components of our life support system but also provide raw materials for meeting human needs. India is one of the twelve mega diversity centres and harbours three of the 28 global hotspots (Chauhan, 1996). Thus India is represented by the rich germplasm of plants of agri-horticultural, medicinal and industrial values.

India is considered as the land of spices. In India 53 major spices are grown. Most of them are tropical and they play a major role in the economy of our country. Spices like black pepper, cardamom, ginger, turmeric, cinnamon, chilli, fennel, fenugreek, coriander and cumin form the economic back borne of large number of people in India. Spices are pan tropical in origin and some, especially herbal spices are temperate in distribution and are cultivated in many countries from wide variety of geographical regions.

CARDAMOM

Cardamom is a large perennial, herbaceous rhizomatous monocot, belonging to the family *Zingiberaceae.* It is a native of the moist evergreen forests of the Western Ghats of southern India, which incidentally is also the center of origin and diversity for black pepper (*Piper nigrum* L). The cardamom of commerce is the fried ripe fruit (capsules) of cardamom plant. This is often referred as the "**Queen of Spices**" because of its very pleasant aroma and

taste, and is highly valued from ancient times. It is grown extensively in the hilly regions of South India at elevations of 800-1300 m as an under crop in forest lands. Cardamom is also grown in Sri Lanka, Papua New Guinea, Tanzania and in Guatemala. It is grown on a commercial scale in Guatemala, which incidentally is also now the largest producer of cardamom.

Cardamom belongs to the genus *Elettaria,* and species *cardamomum* (Maton). The genus name is derived from the Tamil root Elettari, meaning cardamom seeds. The genus consists of about six species (Mabberly, 1987). Only *E. Cardamomum* Maton occurs in India, and this is the only economically important species. The closely related *E. ensal* (gaertn). Abeywick. ,a native of Sri Lanka, is a much larger and strudier plant; and is known as the Sri Lanka (Ceylon) wild cardamom, and its taste and flavour are far inferior to the true cardamom. The Malaysian species, *E longituba* (Ridl.) Holttum is a larger perennial herb, its flowering panicles sometimes reaching a length of over 3 m (Holttum, 1950). Its flowers appear singly, and it seems that the cincinnus stops flowering as soon as a fruit is formed. The fruit is large, and is not used. Sakai and Nagamasu (2000) listed seven species from Borneo (Indonesia). The related genera are Elettariopsis and Cyphostigma, both genera occur in Malaysia – Indonesia region.

The genus *Elettaria* consists of about six or seven species distributetd in India, Sri Lanka, Malaysia and Indonesia; only *E. Cardamomum* is economically important (Holttum, 1950; Mabberley, 1987).

Varities

Based on the nature of panicles, three varieties of cardamom are recognized (Sastri, 1952). The var. Malabar is characterized by prostrate panicle and var. Mysore possesses erect panicle. The third type var. Vazbukka is considered a natural hybrid between the two and its panicle is semi-erect or flexuous.

Germplasm

Cardamom, being a cross-pollinated crop and propagated mostly through seeds, natural variability is fairly high. An assembly of diverse genetic stocks of any crop is the raw material from which a new variety can be moulded to suit the requirements of farmers and end users. Hence, collection, conservation, evaluation and exploitation of germplasm deserve utmost importance in breeding strategies. In 1950s, two surveys were conducted in cardamom growing areas in India to record the genetic resources and wild populations (Mayne, 1951) and to understand the geographical distribution and environmental impact on cardamom (Abraham & Thulasidas, 1958). Thereafter, explorations for germplasm collection are made by as many as six

research organizations in India and the total number of accessions presently available with dfferent centers are above 1200 (Madhusoodanan *et al*., 1998; 1999; AICRPS, 2006). Earlier documentation was based on an old descriptor (Dandin *et al*., 1981) and a key for identification of various types has been formulated (Sudharshan *et al*., 1991). During 1994, a detailed descriptor for cardamom was brought out by the International Plant Genetic Resources Institute (IPGRI), Rome, Italy. Among the collections, genotypes having market characters include terminal panicle, narrow leaves, pink coloured tillers, compound panicles, elongated pedicel etc. Asexuality, cleistogamy and female sterility are a few of the variations observed among the collections.

Distribution

Cultivation of cardamom is mostly concentrated in the evergreen forests of Western Ghats in South India. Besides India, cardamom is grown as a commercial crop in Guatemala and on a small scale in Tanzania, Sri Lanka, El Salvador, Vietnam, Laos, Cambodia and Papua New Guinea (PNG). Earlier, India accounted for 70 percent of the world production and now it is 41 percent only, while Guatemala contributes around 48 percent of the present world production. The total area under cardamom in India was around 1.05,000 ha till the 80s but has subsequently reduced to the present level of around 75,000 ha. It is mainly cultivated in

the three southern states of India – Kerala, Karnataka and Tamil Nadu; contributing approximately, 60, 31 and 9 percent of total area respectively.

Cardamom is cultivated mostly under natural forest canopy, except in certain areas in Karnataka (North Kanara, Chickmagalur, Hassan) and Wynad district in Kerala where it is often grown as a subsidiary crop in arecanut or coffee gardens. The important areas of cultivation in India are Uttara Kannada, Shimoga, Hassan, Chickmagalur, and Kodagu (Coorg) districts in Karnataka state; northern and southern foot hills of Nilgiris, hill regions of Madurai, Salem and Tirunelveli, Annamalai and Coimabtore districts of Tamil Nadu state; Wynad and Idukki districts as well as in the Nelliampathy hills of Palghat district of Kerala.

Cardamom cultivation in India is restricted to the Western Ghat regions, which constitute an extensive chain of hills that lie parallel to the West Coast of peninsular India.

Crop losses caused by widespread occurrence of cardamom mosaic virus (car-MV-Katte) is a major production constraint for cardamom in India. Occurrence of cardamom necrosis virus (car-NV-Nilgiri necrosis virus) and cardamom vein clearing disease (car-VCV-Kokke kandu) in some endemic zones are also matters of concern to the cardamom industry. Major diseases such as the Capsule rot (Azhukal) and the Rhizome rot are comparatively

severe and affect crop production while the widespread leaf blight and nematode infections, lead to weakening of plants, and subsequent reduction in productivity.

Conservation of cardamon genetic resources under in situ situation does not exist, through natural population occurs in protected forest areas, especially in the Silent Valley Biosphere Reserve, where a sizable population of cardamom plants in its natural setting exists. Ex situ conservation of cardamom germplasm is being undertaken mainly by four organizations. **Table 1** gives the present holdings in these centers.

Ex situ conservation in cardamom is being maintained as field gene banks, and they are used for preliminary evaluation, maintenance as well as for characterization. Characterization involves morphological, agronomical as well as chemical characters. Many variations in morphological and chemical characters and in yield have been recorded in these collection (Zachariah & Lukose, 1992; Zachariah *et al*., 1998)

Ex situ conservation is always at risk due to a variety of reasons, mainly biotic and abiotic stress factors. The prevalence of virus diseases is a serious threat to ex situ conservation of germplasm. An alternative is *in vitro* conservation and establishment of an *in vitro* gene bank. Protocols have been

developed for *in vitro* conservation and germplasm by slow growth (Geetha, 2002)

***In vitro* conservation**

In vitro conservation is an alternative method for medium-term conservation. *In vitro* gene bank will be a safe alternative in protecting the genetic resources from epidemic diseases. (Geetha *et al*., 1995; Nirmal Babu *et al*., 1994) reported conservation of cardamom germplasm in *in vitro* gene bank by slow growth. The above workers carried out various trials to achieve an ideal culture condition under which the growth is slowed down to the minimum without affecting the physiology or genetical make up of the plant. The slow growth is achieved by the incorporation of agents for increasing the osmotic potential of the medium, such as mannitol. They found that half strength MS without growth regulator and with 10 mg/l each of sucrose and mannitol was the best for *in vitro* storage of cardamom under slow growth. By using the above medium in screw capped vials the subculture interval could be extended to one year or more, when incubated in 22± 2^0C at 2500 lux of light and at 10 h photoperiod. Low temperature storage at 5^0C and 10^0C was found to be lethal for cardamom, as the cultures did not last more than three weeks (Geetha *et al*.,1995)

Cryo conservation

Cryo-conservation of cardamom seed was attempted by Choudhary and Chandel (1995). They tried to conserve seeds at ultra-low temperature by either (i) suspending seeds in cryovials in vapour phase of liquid nitrogen (- 150^0C) by slow freezing or (ii) by direct immersion in liquid nitrogen (-196^0C) by fast freezing. The result showed that seeds possessing 7.7-14.3 percent moisture content could be successfully cryopreserved and showed more than 50 percent germination when tested after one year storage in vapour phase of liquid nitrogen (at – 150^0C)

GINGER

Ginger, botanically known as *Zingiber officinale* Rosc., belongs to the family *Zingiberaceae* and in the natural order Scitamineae (Zingiberales of Cronquist, 1981). The Latin term *Zingiber* was derived from the ancient Tamil root, *ingiver*, meaning ginger rhizome.

Ginger is one of the most important and most widely used spices worldwide. Due to its universal appeal, ginger has spread to most tropical and subtropical countries from the China-India region, where ginger cultivation was prevalent probably from the days of unrecorded history. Ginger is a unique plant-a spice that is used universally. The ancient Indians considered ginger as the *mahaoushadha* (the great medicine), and it is the raw material for certain soft drinks and a variety of sweetmeats. The plant thus

possesses a combination of many attributes and properties. Ginger contains volatile oil, fixed oil, pungent compounds, resins, starch, protein and minerals. The refreshing aroma and the pungent taste makes ginger an essential ingredient of most world cuisine and of the food processing industry. Ginger powder is also an ingredient in many masala mixes. Ginger is one of the most widely used medicinal plants in the traditional Indian, Chinese and Japanese systems of medicine. According to earlier Indian system (Ayurveda), ginger is carminative and digestive. It is believed to be useful in anorexia, in dyspepsia, and for the suppression of inflammation. Dry ginger is useful in dropsy, otalgia, cephalgia, asthma, cough, colic, diarrhea, flatulence, nausea and vomiting. Ginger also has anti-platelet activity, hypolipidemic activity and an anxiolytic effect. It is an ingredient in many Ayurvedic preparations and is a folk cure for indigestion, fever, colic and any ailment associated with the digestive system.

Centres of cultivation

Ginger is not known to occur in the truly wild state. It is believed to have originated in Southeast Asia, but was under cultivation from ancient times in India as well as in China. There is no definite information on the primary center of domestication. Because of the easiness with which ginger rhizomes can be transported long distances, it has spread throughout the tropical

and subtropical regions in both hemispheres. Ginger is indeed the most widely cultivated spice (Lawrence, 1984).

The main ginger growing countries are: India, China, Jamaica, Taiwan, Sierra Leone, Nigeria, Fiji, Mauritius, Indonesia, Brazil, Costa Rica, Ghana, Japan, Malaysia, Bangladesh, Philippines, Sri Lanka, Solomon Islands, Thailand, Trinidad and Tobago, Uganda, Hawaii, Guatemala and many Pacific Ocean islands.

India is the largest producer of ginger, the annual production is about 263,170 tons from an area of about 77,610 hectares, contributing approximately 30 to 40% of the world production. The productivity is low, at about 3,428 kg/ha. Out of the total production, 10 to 15% is exported to about 50 countries around the world. The crop occupies the largest area in the state of Kerala (19 %), followed by Orissa (17 %) Meghalaya (12 %) and Arunachal Pradesh (6%). Kerala and Meghalaya together account for nearly 40% of the country's production. In terms of productivity, Arunachal Pradesh stands first with 7,164 kg/ha, followed by Meghalaya (5,139 kg/ha), Mizoram (5,000 kg/ha), and Kerala (3,428 kg/ha). During 1999-2000 India exported 8,773 tons of ginger valued at Rs. 306 million, out of which dry ginger contributed Rs. 199.2 million.

Genetic resources

The history of domestication of ginger is not definitely known. However, this crop is known to have been under cultivation and use in India and China for the last 2,000 years or even more. China is probably the region where domestication was started, but little is known about the center of origin, although the largest variability exists in China. Southwestern India, known as the Malabar Coast in ancient times, traded ginger with the Western World from ancient times, indicating its cultivation. This long period of domestication might have played a major role in the evolution of this crop that is sterile and propagated solely vegetatively. Ginger has rich cultivar diversity, and most major growing tracts have cultivars that are specific to the area; and these cultivars are mostly known by place names. Cultivar diversity is richest in China. In India the diversity is more in the state of Kerala and in the northeastern region of India. Being clonally propagated, the population structure of this species is determined mainly by the presence of isolation mechanisms and the divergence that might have resulted through the accumulation of random mutations. At present, more than 50 ginger cultivars possessing varying quality attributes and yield potential are being cultivated in India, although the spread of a few improved and high-yielding ones are causing the disappearance of the traditional land races. Some of these cultivars were introduced into India, and

the cultivar Rio de Janeiro, an introduction from Brazil, has become very popular in Kerala. Introductions such as China, Jamaica, Sierra Leone and Taffin Giwa, are also grown occasionally.

Among the ginger-growing countries, China has the richest cultivar density. Less important ones are Zaoyang of Hubei province, Zunji big white ginger of Giuzhou, Chenggu yellow ginger of Shaxi, Yulin round fleshy ginger of Guangxi, Bamboo root ginger and Mian yang ginger of Sichuan, Xuanchang ginger of Ahuii, Yuxi yellow ginger of Yunnan and Taiwan fleshy ginger. Many of these cultivars have unique morphological markers for identification.

In general, the cultivar variability is much less in other ginger growing countries. Tindall (1968) reported that there were two main types of ginger grown in West Africa. These differ in color of the rhizome, one with a purplish red or blue tissue below the outer scaly skin, whereas the other has a yellowish white flesh. Graham (1936) reported that there were five kinds of ginger recognized in Jamaica known as St. Mary, Red eye, Blue Turmeric, Bull blue and China blue. But Lawrence (1984) reported that only one cultivar is grown widely in Jamaica. According to Ridley (1912) three forms of ginger were known in Malaysia in earlier days: halyia beterl (True ginger), halyia bara, or padi, a smaller leavedginger with a yellowish rhizome used only in medicine; and halyia udang, red ginger having red color at the base of the aerial

shoot. A red variety of ginger, *Z. officinale* var. *rubra* (also called pink ginger), has been described from Malaysia, in which the rhizome skin has a reddish color. A variety “withered skin” also has been reported. In Philippines two cultivars are known, the native and the Hawaiian (Rosales, 1938). In Nigeria the cv. Taffin Giwa (Bold, yellow ginger) is the common one, the other being Yasun Bari, the black ginger.

In Japan the ginger types are classified into three groups: (1) small-sized plants with many tillers and a small rhizome, (2) medium-sized plants with an intermediate number of tillers and a medium-sized rhizome, and (3) large-sized plants with fewer tillers and larger rhizomes. The common cultivars included in these groups are Kintoki, Sanshu and Oshoga, respectively. A stabilized tetraploid line of Sanshu (4x Sanshu) is also being cultivated in Japan (Adaniya, 2001). In addition, *z. mioga* (Japanese ginger) is also grown in Japan for spice and vegetable purposes. In Queensland, Australia, ginger was an important crop in earlier times. The ginger cultivars might have been introduced there, although the exact source is not known. The local cultivar, known as Buderim local, is the most commonly grown. Australia earlier introduced cultivars from Japan, Hawaii, and India. Recently the Buderim Ginger Co. (2002) has released the first tetraploid commercial variety, called Buderim Gold, for cultivation in

Queensland. *Z. mioga,* the myoga ginger, introduced from Japan, is also grown commercially for its unopened flower buds, which are a vegetable delicacy.

In many cases, the major production centers are far from the areas of origin of the crop concerned (Simmonds, 1979). This is true of ginger as well: the Indo/Malayan region is very rich in Zingiberaceous flora (Holttum, 1950). Considering the present distribution of genetic variability, it is only logical to assume that the Indo/Malayan region is probably the major center of genetic diversity for *Zingiber*. It may be inferred that geographical spread accompanied by genetic differentiation into locally adapted populations caused by mutations could be the main factor responsible for variations encountered in cultivated ginger (Ravindran *et al.*, 1994). In India the early movement of settlers across the length and breadth of the Kerala state and adjoining regions, where the maximum ginger cultivation is found, and the story of shifting cultivation in northeastern India (the second major ginger-growing sector in India), are well-documented sociological events. The farmers invariably carried small samples of the common crops that they grew in their original place along with them and domesticated the same in their new habitat-in most cases, virgin forestlands. Conscious selection for different needs such as high fresh ginger yield, good dry recovery, and less fiber

content over the years has augmented the spread of differentiation in this crop. This would have ultimately resulted in the land races of ginger of today (Ravindran *et al*., 1994).

Conservation of ginger germplasm

Major collections of ginger germplasm are maintained at the Indian Institute of Spices Research (IISR), Calicut, India, and the Research Institute for Spices and Medicinal Crops, Bogor, Indonesia. In India serious efforts are being made for conservation ginger germplasm. At present, the ginger germplasm conservatory at IISR consists of 645 accessions that include exotic cultivars, indigenous collections, improved cultivars, mutants, tetraploids, and related species (IISR, 2002). In addition, 443 accessions are being maintained at different centers of the All India-Coordinated Research Project Spices and the National Bureau of Plant Genetic Resources (NBPGR), Regional Station, Thrissur (AICRPS, 2001). The major constraints involved in the conservation of the germplasm of ginger are the two soil-borne diseases: rhizome rot caused by *Pythium* spp. (such as *P aphanidermatum, P myriotylum*, and *P. vexans*) and the bacterial wilt caused by *Ralstonia solanacearum* (*Pseudomonas solanacearum*). Added to this, infection by leaf fleck virus is also posing serious problems for conservation. These diseases are extremely difficult to control or prevent under field conditions. Hence, in the National Conservatory for ginger at IISR,

ginger germplasm is conserved in specially made cement tubs under 50% shade, as a nucleus gene bank to safeguard the material from deadly diseases and to maintain the purity of germplasm from adulteration, which is very common in field plantings. Each year, part of the germplasm collection is planted our in the field evaluation and characterization (Ravindran *et al*., 1994). The collections are harvested every year and replanted in the next season in a fresh potting mixture. On harvesting the rhizomes, each accession is cleaned and dipped in fungicide and insecticide for protection and stored in individual brick-walled cubicles lined with sawdust or sand in a well-protected building.

***In vitro* conservation and cryopreservation of ginger germplsm**

As *in vitro* techniques are becoming more important in crop improvement through somatic cell genetics, genetic stocks are assuming more variable forms from *in vitro* plantlets to protoplasts and DNA (Withers, 1985). With due precautions, the genotypes of plants propagated by node or shoot culture can be preserved without change. This type of *in vitro* culture can therefore be used to maintain genotypes over long periods. Fortunately, several ways have been found to reduce the rate of growth of cultured material so that it can be kept unattended for moderate lengths of time.

Modifying the constituents of culture medium by decreasing the carbohydrate/nutrient supply, changing the osmotic potential using the combinations of sucrose and mannitol and withdrawal of growth regulators from the culture medium, storage in reduced light, desiccation combined with cold treatment, reduced oxygen tension, and the use of growth regulators such as absisic acid induces slow growth in many crop species.

At the IISR, ginger plantlets could be successfully conserved for extended periods of over 12 months on half strength MS basal medium supplemented with 15 gl^{-l} each of sucrose and mannitol. The cultures were sealed with aluminum foil and maintained at a temperature of 22 ± 2^0C. Thus, in ginger, minimal growth was induced by minimizing the evaporation loss using aluminum foil to seal the culture vessel, reduction of both the carbon source and the nutrients to half strength, and addition of mannitol. Ginger cultures grew well at 22 ± 2^0C bur deteriorated when kept under lower temperatures of 0^0C and 10^0C. The rate of growth was higher when full-strength MS medium was used. High concentration of sucrose (30 gl^{-l}) increased culture growth substantially, resulting in exhaustion of culture medium. When the concentration of sucrose was reduced to 20 gl^{-l} and nutrient concentration to half, the cultures could be maintained for a much longer period of 200 to 240 days with a survival percentage of 75 to 81 depending upon

the closure used. Addition of mannitol (10 to 15 gl^{-1}) and reduction of sucrose to lower levels (15 to 10 gl^{-1}) induced slow growth, and subsequently 73 to 80 percent of the cultures could be maintained for a period of 360 days when the culture vessels were closed with aluminum foil. Full- or half-strength MS medium supplemented with 10 or 15 gl^{-1} each of sucrose and mannitol and 1/2 MS with 20 gl^{-1} sucrose and 10 gl^{-1} mannitol allowed the cultures to be maintained for 360 days (Nirmal Babu, 1997; Nirmal Babu *et al*., 1999;2000; Geetha, 2002; Peter *et al*., 2002).

According to Balachandran *et al*. (1990), ginger cultures could be maintained up to 7 months without subculture by using polypropylene caps as culture vessel enclosures. Dekkers *et al* (1991) reported that ginger shoots could be maintained for over 1 year at ambient temperatures (24 to 29^0C) in a medium containing mannitol (25 gl^{-1}) with an overlay of mineral oil.

At present, over 100 core collections of ginger are maintained at the IISR in vitro gene bank with yearly subculture. The small-sized plantlets kept in the conservation medium for over 5 years with yearly subculture when transferred to the multiplication medium (MS + 30 gl^{-1} sucrose and 1 mgl^{-1} BA) gave normal growth with good multiplication rate. These planltets were established easily with >80 percent success developed into normal plants similar to the mother plants. Thus, the in vitro conservation

technique is a safe alternative for a vegetativley propagated crop such as ginger for conservation and exchange of disease-free planting material.

Ravindran and coworkers (Anon. 2004) standardized the use of synthetic seeds in conservation. Synthetic seeds, developed with somatic embryos, encapsulated in 5% sodium alginate gel could be stored in MS medium supplemented with 1 mgl^{-l} Benzyl adenine (BA) at 22 ± 2°C for 9 months with 75% survival. The conservation of germplasm through microrhizome production was also investigated and it was found that microrhizomes can be induced *in vitro* when cultured in MS medium supplemented with higher levels of sucrose (9 to 12%). Such microrhizomes can be easily stored for more than one year in culture. Six months-old microrhizomes can be directly planted in the field without any acclimatization. The microrhizomes can thus be used as a disease-free seed material and for propagation, conservation, and exchange of germplasm (Geetha, 2002). This microrhizome technology is amenable for automation and scaling up.

Cryopreservation offers a better alternative when the base germplasm of any crop can be preserved for long durations with minimum effort. However, protocols for viable cryopreservation and post-thaw recovery of cryopreserved material are not available for ginger. Geetha (2002) and Peter *et al* (2002) reported that

ginger in vitro-derived shoot tips could be successfully cryopreserved with limited success by pretreating the shoot tips with 0.75 M sucrose, desiccating for 1 hour, and plunging into liquid nitrogen (-185^0 C). Only 20 percent of the cultures developed into plantlets after cryopreservation. They also reported that encapsulated shoot buds of ginger (Synseeds) could be successfully cryopreserved after preculture on 0.75 M sucrose for 3 days and desiccated for 4 hours on laminar airflow. Plantlets could be regenerated from 20 percent of the cultures after cryopreservation.

BLACK PEPPER

Black pepper, known as the '**king of spices'**, is the most important and most widely used spice in the world. Black pepper is a woody climber, grown in the South Western region of India, comprising of the states of Kerala, parts of Karnataka, Tamil Nadu and Goa. The humid tropical evergreen forest bordering the Malabar Coast (Western Ghats is one of the hot spot areas of plant biodiversity on earth) is the center of origin and diversity for the king of spices.

The genus *Piper* is represented by about 13 species in South India of which *Piper barberi* is very, rare (Gamble, 1925). *Piper barberi* is reported to be almost extinct and recorded in the red data book of Indian plants (Nayar & Sastry, 1988). After the type

collection of C.A. Barber in 1901, it was thought to be extinct. Its occurance was again repoted six decades later by Subramanyam and Henry (1970) and later by Nirmal Babu *et al*., (1992) and Mathew & Mathew (1992). *Piper barberi* is a remarkable species and can be easily distinguished by its pinnately veined leaves and spikes borne on very long filiform peduncles. This species is a slender dioecious climber and has little resemblance with South Indian taxa of *Piper*. It is more alike to Central and Northern South American forms with its reticulately veined leaves, persistant prophylls and long peduncles, and at the same time differs from them in its climbing habit and dioecious nature. Probably *P. barberi* could be sole survivor of an ancestral type that reached India from Central American region (Nirmal Babu *et al*., 1992).

The cultivars of black pepper might have originated from the wild ones through domestication and selection. Over hundred cultivars are known, but many of them are getting extinct due to various reasons like devastation of pepper cultivation by diseases such as, foot rot and slow decline, replacement of the traditional cultivars by a few high yielding varieties etc. Cultivars density is richest in the state of Kerala followed by the state of Karnataka. Most of the cultivars are bisexual forms. The Western Ghats is very high in endemic species; unfortunately it is also one of the most ecologically threatened areas due to large-scale encroachments

and human settlements that have taken place during the past hundred years.

Indian Institute of Spices Research holds the world's largest collection of pepper germplasm,(**Table 1**) which is at present conserved in clonal field repositories, where serious diseases threaten them.

To save the pepper genetic resources, the Indian Institute of Spices Research has established a National Repository for the *ex situ* conservation of Pepper germplasm and is conserved at four stages (Ravindran and Nirmal Babu 1994).

i) In the nursery gene bank, where each accession is trailed in bamboo splits in serial order and are under continuous multiplication and maintenance.

ii) In the clonal repository where 10 rooted cuttings of each accession is maintained.

iii) In the field gene bank where the accessions are planted for preliminary yield evaluation and characterization.

iv) *In vitro* and cryogene banks.

Storage of germplasm in seed banks is not practical as they are vegetatively propagated and seeds are recalcitrant and heterozygous. Hence storage of germplasm in *in vitro* is a safe alternative.

Protocols for *in vitro* conservation by slow growth of pepper and its related species viz., *P.barberi, P.colubrinum, P.betle* and *P.longum* were standardized by maintaining cultures at reduced temperatures, in the presence of osmotic regulators, at reduced nutrient levels and by minimizing evaporation loss by using closed containers. The conserved materials of all the species 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 with out any deformities and were morphologically similar to mother plants.

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^{0}C, with a survival rate of 45% & 10.5% respectively (Chaudhury & Chandel, 1994).

Table 1 Genetic resources of cardamom, ginger & black pepper

Crop/Center	Cultivated	Indigenous Wild and related sp	Exotic	Total
CARDAMOM				
IISR Regional Station, Appangala, Coorg,Karnataka	314	13	-	327
Cardamom Research Station (Kerala Agricultural University), Pampadumpara	141	15	-	156
Indian Cardamom Research Institute, Myladumpara	600	12	-	612
	132	7	-	139

ICRI Regional station, Mudigere	**1187**			**1234**
Total				

Genetic resources of ginger

Crop/Center	Indigenous Cultivated	Wild and related sp	Exotic	Total
GINGER				
High Altitude Research Station Pottangi	167	2	3	172
Department of Vegetable Crops (Dr YS Parmar Univ. of Horticulture & Forestry), Solan	286		2	288
Department of Horticulture Tirhut College of Agriculture (Rajendra Agrl. University) Dholi	43			43
Department of Vegetable Science (Narendra Dev University of Agril. & Technology), Narendra Nagar Post, Kumarganj	45			45
Department of Horticulture (Uttar Banga Krishi Viswa Vidyalaya, North Bengal Campus) Pundibari	38			38
Regional Agril. Research Station (Indira Gandhi Krishi Vishwa Vidyalaya), Boirdadar Farm, Raigarh	44			44

Department of Horticulture (Konkan Krishi Vidyapeeth) Dapoli	3			3
Indian Institute of Spices Research, Calicut	645			645
National Bureau of Plant Genetic Resources, Regional Station, Thrissur	173			173
Department of Horticulture, Sikkim	58			58
Central Agricultural Research Institute, Port Blair, Andamans	33			33
TOTAL	**1535**	**2**	**5**	**1542**

Source: AICRPS 2006

Genetic resources of black pepper

Crop/Center	Indigenous Cultivated	Indigenous Wild and related sp	Exotic	Total
BLACK PEPPER				
Pepper Research Station (Kerala Agricultural University) Panniyur	164	72	3	289
Agricultural Research Station (Pepper), Sirsi	97	19	1	117
Regional Agrl. Research Station (Acharya N.G. Ranga Agrl. University), Chintapalle	58	-	-	58
Horticultural Research Station Yercaud	122	10	-	132
Department of	16	1	-	17

Horticulture (Uttar Banga Krishi Viswa Vidyalaya, North Bengal Campus) Pundibari				
Department of Horticulture (Konkan Krishi Vidyapeeth) Dapoli	67	-	-	67
Indian Institute of Spices Research, Calicut	2322	15		2337
TOTAL	**2846**	**117**	**4**	**3017**

Source: AICRPS 2006

CONSERVATION OF GENETIC RESOURCES

It is estimated that up to 100,000 plants, representing more than one third of all the world's plant species, are currently threatened or face extinction in the wild (BGCI, 2005). Preservation of the plant biodiversity is essential for classical and modern (genetic engineering) plant breeding programmes. Moreover, this biodiversity provides a source of compounds to the pharmaceutical, food and crop protection industries. Since the 1970s, large numbers of landraces and wild relatives of cultivated crops have been sampled and stored in ex situ gene banks. It is estimated that 6 million samples of plant genetic resources are held in national, regional, international and private gene bank collections around the world (Correidoira *et al*., 2004). Storage of desiccated seeds at low temperature, the most convenient method to preserve plant germplasm, is not applicable to crops that do not

produce seed (e.g., bananas) or with recalcitrant seed (i.e., non-orthodox seed that can not be dried to moisture contents that are low enough for storage, as for instance many tropical trees), as well as to plant species that are propagated vegetatively to preserve the unique genomic constitution of cultivars (such as fruit and several timber and ornamental trees). Preservation only in field collections is risky, as valuable germplasm can be lost (genetic erosion) because of pests, diseases and adverse weather conditions. Moreover, the maintenance of clonal orchards is labour-intensive and expensive. The maintenance of *in vitro* collections (established for some vegetatively-propagated species) is labour-intensive as well, and there is always the risk of losing accessions due to contamination, human error or somaclonal variation (i.e., mutations that occur spontaneously in tissue culture, with a frequency that increases with repeated subculturing).

Hence, cryopreservation or freeze-preservation at ultra-low temperature (-196°C, i.e., the temperature of liquid nitrogen) is a sound alternative for the long-term conservation of plant genetic resources, since under these conditions, biochemical and most physical processes are completely arrested. As such, plant material can be stored for unlimited periods. Moreover, besides its use for the conservation of genetic resources, cryopreservation proved to be extremely useful for the safe long-term storage of

plant tissues with specific characteristics, such as medicinal and alkaloid producing cell lines, hairy root cultures, genetically transformed and transformationcompetent culture lines (Cyr, 2000). Recently, it was also proven that cryotherapy can be successfully applied to eradicate viruses from plum, banana and grape (BGCI, 2005).

However, despite the fact that cryogenic procedures are now being developed for an increasing number of recalcitrant seeds and *in vitro* tissues/organs, the routine utilization of cryopreservation for the preservation of plant biodiversity is still limited.

Theoretical basis of plant cryopreservation

Cryopreservation of biological tissues can be successful only if intra-cellular ice crystal formation is avoided, since this causes irreversible damage to cell membranes thus destroying their semi permeability. In nature, some plant species adopted systems where ice crystal formation at sub-zero temperatures can be avoided through the synthesis of specific substances (such as sugars, proline and proteins) that lower the freezing-point in the living plant cells, resulting in ”supercooling”. Such ‘avoidance’ of crystallization, while still maintaining a minimal moisture level needed to maintain viability, it is not possible when dealing with ultra-low temperatures of cryopreservation (-196°C). Crystal

formation, without an extreme reduction of cellular water, can only be prevented through “vitrification”.

Vitrification refers to the physical process of transition of an aqueous solution into an amorphous and glassy (i.e., non-crystalline) state (Dumet *et al*., 1994). Two requirements must be met for a cell to vitrify: (i) rapid freezing rates, and (ii) a concentrated cellular solution. Rapid freezing rates (6°C/sec) are normally obtained by plunging explants enclosed in a cryovial into liquid nitrogen. Higher cooling rates can be obtained by enclosing the meristems in semen straws, resulting in cooling rates of about 60°C/sec, or using a “droplet freezing protocol” where the material is placed on aluminium foil strips that are plunged directly into liquid nitrogen, giving rise to cooling rates of 130°C/sec (De Carlo *et al*., 2000; Engelmann, 2004). The cell cytosol can also be concentrated through air drying, freeze dehydration, application of penetrating or non-penetrating substances (cryoprotectants), or adaptive metabolism (hardening). For a solution to be vitrified at high cooling rates, a reduction in water content to at least 20-30% is required. For dehydration, the following techniques are applied:

1. Air Drying

Usually, samples are dried by the sterile airflow of a laminar airflow cabinet. Doing so, there is not any control of temperature and air humidity, both influencing strongly the evaporation rate.

More reproducible is the air-drying method that uses closed vials containing a fixed amount of silica gel (Fabre & Dereuddre, 1990).

2. Freeze Dehydration

Because plant cells rarely contain ice-nucleating agents, during slow cooling crystallization is initiated in the extra-cellular spaces. Since only a proportion of the water that contributes to the extra-cellular solution undergoes transition into ice, the remaining solution becomes more and more concentrated and thus hypertonic to the cell. To restore the osmotic equilibrium, cellular water will leave the protoplast, resulting in cell dehydration. Generally, freezing rates of 0.5 to 2°C/min, depending on the type and physiological state of the plant material, are applied. These slow-cooling rates are generally obtained using computer-driven cooling devices, stirred methanol baths, and propanol containers held at –70°C.

3. Non penetrating cryoprotective substances

Osmotic dehydration can be obtained through the application of non-penetrating cryoprotective substances, such as sugars, sugar alcohols and high molecular weight additives like polyethylene glycol (PEG).

4. Penetrating cryoprotective substances

Commonly used penetrating cryoprotective agents are dimethyl sulphoxide (DMSO) and glycerol. For many applications,

DMSO is preferred because of its extreme rapid penetration into the cells. Where DMSO toxicity is a problem, glycerol or amino acids (e.g., proline) are often applied.

5. Adaptive metabolism (Hardening)

Hardening is a process that increases plants ability to survive the impact of unfavorable environmental stress. This is triggered by environmental parameters, like reduction in temperature and shortening of daylength. Also osmotic changes and abscisic acid (ABA) treatments can have similar effects. Hardening can result in a considerable increase of, for instance, proteins, sugars, glycerol, proline and glycine betaine which will all participate in the increase of osmotic value of the cell solutes.Most hydrated tissues, however, do not withstand dehydration to moisture contents needed for vitrification (20-30%) due to solution and mechanical effects. Exceptions are pollen, seeds and somatic embryos of most orthodox seed species.

The key for successful cryopreservation is thus shifted from freezing tolerance to dehydration tolerance. This tolerance can be induced by chemical cryoprotection with substances like sugars, amino acids, DMSO, glycerol, etc. The mode of action of most of these substances is, however, still far from being understood. Alternatively, tolerance to dehydration can also be induced by adaptive metabolism. For example, it has been observed that cold

acclimation in nature often leads to the accumulation of specific proteins, sugars, polyamines and other compounds that can protect cell components during drying. Also alterations in membrane composition are reported, influencing both their flexibility and permeability (Dumet *et al*., 1993).

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, 1985a; b; c; Kartha, 1985).

Plant cells contain high amounts of cellular water and freezing of plant cells implies conversion of some or all of their liquid water to ice, whereas thawing is reversal of this transition. Since most of the experimental systems (meristems, shoot tips, cultured cells etc.) contain high amount of cellular water and hence extremely sensitive to freezing injury, protection from freezing and thawing has to be imposed artificially. This involves various strategies such as the use of cryoprotectants or other manipulations (Kartha & Engelmann, 1994).

However, only by freezing at ultra low temperatures such as -196^{0}C, the temperature of liquid nitrogen, growth in plant tissue and cell cultures can be entirely suspended. Since very few cultures have a natural resistance to freezing, special

manipulations of explant and culture conditions are required to prepare a specimen with maximum freeze tolerance. Application of certain cryoprotectants like dimethyl sulphoxide (DMSO), glycerol in combination with other compounds like mannitol, sorbitol, glucose, sucrose, proline or polyethylene glycol (PEG) will bring about changes in cell permeability, freezing point and response to the stresses of freezing and thawing essential for survival.

Cryopreservation involves essentially three steps namely, specimen treatment and freezing, storage at ultra-low temperature and thawing and recovery. Before commencing proper cryopreservation procedures different culture systems need to be given specific manipulations and or culture conditions in order to present a specimen with maximal freeze tolerance. An ideal cryoprotectant should protect cells from all the factors, which would affect the viability of the frozen biological sample during all these stages. Since viability loss is not expected to occur at ultra-low temperatures, protection of the cells during freezing and thawing is of paramount importance. A number of compounds such as glycerol, dimethyl sulphoxide (DMSO), ethylene glycol, polyethylene glycol (PEG), sugars and sugar alcohols either alone and in combination protect living cells against damage during freezing and thawing. Such compounds can lower the temperature at which freezing first occurs and can alter the crystal habit of ice

when it separates (Withers, 1980; Kartha and Engelmann, 1994). Although the exact mechanism of action of cryoprotective compounds is still poorly understood, the colligative properties of the cryoprotectants can minimize the deleterious action of excessive electrolyte concentration resulting from removal of water and conversion of water to ice (Nash, 1966).

Cryoprotectants generally used for freezing biological specimens fall into two categories namely, permeating and non-permeating. The most commonly used permeating additives are DMSO and glycerol; the former permeates rapidly and is more toxic when compared to the latter. Generally a concentration of 5–10% for DMSO and 10–20% for glycerol is adequate for most material. In instances where application of a single cryoprotectant does not result in survival, a mixture of cryoprotectants has been beneficial (Finkle & Ulrich, 1979; Chen *et al.*, 1984, Finkle *et al.*, 1985; Withers, 1985a; b; c). Application of cryoprotectant to the cells and the removal of cryoprotectants from the thawed samples should always be a gradual process to alleviate the problems associated with plasmolysis and deplasmolysis, respectively (Kartha & Engelmann, 1994). Cryoprotectants are usually prepared in culture medium and the material to be cryopreserved is incubated prior to their freezing (Withers, 1985a; b; c; Kartha & Engelmann, 1994).

There are different freezing methods such as slow freezing, rapid freezing, droplet freezing, vitrification, etc. A number of factors such as cooling rates, pretreatment and cryoprotection, type and physiological state of the experimental material and the terminal freezing temperature, influence the success of slow freezing method. The most commonly used method of freezing meristems and cell cultures is by regulated slow cooling at a rate of 0.5 to 1.0 ^{0}C /min down to either –30^{0}C, –35^{0}C or –40 ^{0}C with the help of a programmable freezer, followed by storage in liquid nitrogen. Meristems, cell cultures and somatic embryos of a number of species have been cryopreserved using slow freezing methods (Kartha, 1985; Withers, 1985a; b; c).

New cryopreservation techniques offer practical advantages in comparison to classical ones (Sakai, 1995) Rapid freezing is the simplest form of cryopreservation since the procedure does not require sophisticated and expensive equipment. Rapid freezing has successfully cryopreserved meristems and somatic embryos of a few plant species. It is suggested that the viability of cells may be maintained by preventing the growth of intracellular ice crystals formed during rapid cooling by rapidly passing the tissue through the temperature zone in which lethal ice formation occurs (Sakai, 1995; Engelmann, 1997).

The technique of droplet freezing was originally developed for cryopreservation of cassava meristems (Kartha *et al.*, 1982). After dissection, apices are precultured with DMSO for a few hours and frozen rapidly in droplets of cryoprotective medium placed on aluminium foils. This procedure has been applied successfully to more than 150 varieties of potato with an average recovery rate of 40% (Schäfer-Menuhr, 1996).

The development of vitrification technique for the cryostorage of cultured plant cells and organs is of recent origin although Sakai (1958) succeeded in obtaining the survival of hardy mulberry cortical tissue in liquid nitrogen by vitrification. In the vitrification method, cells or meristems must be sufficiently dehydrated with a highly concentrated vitrification solution at 25 ^{0}C or 0 ^{0}C without causing injury prior to immersion in liquid nitrogen. Vitrification method of cryopreservation was reported in 5 apple species or cultivars, 8 pear cultivars (Niino *et al.*, 1992a) and 13 mulberry species or cultivars (Niino *et al.*, 1992b; 1992c).

For freezing of differentiated tissues and organs such as apices zygotic and somatic embryos, new techniques have been developed (Deruddre, 1992; Engelmann, 1997; 2000). They are based on the removal of most or all freezable water by physical or osmotic dehydration of explants followed by ultra-rapid freezing which results in vitrification of intracellular solutes, i.e. the

formation of an amorphous glassy structure without occurrence of ice crystals which are detrimental to cellular structural integrity. Their main advantages in comparison to classical procedures are their simplicity, since they do not require the use of a programmable freezer, and their applicability to a wide range of genotypes. They include encapsulation-dehydration, vitrification, encapsulation-vitrification, desiccation, pregrowth, pregrowth-desiccation and droplet-freezwth, pregrowth-desiccation and droplet freezing (Engelmann, 1997; 2000).

The encapsulation-dehydration procedure is based on the technology developed for the production of artificial seeds. Explants are encapsulated in alginate beads, pregrown in liquid medium enriched with sucrose for several days, partially desiccated down to a water content around 20% (fresh weight basis), then frozen rapidly. Survival rates are high and growth recovery of cryopreserved samples is generally rapid and direct, without callus formation. This technique has been developed for apices of various species from temperate origin such as apple, pear, grape, eucalyptus, and of tropical origin such as sugarcane and cassava (**Table 2**).

Table 2 List of plant species whose shoot tips (or other explants – specified in the column Plant species) have been cryopreserved using the encapsulation- dehydration technique.

Crop	**Reference**	**Crop**	**Reference**
Actinidia	Wu *et al*., 2001	*Fragaria x*	Clavero-Ramirez

chinensis		*ananassa*	*et al*., 2005
Actinidia spp.	Bachiri *et al*., 2001	*Holostemma annulare*	Decruse *et al*., 1999
Anthirrinium microphyllum	Gonzalez-Benito *et al*., 1998	*Iopomea batatas*	Pennycooke and Towill, 2001
Armoracia rusticana	Hirata *et al*., 1995	*Iris nigricans*	Shibli ,2000
Auricularia	Hirata *et al*., 1996	*Lilium*	Matsumoto & Sakai ,1995
Beta vulgaris	Vandenbussche & Proft ,1996	*Malus* spp.	Zhao *et al*., 1999
Brassica napus microspore embryos	Uragami *et al*., 1993	*Mentha spicata*	Sakai *et al*., 2000
blackberry and raspberry	Sandhya gupta & Reed, 2006		
Chichorium intybus	Vandenbussche *et al*., 1993	microalgae	Hirata *et al*., 1996
Chrysanthemum morifolium	Sakai *et al*., 2000	*Morus bombysis*	Niino *et al*., 1992
Citrus madurensis	Cho *et al*., 2002	*Olea europea* somatic embryos	Shibli & Al-Juboory ,2000
		Oncidium bifolium sims. (seeds & protocorms)	Flachsland *et al*., 2006
Citrus spp.	Gonzalez-Arnao *et al*., 1998	Orchid seeds + fungal symbiont	Wood *et al*., 2000
Citrus spp. Ovules and somatic embryos	Gonzalez-Arnao *et al*., 2003	*Polygonium aviculare* cell suspension	Swan *et al*., 1998
		Peach palm(ZE)	Douglas *et al*., 2007
Cocos nucifera	Hornung *et al*., 2001	*Prunus dulcis*	Shatnawi *et al*., 1999
Coffea racemosa	Mari *et al*., 1995	*Pyrus communis*	Dereuddre *et al*., 1990
Coffea Arabica(s)	Dussert and Engelmann, 2006		

Crop	**Reference**	**Crop**	**Reference**
C. sessiliflora	Mari *et al*., 1995	*Ribes*	Reed & Yu ,1995
Cosmos atrosanguineus	Wilkinson *et al*., 1998	*Saccharum* spp.	Gonzalez-Arnao *et al*., 1993

Daucus carota somatic embryos	Dereuddre *et al*., 1991	*Solanum* spp.	Fabre& Dereuddre,1990
Dioscorea alata	Malaurie *et al*., 1998	*Solanum tuberosum* Strawberry	Grospietch *et al*., 1999 Clavero-Ramírez *et al*., 2005
D. *bulbifera*	Malaurie *et al*., 1998	*Vitis vinifera*	Plessis *et al*., 1991, Zhao *et al*., 2001
Dioscorea spp.	Mandal *et al*., 1996	*Wasabia japonica*	Sakai *et al*., 2000

Vitrification consists of placing explants in the presence of a highly concentrated cryoprotective solution, then freezing them rapidly. This technique has been experimented with cell suspensions, apices and somatic embryos of around 135 plant species (**Table 3**).

Table 3 List of plant species cryopreserved using the vitrification technique. Protocols developed for shoot tips, if not specified otherwise. C: calluses; CS: cell suspension; EA: embryonic axes; EC: embryogenic cultures; HR: hairy roots; IF: inflorescences; M: microspores; NC: nucellar cells; NS: nodal segments; P: protocorms; PP: protoplasts; R: roots; S: seeds; SD: seedlings; SE: somatic embryos; ZE: zygotic embryos.

Crop	**Reference**	**Crop**	**Reference**
Actinidia chinensis	Xu *et al*., 2006	*Gentiana tibetica* (CS)	Mikula, 2006
Actinidia deliciosa	Xu *et al*., 2006	*Gypsophila paniculata*	Hanada *et al*., 2002
Aesculus hippocastanum (EC)	Lambardi *et al*., 2005	*Grevillea scapigera*	Touchell & Dixon, 1996
Allium porrum	Niino *et al*., 2003	*Helianthus tuberosus*	Volk & Richards,2006
Allium sativum	Niwata, 1995;Makowska *et al*.,1999 Baek *et al*.,2003;Kim *et al*.,2004; Volk *et al*.,2004;; Ellis *et*	*Hevea brasiliensis*	Sam & Hor, 1999

	al.,2006; Wang *et al*., 2005		
Allium wakegi	Kohmura *et al*.,1994	*Hordeum vulgare*	Wang *et l*.,1996
Amorphophallus	Zhang *et al*., 2001	*Hyoscyamus niger* (R)	Jung *et al*.,2001
Amygdalus communis	Al-Ababneh *et al*., 2003	*Ipomoea batatas*	Hirai & Sakai, 2003; Towill & Jarret , 1992
		juncus spp	Niino *et al*., 2007
Ananas comosus	Gonzalez-Arnao *et al*., 1998; Thinh *et al*., 2000	*Lilium japonicum*	Matsumoto *et al*.,1995

Crop	**Reference**	**Crop**	**Reference**
Anigozanthos humilis	Turner *et al*., 2001	*Lilium* sp.	Bouman *et al*.,2003; Zhang *et al*.,2004
Anigozanthos kalbarriensis	Turner *et al*., 2001	*Limonium*	Matsumoto *et al*.,1998
Anigozanthos viridis	Turner *et al*., 2001	*Limonium altaica x L. caspium*	Matsumoto *et al*.,1997
Arabidopsis thaliana(SD &shoot tips)	Liu *et al*., 1999; Towill *et al*., 2006	*Macropidia fuliginosa* (SE)	Turner *et al*., 2000
Arachis spp (ZE & shoot tips).	Gagliardi *et al*., 2002; 2003	*Malus* spp.	Liu *et al*., 2004; Wu *et al*.,1999;2001
Armoracia rusticana (HR)	Phunchindawan *et al*., 1997	*Malus pumila*	Previati *et al*., 2002
Artocarpus heterophyllus	Thammasiri, 1999	*Mangifera indica* (EC)	Wu *et al*., 2003
Asparagus officinalis(EC, shoot tips & SE)	Uragami *et al*., 1989; Kohmura *et al*., 1992; Nishizawa *et al*., 1993	*Mangifera indica*	Drew *et al*., 2005
Atropa belladonna (HR)	Kamiya *et al*., 1995; Touchell *et al*.,2002	*Manihot esculenta*	Charoensub *et al*.,1999; 2003
Beta vulgaris	Vandenbussche *et al*.,2000	*Melia azeradach* (SE)	Deb, 2002
Bletilla striata (ZE)	Ishikawa *et al*.,1997	*Mentha spicata*	Hirai & Sakai, 1999
Bletilla striata (S)	Hirano *et al*.,2005	*Mentha aquatica x M. spicata*	Towill , 1990

Brassica campestris (CS)	Langis *et al*.,1989	*Mentha* spp.	Towill & Bonnart, 2003
Bratonia (P)	Popova ,2007	*Morus bombycis*	Niino *et al*.,1992
Bromus inermis (CS)	Ishikawa *et al*.,1996; 2006	*Musa* spp.	Agrawal *et al*.,2004; Thinh *et al*.,1999; Takagi *et al*.,1998; Tannoury, 1993
Camellia sinensis	Lambardi *et al*.,2005	*Nicotiana tabacum* (CS)	Reinhoud , 1996; Reinhoud *et al*.,1995
		Oxalis tuberosa mol	González-benito *et al*., 2007
Carica papaya (shoot tips & S)	Baek *et al*.,2003	*Olea europaea* (EC)	Benelli *et al*.,2001 Lambardi *et al*.,2001
Castanea sativa (SE)	Corredoira *et al*.,2004	*Olea europaea* (NS)	Benelli *et al*.,2001
Castanea sativa	San Jose *et al*.,2005 ; Vidal *et al*.,2005	*Oryza sativa* (EC)	Watanabe & Steponkus, 1995; Huang *et al*.,1995; Wang *et al*.,1998; 2001

Crop	**Reference**	**Crop**	**Reference**
Centorium	Gonzalez-Benito & Perez, 1994	*Panax ginseng*	Yoshimatsu *et al*.,1996
Ceratonia siliqua (M)	Custodio *et al*.,2004	*Picea mariana* (EC)	Touchell *et al*.,2002
Citrus aurantium	Al-Ababneh *et al*.,2002	*Picrorhiza kurroa*	Sharma & Sharma , 2003
Citrus junios x C. grandis (SE)	Oh, 1997	*Poncirus trifoliata x Citrus sinensis*	Wang *et al*.,2003
Citrus grandis x C. junios (SE)	Oh, 1997	*Ponerorchis graminifolia* (S)	Hirano *et al*.,2005
Citrus madurensis	Cho *et al*.,2002	*Populus alba*	Lambardi *et*

(EA)			*al*.,2000
Citrus platymamma x C. junios(SE)	Oh, 1997	*Populus canescens*	Lambardi *et al*.,2001
Citrus sinensis (NC)	Sakai *et al*.,1990	*Populus nigra*	Lambardi *et al*.,2001
Citrus sinensis (C)	Hao *et al*.,2003	*Populus tremula x P. tremuloides*	Jokipii *et al*.,2004
Citrus spp. (CS)	Hao *et al*.,2002	*Porphyra yeozensis* (PP)	Liu *et al*.,2004
Citrus spp.	Wang & Deng, 2004	*Prunus avium*	Tashiro *et al*.,1995
Chrysanthemum	Schnabel-Preikstas *et al*.,1992	*Prunus domestica*	De Carlo *et al*.,2000
Cnidium (SE)	Cho *et al*.,1998	*Prunus dulcis*	Shatnawi *et al*.,1999; Channuntapipat *et al*.,2001
Colocasia esculenta	Takagi *et al*.,1997; Ribeiro *et al*.,2004;Sant *et al*.,2006	*Prunus persica*	Paulus *et al*.,1993
Conostylis dielsia	Turner *et al*.,2001	*Prunus* spp.	Niino *et al*.,1997
Conostylis micrantha	Turner *et al*.,2001	*Pyrus* spp.	Niino *et al*.,1992
Conostylis wonganiensis	Turner *et al*.,2001	*Pyrus communis*	Previati *et al*.,2002
Crateva nurvala	Sanayaima *et al*.,2006	*Quercus robur* (EC)	Martinez *et al*.,2003
Cymbidium spp.	Thinh & Takagi, 2000	*Quercus suber* (SE)	Valladares *et al*.,2004
Cymbopogon	Thinh, 1997	*Rehmania glutinosa*	Xue *et al*.,2003
Daucus carota (CS)	Chen & Wang, 2003	*Rhodolia fastigiata* (CS)	Guo & Liu, 2006
Daucus carota (PP)	Chen & Wang, 2003	*Ribes*	Reed, 1992
Dendranthema	Fukai ,1992	*Ribes nigrum*	Reed *et al*.,2001
Dendranthema grandiflorum	Ahn & Sakai, 1994, Halmagyi *et al*.,2004	*Robinia pseudoacacia*	Verleysen *et al*.,2005
Dendrobium candidum	Chen *et al*.,2001	*Rosa*	Halmagyi & Pinker ,2006

Crop	**Reference**	**Crop**	**Reference**
Dianthus caryophyllus	Fukai ,1992, Langis *et al*.,1990	*Saintpaulia ionantha*	Moges *et al*.,2004
Dimocarpus longan (C)	Guo *et al*.,2006	*Secale cereale* (PP)	Langis & Steponkus,

			1990;1991
Dioscorea deltoidea	Sonali-Dixit *et al*.,2005	*Solanum* spp.	Golmirzaie & Panta ,2000
Dioscorea floribunda	Sangeeta *et al*.,2002	*Solanum tuberosum*	Debrabata & Naik , 1998;2000; Criel *et al*.,2005; Zeng *et al*.,2005; Kryszszuk *et al*.,2006
Dioscorea rotundata	Takagi *et al*.,1998	*Solemostemon rotundifolius*	Niino *et al*.,2000
Diospyros kaki	Matsumoto *et al*.,2001; Ai & Luo 2003; 2004	*Trifolium repens*	Yamada *et al*.,1991;1993
Diospyros lotus	Ai & Luo 2004	*Vitis vinifera*	Matsumoto & Sakai ,2003
Doriteanopsis (CS)	Tsukazaki *et al*.,2000	*Vitis* spp.	Matsumoto T & Sakai A , 2000; Wang *et al*.,2002
Doritis pulcherrima (S)	Thammasiri ,2000	*Vitis vinifera* (SE)	Miaja *et al*.,2004
Eriobotrya japonica	Wang *et al*.,2006	*Wasabia japonica*	Matsumoto *et al*.,1998
Fragaria x ananassa	Hirai & Sakai, 2001; Niino *et al*.,2003 ; Zhao *et al*.,2006	*Xanthosoma* spp.	Thinh , 1997
Fraxinus excelsior	Schoenweiss *et al*.,2005		
Gentiana spp.	Tanaka *et al*.,2004		

Encapsulation-vitrification is a combination of the above techniques, where explants are encapsulated in alginate beads and treated with vitrification solutions before freezing. It has been applied to apices, somatic embryos etc (**Table 4**)

Table 4 List of plant species cryopreserved using the encapsulation-vitrification technique. Protocols developed for shoot tips, if not specified otherwise. SE: somatic embryos; HR: hairy roots.

Crop	**Reference**	**Crop**	**Reference**

Ananas comosus	Gamez-Pastrana *et al*., 2004	*Malus domestica*	Paul *et al*., 2000
Armoracia rusticana (HR)	Phunchindawan *et al*., 1997	*Manihot esculenta*	Hirai, 2001; Charoensub *et al*., 2004
Citrus aurantium	Al-Ababneh *et al*., 2002	*Mentha spicata*	Hirai & Sakai, 1999; Hirai, 2001; Hirai & Sakai, 2001
Crop	**Reference**	**Crop**	**Reference**
Daucus carota	Hirai ,2001	*Olea europea* (SE)	Shibli & Al-Juboory, 2000
Daucus carota (SE)	Tannoury, 1993	*Poncirus trifoliata x Citrus sinensis*	Wang *et al*., 2002
Dianthus caryophyllus	Tannoury *et al*., 1991; Tannoury, 1993	*Prunus domestica*	De Carlo *et al*., 2000
Dioscorea	Hirai, 2001; Hirai & Sakai, 2001	*Rubus idaeus*	Wang *et al*., 2005
Diospyros kaki	Zhang & Luo, 2004	*Saintpaulia ionantha*	Moges *et al*., 2004
Fragaria x ananassa	Hirai, 2001; Hirai & Sakai, 2001	*Solanum tuberosum*	Hirai & Sakai, 1999;Hirai, 2001; Hirai & Sakai, 2001
Gentiana spp.	Tanaka *et al*., 2004	*Vitis* spp.	Wang *et al*., 2004
Ipomoea batatas	Hirai & Sakai, 2003	*Vitis berlandieri x V. riparia*	Benelli *et al*., 2003
Lilium	Hirai & Sakai, 2001	*Wasabia japonica*	Matsumoto *et al*., 1995

Desiccation is the simplest procedure since it consists of dehydrating explants, then freezing them rapidly by direct immersion in liquid nitrogen. This technique has been applied to embryos of a large number of recalcitrant and intermediate seed species (Engelmann, 1992; Engelmann *et al*., 1995, Dumet *et al*., 1997). Optimal survival rates are generally obtained when samples

are frozen with a water content comprised between 10 and 20% (fresh weight basis).

Pregrowth involves preculturing the plant material on a medium containing cryoprotectants, then freezing explants rapidly. An efficient pregrowth procedure has been developed recently for meristematic clumps of *Musa* sp. (Panis, 1995a; b; Panis & Thinh, 2001).

In a pregrowth-desiccation procedure, explants are pregrown in the presence of cryoprotectants (generally sugars such as sucrose or glucose), dehydrated under the laminar airflow cabinet or with silica gel, then frozen rapidly. This method has currently been applied to coconut zygotic embryos and oil palm somatic embryos only (Assy – Bah & Engelmann, 1992b; Dumet *et al*., 1993). Survival rates obtained after freezing are high and growth recovery is rapid and direct.

Cryopreservation methods have been developed for more than 200 different plant species in various forms like cell suspensions, calluses, apices, somatic and zygotic embryos (Kartha & Engelmann, 1994; Engelmann, 1997). However, their routine utilisation is still restricted almost exclusively to the conservation of cell lines in research laboratories. The method is routinely used in oilpalm for which more than 80 clones of somatic embryos are

stored for the long term in liquid nitrogen (Dumet, 1994) and potato (Schäfer-Menuhr, 1996).

Technique of *in vitro* conservation has been made dramatic progress in the last 10 years and methods have been standardized for quite a lot of plant species. Slow growth techniques are immediately applicable in most cases, whereas cryopreservation techniques often require further improvement before they can be applied routinely. Many recent reports are available on slow growth storage and cryopreservation of crops such as cassava (Mabanza *et al.*, 2001); rice (Watanabe *et al.*, 1999; Wang *et al.* 2001), kiwifruit (Bachiri *et al.*, 2001; Wu, *et al.*, 2001), *Rubus* (Chang & Reed, 1999), *Zoysia* sp. and *Lolium* sp. (Chang *et al.* 2000); orchid seed and its fungal symbiont (Wood *et al.*, 2000), *Eucalyptus* hybrid (Blakesley & Kiernan, 2001), *Auricula* (Hornung, *et al.*, 2001) etc.

Issues involved in implementing liquid nitrogen storage

Now that many successful techniques are available and already tested on numerous gtenotypes, the time has come to start storing germplasm. Implementing storage requires many considerations. The present selection of cryopreserved plants very from randomly chosen selections to carefully selected clones that represent morphological variation and unique geographic criteris sometimes referred to as core collections (Golmirzaie & Panta,

2000; IPGRI/CIAT, 1994; Reed *et al*., 1998; Reed *et al*., 1998). The curatorial decision regarding what to store generally starts with at-risk plants i.e. those that are likely to be lost to disease, insect pests, or environmental conditions at the field site. These decisions will vary with crop type, location of the field genebank, and economic importance of each crop. The form of plant to store is also an issue, and pollen, shoot apices, dormant buds, excised embryonic axes, or somatic embryos may be the form of choice depending on the species involved (Ashmore, 1997). The next decisions involve logistics of storage. How many samples of each accession should be stored? The number of propagules will vary with the plant type and the recovery potential. Ideally enough propagules should be stored in each container to produce several living plants and enough containers to allow for several recoveries over time. If periodic testing is anticipated, then additional vials should be included for that purpose so that the vials in the base collection remain untouched

Developing a testing and storage protocol

Once the initial decisions are made on accessions to be stored and how many of each, the testing protocol must be established. The amount of intital testing varies greatly from facility to facility. If the development of methods involved several species and cultivars, then less testing may be needed at the

storage phase. There are two schools of thought on testing. The first system uses several storage trials and their controls as the testing phase. In this scenario a lab might store five vials of 10 propagules and use one as the control. If the control recovers with a high percentage, then four vials remain in storage. If recovery of the control is low, then another group is stored to increase the number of viable propagules in storage (Schafer-Menuhr, 1996). The second method stores a small number of propagules in two vials and thaws both after a short time. If the regrowth percentage is >40%, then another group is processed and stored. If the percentage is lower, improvements are made to the culture of cryopreservation protocols to improve performance before storate (Golmirzaie & Panta, 2000; Reed *et al*., 1998).

Storage location

For long-term storage the physical location of the storage dewar is very improtant. As a base collection of important germplasm, the dewar should be located at a site remote from the field genebank if at all possible. The storage site needs to be secure and under the control of dependable management to insure that LN is added to the dewar as needed. Alarms should be installed to monitor the LN level in the dewars and ensure constant temperature in the storage container. The use of a remote storage location is especially important when field collections are at risk

due to environmental or political problems (earthquake, hurricane, flood, volcano, or civil unrest.)

Long-term monitoring

Monitoring of viability of cryopreserved collections is problematic. Ideally storage would be in dewar that is not distrubed, filled regularly, and accessed only rarely. In this ideal case viability should remain similar to control percentages and little testing is required. If storage is in a general purpose dewar that is often used, regular monitoring may be required. Since little data is available on the effect of dewar use on the life of stored propagules, it is difficult to propose an adequate testing scheme. The best advice might be to designate a dewar for storage and ensure that it is not used for other purposes. Dewars with long holding times (one to two years) are now available and would be ideal for base collection.

Cryopreservation and storage records

Germplasm systems require information on the origin of each plant (passport information) and cryopreserved propagules must be linked to that original plant accession. Important cryogenic information must be linked as well since the propagules are to be retrieved 50, 100 or 500 years in the future. Each accession must have information on preparation, pretreatment, cryopreservation method, thawing method, and the recovery

medium. Especially critical to recovering the germplasm are thawing methods and recovery media. These two items should be readily accessible in the accession database for easy access by future curators wishing to recover plants. Complete protocol information could be in a secondary databse as it is not critical to recovering plants, but may be of scientific interest.

GENETIC INTEGRITY OF CRYOPRESERVED PLANT CELLS

Plant cryopreservation has a role to play in the development of long-term storage procedures for the *in vitro* conservation of genetic resources (Ashmore, 1997*)*, which is achieved at ultralow temperatures by placing appropriate explant samples into liquid nitrogen (Harding *et al*.,1997)). This process comprises many stages ranging from tissue culture, pre-growth, cryoprotection, freezing, thawing, recovery (re-growth) and regeneration. Successful cryopreservation is often judged by the survival of the plant tissue and its ability to regenerate into complete plants. Unlike mammalian animal cells, plant cells exhibit the phenomenon of totipotency, that is the ability of a single cell to regenerate to a whole plant (Haberlandt, 1902). Larkin & Scowcroft (Larkin & Scowcroft, 1981). described this process, whereby fully differentiated, organised tissues taken from a plant are introduced into culture, where they are forced into a phase of cell division by growth hormones, which can also result in genetic

change. This phenomenon is collectively known as somaclonal variation, which can manifest itself as variation in: plant morphology (Harding, 1996), numbers of chromosomes (Scowcroft, 1984), accumulation of gene mutations (Scowcroft *et al*., 1984), levels of gene expression in ribonucleic acid, protein profiles and molecular changes in DNA sequences (Semal, 1986).

Moreover, cryopreservation techniques for the long-term conservation of *in vitro* germplasm have been developed using a range of methodologies including rapid and controlled cooling rates, those employing liquid-meristem droplets, protective encapsulation dehydration and chemical cryoprotective vitrification; consequently exposing tissues to physical, chemical and physiological stresses may well result in cryoinjury (Dumet & Benson, 2000). The effects of cryoinjury upon the genome of an organism are often unknown. Because of the cryopreservation-tissue culture-regeneration process, surviving plants may well be subject to the effect of somaclonal variation thereby producing distinct differences in their genotype, phenotype profiles. Obviously, this has significance for *in vitro* conservation and other tissue culture related technologies; consequenlty, the genetic stability of plant tissues cultures has been reviewed (Karp, 1993; Phillips *et al*., 1994; Potter & Jones, 1991). It is important to assess whether plant germplasm surviving cryogenic storage is

genetically identical to the material prior to storage. Therefore, there is an increasing requirement to determine whether plants derived from cryopreservation are true to type and to measure the extent of this near normal phenotype in cryopreserved plants and estimate the degree of closeness to the true parental genotype. Scowcroft, 1984; 1985 indicated that some of this variation may well pre-exist in natural populations of plants taken from field collections or genebanks, moreover, variation itself may be generated de novo as a result of culture techniques. An important application of tissue cultures is the use of differentiated explants, which comprise organised structures like shoot-tips, roots and embryos. These are genetically programmed to develop into true to type plants. If precautions are taken to avoid the dedifferentiated callus phase, it is recognised that the induction of variation in regenerating plants is minimal (D'Amato , 1985)

Tissue culture continues to play a vital role in the development of cryopreservation techniques and there remain many scientific challenges regarding the detection of genomic change. Current assessments of phenotypic variation can be achieved with descriptors for a given species, whereby a range of multi-variate statistical techniques can examine a large number of characters per plant. There is a wide range of cytological techniques available to detect various types of chromosomal

instability. Variation cells with gross chromosomal changes may include: polyploidy, aneuploidy and other mitotic abnormalities. Biochemical metebolite/protein (isozyme) profiles are useful to compare plants recovered from cryopreservation with the original parental material for changing patterns in gene expression. Analysis of genomic DNA sequences has been performed using a range of hybridziation and polymerase chain reaction (PCR) techniques; several investigations report evidence of stability after cryopreservation. Epigenetic variation in chromatin and DNA methylation of gene sequences has been found in plants after cryopreservation suggesting altered patterns of gene expression. The approaches to assess genetic integrity present a unique opportunity for research into cryopreservation and other related activities.

Techniques to assess genetic stability

The analysis of plants regenerated from *in vitro* conservation procedures can be performed at the phenotypic, cytological biochemical and molecular level with a range of techniques (Gonzalez-Benito *et al*., 1999; Harding, 1996; Harding 1999), as described below:

Phenotypic variation

There are reports of morphological changes during the regeneration of *in vitro* plantlets (Scowcroft, 1984; 1985). These plant characters can result from phenotypic plasiticity and therefore such unstable phenomena can be misleading in stability assessments. The evaluation of phenotypic variation can be done with an approved list of plant descriptors (IBPGR, 1977), whereby the characters can be analysed by principal component analysis (Demon *et al*., 1977) using a range of multi variate statistical techniques.

Biometric studies

Where phenotypic studies (Bajaj 1983;1986;1995;Engelmann, 1991; Hao *et al* 2001; Kobayashi & Sakai ,1997) report similarity in control plants compared to those after cryopreservation but lack a detailed examination, the application of biometrics can be useful with phenotypic characters to evaluate variation, as this is more indicative of phenotypic changes resulting from total genomic interactions and temporal changes in gene expression an epigenetic phenomenon (Harding , 1996).

Histological/cytological analysis

Histological studies to evaluate regenerated plants after cryopreservation have shown stability in pea (Haskins & Kartha , 1980), oil palm (Mari *et al* .,1995), silver birch (Ryynanen, 1998)

and *Rubus* species (Chang & Reed, 1999). Cytological studies revealed that the frequency of polyploidization was minimal in plants regenerated from cryopreservation, leading to the conclusion that cryopreservation does not appear to induce ploidy changes in sensitive dihaploids of *S. tuberosum* (Ward *et al*., 1993). Similar observations were made from 20 cultivars of potato after cryopreservation (Schafer *et al* .,1996). Moukadiri *et al.* (Moukadiri *et al* ., 2002) using flowcytometric analysis reported that cryopreservation maintained the characteristics of the rice cell population with an increase in metabolic activity.

Biochemical (metabolite/protein) analysis

The principle of biochemical analysis is to compare the products of gene expression (metabolites/proteins) of the plants recovered from in *vitro* culture with the original parental material which include Metabolite analysis, Protein/enzyme analysis. There are cellular attributes, for example chlorophyll fluorescence (McMichael *et al* .,1989) and other secondary pigments that are useful biochemical markers for genetic stability assessments (Hitmi *et al* .,1999). These pigments can be readily detected with a range of spectrophotometric and chromatographic separation techniques (Charlwood & Rhodes, 1990).

Protein/enzyme analysis

Gel electrophoresis procedures have been instrumental for the classification and identification of plant species in agriculture and horticulture. The application of these electrophoretic techniques also plays an important role in *in vitro* conservation and stability assessments. There are some important considerations in the use of protein/enzyme analysis for genetic stability assessments of plants after cryopreservation: (a) the expression of some proteins enzymes can be subject to alterations by the environment and cellular development of the plant and (b) the genetics of the breeding systems (Cooke ,1989; Gilliland, 1989)). This variation is maintained in cycles of seed production as a dynamic equilibrium within the genepool. Therefore, where a genotype is unique to one cultivar the same protein profile is unlikely to occur in every plant of that cultivar. In contrast, a vegetatively propagated cultivar is a clone of identical (phenotypically and genotypically) individuals each containing the same fixed combination of homozygous and heterozygous genes. Therefore, a cultivar with a distinct electrophoretic profile should contain individual plants expressing the same discriminating genotype, which is different from individual plants of another cultivar. Although, there is a wide range of metabolitel protein markers available, before plant species are introduced into tissue culture, the source and genetic background should be known. This

knowledge is likely to avoid erroneous interpretation of biochemical profiles and make assessments of stability less complex. The stability in the composition of polymorphic proteins in potato plants has been shown in meristems after cryostorage (Donets *et al*., 1991). Electrophoretic profiles of two enzymatic systems, aminoleucine and amylase, were comparable in plants regenerated from *in vitro* cultures, control meristems and cryopreserved apices of sugar-cane (Paulet *et al*., 1993; 1994) in contrast to isozymic variation in plants regenerated form cryopreserved callus cultures (Eksomtramage *et al* .,1992). Also, total soluble proteins, peroxidase and esterase isoenzyme analysis showed no differences between treated plants and non-frozen controls of sweet orange somatic embryos (Marin *et al* .,1993); similarity was also reported in the peroxidase zymograms of cryopreserved kiwi and their controls (W u *et al*., 2001).

Molecular genetic analysis

The frequency of mutation is an important consideration in the detection of genetic stability, as it can influence the sample size in experiments. For example, the natural mutation rate for some microorganisms are recognised to be -10^{-6}-10^{-9} bp/generation (Mayard-Smith, 1998), whereas it is known that field grown potato plants have to be rogued for aberrant forms, particularly as

mutations (bolters) can occur at a relatively high frequency of 10^{-3} (Heiken, 1960). This has significance for stability assessments, as these mutation rates suggest the need to use a sample size of about 1000 plants for potato, but this is also dependent on the techniques used to detect DNA sequence changes. The occurrence of variation, in some cases, can be at high frequencies (>1 per 100). For example, a reduction in the number of ribosomal RNA gene (rDNA) copies was shown to occur in tissue cultures (Landsmann & Uhrig, 1985; Potter & Jones**,** 1991) repetitive DNA sequence probes showed variation in hybridisation signals in two somaclonal variants (Ball & Seilleur , 1986) and variation in rDNA was observed in cryopreserved genomic DNA samples (Harding, 1997).

There is a wide range of molecular techniques available to analyse DNA (Ayad *et al* 1997; Karp *et al* 1997; Westman & Kresovich , 1997). The two general approaches used to study genetic stability are: (a) DNA-DNA hybridisation and (b) Polymerase Chain Reaction (PCR) described below to study genetic stability, from a practical viewpoint, these should be simple, easy, rapid, non-hazardous and cost-effective. However*,* it is important to consider the concept of genomic complexity prior to the selection of an analytical technique.

Genome structure

The molecular architecture of the plant genome is highly complex, it comprises several levels of DNA sequence organisation shown by the variation in the size of the genome in a range of plant species (Arumuganathan & Earle, 1991). The variability in genome size is largely due to the amount of repetitive DNA within the genome; for example, high to moderately reiterated sequences include the genes encoding ribosomal RNAs and proteins essential for functional ribosomes, present in several hundreds to thousands in plant species. The 'house keeping' genes that encode protein products for key metabolic pathways are often found as single/low copy DNA sequences (Dean & Schmidt 1995). There are several classes of reiterative DNA sequences described as long tandem repeats or short nucleotide repeats and many other unique elements within plant genomes (Harding, 1999) there are also well-documented methods described for their detection, these fall into the two approaches described below.

DNA-DNA hybridization

This is an established technique for genome analysis; it is a multistep procedure, which has been applied to germplasm conservation (Harding, 1992; Harding & Benson,1995). This

analysis requires the correct restriction enzyme and hybridisation probe combination to produce an informative DNA fragment profile. Prior to hybridisation, the DNA probe is labelled either radioactively or preferably with a non-radioactive chemical biotin tag (Harding, 1992). The detection of homologous genomic DNA sequences after DNA probe hybridisation generates a characteristic DNA profile described as a restriction fragment length polymorphism (RFLP). This is an informative diagnostic procedure for the identification of plant species and cultivars (Ainsworth & Sharp, 1989; Harding, 1991; Harding & Millam, 1999) and is a proven technique for genetic stability assessments of cryopreserved germplasm (Harding, 1991; 1997; Harding & Benson, 2000; Harding *et al* 2000).

Polymerase chain reaction (PCR) techniques

This is an invaluable 'molecular tool' for genetic analysis. It has numerous applications. Characteristically, PCR involves the use of a thermostable enzyme, Taq DNA polymerise, to cyclically amplify genomic DNA. Typically, a single reaction requires a heat denaturation step to melt the double stranded DNA molecules, which allows the oligonucleotide primers to anneal to their complementary genomic sequences. DNA single strands attached with their primers act as templates for the DNA amplification reaction. PCR oligonucleotide primers of arbitrary nucleotide

sequence or arbitrarily primed oligonucleotides produce randomly amplified polymorphic DNA (RAPDs) fragments (Welsh *et al* 1991; Williams *et al* 1991). The controlled repetition of this reaction sequence will selectively amplify the target DNA sequences specified by the DNA primers, PCR technology is relevant to genetic stability studies. The techniques arc reliable, requiring as little as 5-10 ng of genomic DNA per reaction but they have their limitations (Hallden *et al*.,1996; Lowe *et al* ., 1996). Within a given set of PCR reactions, it is not uncommon to find DNA fragment profile changes between: DNA preparations of the same sample; thereto-cycling machines; operators and laboratories and preparations of Taq DNA polymerase (Harding, 1996).

RAPD analysis

Several studies have reported the detection of genetic stability after cryopreservation by the use of RAPD-DNA fingerprinting, for instance in the endangered shrub species, *Grevillea scapigera* offering support to the use of cryogenic storage methods for the conservation of plant genetic resources (Touchell & Dixon, 1996). The genetic fidelity of the cryopreserved cell lines of embryogenic cultures of open-pollinated *Abies cephalunica* was tested with RAPD markers (Aronen *et al* ., 1999). Dimethylsulfoxide and two

mixtures of polyethylene glycol, glucose and DMSO (PGD I and PGD II), were used as cryoprotectants in treatments with and without storage in liquid nitrogen. The RAPD assays revealed considerable genetic variation in the DMSO treated non-frozen samples, i.e. 16.8% of the RAPD profiles showed intra-clonal variation while background variation was seen in 1.7% of the control amplifications. These results indicate the cryoprotectants may cause a risk for genetic fidelity but this was reduced after cryopreservation. There was no effect of cryoprescrvation on the genetic fidelity of PGD treated somatic embryo cultures of Scots pine (Haggman *et al*., 1998) based on RAPDs profiles. In cryopreserved cultures of white spruce, no genetic differences could be attributed to cryopreservation (DeVerno *et al*., 1999). Among 42 RAPD primers, only one primer produced a polymorphic fragment present in 42 plants (out of 59) regenerated from cryopreserved *Prunus* cultures but it was also found in 44 non-frozen controls taken from 99 plants (Helliot *et al*., 2002). RAPD analysis was performed to detect DNA sequence variation in 8 single-cell sibling lines derived from Citrus callus cultures. In all non-cryopreserved and cryopreserved samples, no aberration in band number and size was observed, which indicated that no DNA sequence variation was detected with the primers used following cryopreservation (Hao *et al*., 2002b). The genetic stability of

plants of *Dioscorea floribunda* derived from cryopreserved shoot tips was evaluated using RAPD analysis of 60 cryopreserved-derived and 20 *in vitro* grown (control) plantlets. For all the plantlets tested, results showed the amplification products were monomorphic with only one polymorphic exception (Ahuja *et al*., 2002). Genetic stability was reported after RAPD analysis of the genomic DNA of *Atropa belladonna* hairy roots following cryopreservation (Yoshimalsu *et al*., 2000).

Simple Sequence Repeat (SSR) analysis

Simple sequence repeats or microsatellites are short, tandemly repeated DNA motifs of I to 4 nucleotides, which are highly abundant in plant genomes, characteristically they are co-dominant, single-locus, simple, robust PCR markers, highly polymorphic. These sequences have been used to develop a simple and rapid technique to assist the post-cryopreservation assessment of genetic stability in vegetatively propagated germplasm (Harding & Benson, 2001). Microsatellite analysis was used to detect DNA sequence length polymorphisms in encapsulation-dehydration cryopreserved *S. tuberosum* cultivars Brodick and Golden Wonder. As the basis of stability assessments, Harding and Benson (2001) reported the reproducibility of microsatellite profiles in: (i) individual, field-grown, plants of

Golden Wonder; (ii) individual Golden Wonder plants derived from a single parental tuber progeny; (iii) plantlets derived from *in vitro* cultures of Golden Wonder and Brodick and (iv) Golden Wonder and Brodick plantlets derived from cryopreserved germplasm.

Amplified Fragment Length Polymorphism (AFLP) analysis

This is a multi-locus DNA fingerprinting technique that has been used to assess the genetic stability of several plant species after cryopreservation (Turner *et al*., 2001; Vos *et al*.,1995). Typically, it requires the use of fluorescent markers, an automated sequencer (ABI Prism 377 automated genetic analysis system, AGAS) and dedicated software (ABI GeneScan) to detect polymorphic DNA fragments after DNA-primer amplification. AFLP analysis of cryopreserved Prunus plants and their non-frozen controls revealed two polymorphic fragments characterised by a 135bp fragment and a missing 90bp fragment. The frequency of polymorphism was 18% in the non-frozen plants and 37% for the *in vitro* plants regenerated from cryopreservation (Helliot *et al*., 2002). AFLP analysis of DNA samples from eight apple single-bud sibling lines revealed equal numbers of amplified fragments from the non-cryopreserved samples and cryopreserved samples of

each line; no change in DNA fragment pattern and number was observed during the period of cryopreservation (Hao *et al*., 2001). AFLP analysis was performed with non-cryopreserved and cryopreserved single-cell sibling lines derived from *Citrus* callus cultures (Hao *et al*., 2002b); analysis showed that the DNA fragments amplified were identical in number and size, with one additional different fragment detected in the cryopreserved samples. The difference was contributed by a change in DNA methylation status; similar DNA methylation may account for the AFLP polymorphisms seen in the analysis of *Prunus* cultures (Helliot *et al*., 2002) and those derived from cryopreserved potato (Harding, 1997). The effects of three storage methods (tissue culture, cold storage and cryostorage) on genetic fidelity and shoot apex viability were evaluated for *Anigozanthos viridis* after 12 months of storage (Turner *et al*., 2001). The AFLP technique generated a total of 95 fragments for three primer pairs, and no differences were detected across treatments. Although no qualitative (presence/absence) differences were detected among the 45 DNA fingerprints (a total of 95 DNA fragments scored between 60 and 500 by in length), some `lane to lane' experimental variation was observed in peak height, but replicate runs confirmed that these were due to subtle PCR effects and/or gel loading artefacts. These results show that genetic fidelity and

shoot apex viability (for cryopreserved material) were maintained following tissue culture, cold storage and cryostorage of *A. viridis* for up to 12 months .Wilkinson *et al* (Wilkinson *et al* ., 2003*)* reported there were no genetic differences in the AFLP analysis of *Cosmos atrosanguineus* tissue cultures and cryopreserved material after 12 months:

Cryopreseryation techniques have been developed for more than 100 species derived from various tissues cultures (cell suspensions, calluses, apices, somatic and zygotic embryos (Bajaj, 1995; Engelmann, 1997), with current publications reporting numerous methodological improvements and applications of the alginate encapsulation-dehydration and vitrification procedures to an increasing range of plants. Commensurate with the advancement of cryopreservation techniques has been the steady increase in genetic stability assessments to validate newly established cryoprservation protocols (Martinet *et al*., 1998).

It has been 29 years since the first reports of genetic stability after cryopreservation (Bajaj, 1978) and during the last decade, there have been some 80 reports of genetic stability after cryopreservation. The literature is overall `positive' regarding the outcome of stability assessments from cryopreservation with studies falling into four main areas: phenotypic/morphological (45 reports); cytological (9 reports); biochemical (13 reports) and

molecular biological (35 reports) using the following molecular techniques: RFLPs, RAPDs, SSRs and AFLPs/MSAPs and other PCR techniques. The increase in genetic stability studies has paralleled advances in molecular biology, especially through various training initiatives aimed at improving the utilisation and application of current techniques.

There is a need for guidance, before the selection and application of specific techniques; guidelines exist for the selection of markers (Westman and Kresovich, 1997) and their application to assess genetic diversity in conservation (Ayad *et al*., 1997; Karp *et al*., 1997) but there is little assurance that these techniques provide adequate information in stability assessments. Paradoxically, before the establishment of molecular techniques, the plants phenotype and morphological descriptors were commonly used criteria to assess stability (Bajaj, 1978; 1995) and, during their development, it was claimed these techniques would provide simple, rapid screening procedures as future alternatives to existing morphological markers. Indeed, comparisons of phenotypes provide valuable information in contrast to molecular techniques, which sample a small fraction of the genome, perhaps, in most cases. Thus, it is not unusual to expect the detection of stability rather than instability. However, where plants regenerate from cryopreservation, it is generally considered, that appropriate

estimates of stability can be made from the phenotypic and cytological approaches (Helliot *et al*., 2002) and further supportive evidence can he obtained from the biochemical and molecular investigations.

RELEVANCE OF THE PRESENT STUDY

Spices like Cardamom, Ginger and Black pepper are commercially important crops in India. Since these crops are propagated vegetatively, in living collections working for ex situ preservation of these plant genetic resources always imposes special needs on the staff and requires much more labour input. In these crops, both field culture and *in vitro* preservation suffer from many risks for the maintenance, such as virus infections and difficulties in permanent micropropagation and storage regimes. Cryopreservation is, therefore, an increasing conservation strategy in these crops and can be an excellent alternative to both field and *in vitro* gene banks. Hence, cryopreservation or freeze-preservation at ultra-low temperature (-196°C, i.e., the temperature of liquid nitrogen) is a sound alternative for the long-term conservation of plant genetic resources, since under these conditions, biochemical and most physical processes are completely arrested. As such, plant material can be stored for unlimited periods. Moreover, besides its use for the conservation of genetic resources, cryopreservation proved to be extremely useful

for the safe long-term storage of plant tissues with specific characteristics and can also be used for disease – free exchange of germplasm from one location to another. So far for the long-term conservation of the germplasm of spices like black pepper and cardamom, have used only seeds (Chaudhury & Chandel, 1994; 1995) which are recalcitrant and heterogenous and ginger shoot buds with encapsulation dehydration,(Geetha, 2002) where recovery rate is very low, Therefore, for both practical and scientific reasons, there is a need to find alternative cryopreservation strategies like encapsulation dehydration, encapsulation vitrification and vitrification which reduce demands on time and resources and results in maximum recovery. In addition, the molecular techniques standardized to estimate the genetic fidelity can be used to characterize the valuable germplasm.

The present study is taken up keeping all the above in view.

MATERIALS

PLANT SPECIES STUDIED

In the present study, vegetatively propagated spice crops namely, Cardamom (*Elettaria cardamomum* Maton) ,Ginger (*Zingiber officinale* Rosc. var. Maran), Black pepper (*Piper nigrum* L.) with its related and an endangered species *Piper barberi* Gamble., were used. The *in vitro* plants already established and maintained in the *In Vitro* Gene Bank of Indian Institute of Spices Research, Calicut, were used as mother plants for source of explants which in turn facilitates the reduction in size of the plantlets, made them suitable for cryopreservation.

CULTURE MEDIUM

Murashige and Skoog (1962), Woody Plant (McCown and Amos, 1979) and Schenk and Hildebrandt (1972) media with modifications wherever required, were used in the present study (Table 5). Plant growth substances namely 6-Benzyl adenine(BA), kinetin (Kin), α-naphthalene acetic acid (NAA), and Indole-3-butyric acid (IBA, were used in various permutation and combination. Analytical grades of the chemicals were used in the preparation of various media.

Composition of culture media

Macro and micronutrients were obtained from 'Hi-Media', Bombay, while vitamins and growth regulators were procured from 'Sigma', USA. The required quantities of chemicals were weighed in an analytical balance (Sortorius) and dissolved in double distilled water. For convenience, the stocks of macronutrients (20X),

micronutrients, myo-inositol and vitamins (100X) were prepared and stored in reagent bottles. Separate stock solutions for each hormone required for the establishment of cultures, initiation of growth and maintenance, proliferation, and rooting were prepared and stored and supplemented to basal media in various concentrations and combinations. All stock solutions were stored in refrigerator.

Table 5 Composition of MS*, WPM* and SH* basal media

Composition	Molecular formula	Concentration (mgl^{-1}) MS	Concentration (mgl^{-1}) WPM	Concentration (mgl^{-1}) SH
Macronutrients				
Ammonium nitrate	NH_4NO_3	1650.00	400.00	-
Ammonium phosphate	$NH_4H_2PO_4$	-	-	300.00
Potassium nitrate	KNO_3	1900.00	-	2500.00
Calcium chloride	$CaCl_2.2H_2O$	440.00	-	-
Calcium chloride	$CaCl_2$	-	72.50	151.00
Calcium nitrate	$Ca(NO_3)_2.4H_2O$	-	386.00	-
Potassium di hydrogen orthophosphate	KH_2PO_4	170.00	170.00	-
Potassium sulfate	K_2SO_4	-	990.00	-
Magnesium sulphate	$MgSO_4.7H_2O$	370.00	180.70	195.40
Micronutrients				
Sodium EDTA	Na_2EDTA	37.30	37.30	20.00
Ferrous sulphate	$FeSO_4.7H_2O$	27.80	27.800	15.00
Boric acid	H_3BO_3	6.20	6.20	5.00
Manganese sulphate	$MnSO_4.4H_2O$	22.30	22.30	10.00
Potassium iodide	KI	0.83	-	1.00
Zinc sulphate	$ZnSO_4.7H_2O$	8.60	8.60	1.00
Sodium molybdate	$Na_2MoO_4.2H_2O$	0.25	0.25	0.10
Copper sulphate	$CuSO_4.5H_2O$	0.025	0.25	0.20
Cobalt chloride	$CoCl_2.6H_2O$	0.025	-	0.10
Vitamins				
Myo-inositol	$C_6H_{12}O_6$	100.00	100.00	1000
Thiamine HCl	$C_{12}H_{17}ClN_4OS.HCl$	0.10	0.50	100
Nicotinic acid	$C_6H_5NO_2$	0.50	0.025	1.00
Pyridoxine HCl	$C_6H_{11}NO_3.HCl$	0.50	0.025	1.00
Amino acid				
Glycine	$C_2H_5NO_2$	2.00	1	-

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

Distilled water

Double distilled water was used for washing glasswares and preparation of stock solutions, growth regulators, media, buffers etc.

Glassware

Borosil or Duran Scoth glassware was used in all the experiments, unless otherwise mentioned. Culture tubes (15 x 200 mm) were used for culture initiation. For plantlet growth and multiplication, Duran Scoth conical flasks (100-500 ml) and borosilicate glass bottles were used. Duran conical flasks (250 ml) were used for liquid cultures and Tarson petriplates were used for plating after cryopreservation.

Cryovials

Sterile Vangard cryogenic vials (Sumitomo Bakelite Co, Ltd, Japan) of quantity 2 ml were used for cryopreservation experiments.

Closures

Test tubes, bottles and flasks were closed with cotton plugs made of non-absorbent cotton covered with cheesecloth, or polypropylene caps or aluminium foils wrapped with cling films were used as closures, depending on the nature of the experiments.

METHODS

Experiments were conducted to standardize cryopreservation protocols and to assess the genetic fidelity of the conserved material through morphological and molecular characterization.

MEDIA PREPARATION AND STERILIZATION

Stock solutions of macronutrients, micronutrients, vitamins and amino acids were prepared using double distilled water. During macronutrient preparation, calcium chloride was separately dissolved and added to the solution of other chemicals. Concentration of stock solutions for all three media and growth regulators are given in the Table 6.

Table 6 Details of various stock solutions for # MS,# WPM and #SH media.

Stock composition & Strength - MS	Quantity for 1 liter medium(MS)	Stock composition & Strength - WPM	Quantity for 1 liter medium(WPM)	Stock composition & Strength - SH	Quantity for 1 liter medium(SH)
A . Macronutrients X20		**A . Macronutrients** X100	10 ml	**A . Macronutrients** X20	
NH_4NO_3		NH_4NO_3		KNO_3	
KNO_3		$Ca(NO_3)_2.4H_2O$		$MgSO_4.7H_2O$	
$CaCl_2.2H_2O$*				$NH_4H_2PO_4$	50 ml
KH_2PO_4		X50		$CaCl_2.2H_2O$*	
$MgSO_4.7H_2O$	50 ml	K_2SO_4	20 ml		
		X100			
		$CaCl_2.2H_2O$*	10 ml		
		X100			
		KH_2PO	10 ml		
B. Micronutrients X100		**B. Micronutrients** X100		**B. Micronutrients** X200	
H_3BO_3		H_3BO_3		H_3BO_3	
$MnSO_4.4H_2O$		$MnSO_4.4H_2O$		$MnSO_4.4H_2O$	
KI		$ZnSO_4.7H_2O$		KI	5 ml
$ZnSO_4.7H_2O$	10 ml	$Na_2MoO_4.2H_2O$	10 ml	$ZnSO_4.7H_2O$	
$Na_2MoO_4.2H_2O$		$CuSO_4.5H_2O$*		$Na_2MoO_4.2H_2O$	
$CuSO_4.5H_2O$*				$CuSO_4.5H_2O$*	
$CoCl_2.6H_2O$*				$CoCl_2.6H_2O$*	

C. Micronutrients		**C. Micronutrients**		**C. Micronutrients**	
X100 Na_2EDTA* $FeSO_4.7H_2O$	10 ml	X100 Na_2EDTA* $FeSO_4.7H_2O$	10ml	X200 Na_2EDTA* $FeSO_4.7H_2O$	5 ml
D. Vitamins X100 Thiamine HCl Nicotine acid Pyridoxine HCl	10 ml	**D. Vitamins** X100 Thiamine HCl Nicotine acid Pyridoxine HCl	10 ml	**D. Vitamins** X100 Thiamine HCl Nicotine acid Pyridoxine HCl	10 ml
E. Amino acid X100 Glycine	10 ml	**E. Amino acid** X100 Glycine	10ml		
F. Myo-inositol X100	10 ml	**F. Myo-inositol** X100	10ml	**F. Myo-inositol** X100	10 ml
Growth regulators					
NAA	100mg/100ml				
BA	100mg/100ml				
Kin	100mg/100ml				
IBA	100mg/100ml				

*Dissolve separately before mixing in the final stock.

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

To prepare media, the required amount of each of the above stock solutions of macronutrients, micronutrients, vitamins, hormones and carbon source (sucrose, Merck) and other supplements were added in appropriate quantities and the final volume was made to the required quantity by adding double distilled water. The pH of the medium was adjusted to 5.8 using 0.1 N NaOH or HCl. For gelling the medium, agar agar (Qualigens) was used at 0.7-0.8% (w/v). After melting the agar, the media were dispensed into appropriate containers and sterilized by autoclaving at 121^0C and 15 psi (1.08 kg/cm^2) for 20 minutes. Alternatively, for

media containing thermolabile chemicals Millipore filter sterilized system with 0.22 µ pore size was used in laminar airflow station and dispensed into appropriate sterile containers.

Aseptic inoculation

Surface sterilization of explants, preparation and inoculation and further subculturing were carried out under a laminar airflow station (Klenzoids). Before using the laminar airflow station it was wiped with alcohol and sterilized under UV light. All the surgical instruments were autoclaved and dried in oven. These were dipped in alcohol, flamed and cooled before using.

Culture conditions

The cultures were generally maintained in an air conditioned room with temperature at 25±2^{0}C and relative humidity around 60%, photoperiodic regime of 16 hours light and 8 hours dark cycle provided by 'Phillips' cool white fluorescent tubes at an intensity of 130 µEm^{-2}S^{-1}. The cultures were incubated in dark for somatic embryo induction from black pepper seeds and immediately after cryopreservation.

Micropropagation

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 were used.

IN VITRO CULTURE INITIATION IN CARDAMOM AND GINGER

Collection, surface sterilization and inoculation of explants

Healthy rhizomes of cardamom and ginger were collected from the field and were thoroughly washed in running water. The scales on the rhizome surface were removed and washed. These rhizomes were cut into bits of 2-3 cm having buds, soaked in distilled water containing copper oxychloride and a few drops of Tween-20 for 30 minutes and then thoroughly washed with distilled water. Further steps were carried out under laminar flow station. Buds were surface sterilized with 0.1% mercuric chloride solution for 8 minutes, followed by thorough washing with sterile distilled water for three times to ensure complete removal of the disinfectant. These were placed on a sterile petriplate and the rhizome segments containing buds with a portion of rhizome tissue were excised carefully using sterile forceps and scalpel and transferred to basal MS medium supplemented 0.5 mg/l kinetin for culturing. The cultures were initially placed in dark for 2 days and then transferred to light.

Shoots obtained *in vitro* were used as explants for further studies. Individual shoots were trimmed to 1-2 cm long in a sterile pertriplate using sterile scalpel and forceps. These were cultured

on MS medium, supplemented with BA (2.2-13.3 µM) These cultures were incubated in a culture room. Observations were recorded at 15 days interval.

INDUCTION OF SOMATIC EMBRYOS IN BLACK PEPPER

Mature seeds of black pepper (*Piper nigrum* L.) cv. "Karimunda" collected from a plant grown in the germplasm repository at the Indian Institute of Spices Research, Calicut were used for establishing primary somatic embryogenic cultures.

Establishment of primary somatic embryogenic cultures

Primary embryogenic cultures were established following the method described by Nair and Dutta Gupta (2003). The surface sterilized seeds were cultured 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 d were 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, Hi-media). 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.

IN VITRO CULTURE INITIATION IN *PIPER BARBERI*

Shoot tips, approximately 2-3 cm long, were excised from field grown plants of *Piper barberi* and were washed in detergent (teepol) for 15 minutes. They were surface sterilized with 0.1% $HgCL_2$ for 5 minutes and were rinsed in 3 times in sterile water and transferred to culture media in aseptic condition. Woody Plant Medium (McCown & Amos, 1979) was used as basal medium with 2% sucrose and 0.6% agar supplemented with 0.5 mg/l kinetin.

CRYOPRESERVATION

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,

the approach is different and 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^{0}C) can be achieved by different techniques like encapsulation dehydration, encapsulation vitrification and vitrification.

CARDAMOM

Plant material and culture conditions

Stock *invitro* shoot cultures of cardamom (*Elettaria cardamomum* Maton) were maintained on MS medium that contained 3% sucrose and 7% (w/v) Hi media agar at pH 5.8 (Basal medium) and was supplemented 1 mg/l BA and 0.5 mg/l IBA (proliferation medium).

Production of starting material

All cardamom cryopreservation experiments revealed that regrowth after cryopreservation could only succeed with 1-2 mm long shoots. To minimize the effect of somaclonal variation and to maintain genetic stability, the proliferated cardamom cultures were maintained in growth regulator free medium.

Encapsulation Dehydration

1. Cardamom shoots were collected and suspended in MS basal medium supplemented with 4% (w/v) Na alginate, 2M Glycerol and 0.4 M sucrose.
2. The mixture including shoots, was dropped with a sterile pipette into 0.1M $CaCl_2$ 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. The encapsulated shoots were then stepwise precultured 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. The precultured beads were then placed on sterile fitter 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. The water content of the beads was measured by weighing them prior and after drying in an oven at 80^0C for 48h.
6. Dehydrated beads were then transferred into a 2 ml cryovial (ten beads per tube) and directly immersed in liquid nitrogen for 24h.

Encapsulation Vitrification

Encapsulation, osmoprotection and vitrification

1. Cardamom shoots (1-2mm) with 2-3 apical domes pre-cultured on 0.3M sucrose for 16h were suspended in MS basal medium supplemented with 4% sodium alginate and 0.3 M sucrose.
2. The mixture including shoots, were dispensed with a sterile pipette into MS medium supplemented with 0.1M $CaCl_2$ and 0.4 to 1.0M sucrose, with or without 2M Glycerol gently shaken (20 rpm) on a rotary shaker for 1h at 25^0C.
3. Beads containing one shoots were about 4 mm in diameter. In this procedure, osmoprotection was carried out simultaneously with the encapsulation process.

4. Ten to 15 encapsulated and osmoprotected cardamom shoots were dehydrated with 20 ml PVS2 in a 100 ml Erlenmeyer flask at 25^0C and plunged into LN and held for at least 24 h at -196^0C.
5. 15 beads were treated in each of 3 replicates.

Vitrification

1. Shoots (1-2mm in diameter with at least two meristematic domes) were excised and cultivated on MS medium supplemented with 0.3 M sucrose for 24h at 25^0C.
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 cryocial 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 (10, 20, 40, 60, 80, 100 minutes) were tested for osmoprotected explants
5. Cryovials containing 8-10 explants were directly immersed in liquid nitrogen and kept for 24 h.

Thawing and plating

In a first trial after PVS2 treatment and cooling in LN, explants were thawed by removing the vials from the LN maintaining them for 5-10 s at air temperature and then plunging them for 2-3 minutes in a water bath adjusted to one of the following temperature 30^0C, 35^0C, 40^0C, 45^0C and 50^0C. In all subsequent experiments, the explants were thawed at 40^0C.

Dilution process and percentage survival

After LN storage, cryovials were rewarmed rapidly in a 40 ^{0}C water bath for 2 min in all three experimental conditions, PVS2 was drained from the cryovials and replaced twice at 10 min intervals with 1 ml 1.2 M sucrose solution in the case of encapsulation vitrification and vitrification methods. In two additional experiments, the composition of recovery medium (Basal medium supplemented with 2.22 µM BA, 2.69 µM NAA, BA (2.22 µM) + NAA (2.69 µM), BA (4.44 µM) + NAA (2.69 µM), NAA (5.37 µM) + BA (2.22 µM), NAA (5.37 µM)+ BA (4.44 µM) and the effect of the subculture period of stock shoots (3 or 5 wk) were examined.

Viability tests using TTC (Stponkus & Lamphear, 1967; Harding & Benson, 1995)

The solution contained 0.6% (w/v) TTC and 0.05% Tween 80 in a 0.05 M Na_2 HPO_4/KH_2PO_4 buffer at pH 7.4. Cryopreserved shoots were placed in a test tube and 1.5ml of TTC – solution was added and allowed to penetrate the plant tissues (Vaccaum, 30 min); subsequently the shoots were incubated overnight at 28^{0}C (atmospheric pressure)

TTC microscopic test

During the incubation in the TTC solution, the colour of the shoots were closely monitored (every hour, until results of scoring were stable). After 24 h the colour of the meristemaic clumps were evalauated under a microscope. Red tissue was regarded as living tissue with other colours was presumed to be dead. Shoots were

regarded as being alive if more than 50% of the meristematic tissue was coloured red.

Plant regeneration

In the En De, En Vi and vitrification procedures, surviving shoots were identified by green colour following 2 weeks of post culture. Regrowth was defined as the shoots that regenerated to shoots in 6 weeks of postculture. Elongated shoots were used for micropropagation and rooting and subculture was done every 4 weeks. For rooting shoots longer than 5 mm were transferred to solid MS medium devoid of phytohormones.

GINGER

Plant material and culture conditions

For all cryopreservation experiments shoot buds (0.5-1.0 mm in length) consisting of the apical dome with 3-4 leaf primordia of ginger were used. *In vitro* stock cultures of ginger cv Maran were maintained on MS medium that contained 3% sucrose and 7% (w/v) (Hi Media) Agar at pH 5.8 with 1 mg/l BA (proliferation medium).

Encapsulation Dehydration

1. Ginger shoots consisting of the apical dome with 3-4 leaf primordia were pipetted with 4 % sodium alginate solution and dispensed drop wise in liquid MS medium containing 0.1 M calcium chloride.
2. After encapsulation beads (each containing one or two shoots) were left for 30 min in the calcium chloride solution to ensure polymerization of calcium alginate.

3. The beads were then subsequently precultured in liquid MS medium containing 0.1, 0.3, 0.5, 0.7, 0.9 and 1.0 M sucrose for 24 h each.
4. The precultured beads were placed on sterile filter paper in Petri dishes (90 mm) and dehydrated in laminar flow cabinet (at room temperature and humidity) for periods of 0-10 h to determine the optimal dehydration time.
5. For each dehydration time, half of the beads were then transferred to proliferation medium
6. The other half were placed in 2 ml cryotubes (15 beads to a vial), which were immediately plunged into liquid nitrogen and kept for 24 h.
7. The beads of a control group that had received no sucrose or dehydration treatment were similarly dealt with half being cooled and the other half left uncooled.
8. The water content of the beads was determined as follows: total fresh weight of 15 beads with three replicates, was measured after sucrose preculture and during the dehydration period (0-10 h). After the final period of dehydration, the beads were oven-dried at 85 ºC for 24 h to determine the dry weight. Bead water content on each period was calculated from these values and expressed as the percentage of water weight over fresh weight.

Encapsulation Vitrification

1. Shoots were encapsulated following the above procedure and precultured in 0.1 M sucrose for 24 h and osmoprotected with 0.3 M sucrose for 16 h.
2. Osmoprotection was followed by treatment with 2 M glycerol with 0.4 M, 0.6 M, 0.8 M, 1.0 M, 1.2 M, 1.4 M 1.6 M, or 1.8 M sucrose for 3 hours on a shaker at 60 rpm at 25ºC.

3. The beads were then placed in a 2 ml cryotube and then osmotically dehydrated with 1.5 ml PVS2 at 25 ºC for 40 min.
4. The encapsulated beads in the cryotube were suspended in 1.0 ml fresh PVS2 (15% (w/v) DMSO, 15% (w/v) ethylene glycol and 30% (w/v) glycerol in liquid MS medium with 0.4 M sucrose) solution (37) prior to a plunge into LN for 24 h.

Vitrification

1. Naked shoots (0.5-1.0 mm) were precultured in 0.3 M sucrose for 72 h.
2. The precultured shoots were immersed for 20 min at room temperature in five cryoprotectant mixtures containing 5% DMSO, 10% DMSO, 5% glycerol, 10% glycerol, 5% DMSO + 5% glycerol.
3. Further, the shoots were treated with loading solution (2 M glycerol + 0.4 M sucrose) for 20 min at 25ºC.

Naked, precultured and osmoprotected shoots were then dehydrated in 20 ml PVS2 in a 50 ml Erlenmeyer flask in a rotary shaker (60 rpm) at 25 ºC for various periods of time (0-60 min).

After dehydration shoots were suspended in 1 ml of PVS2 in cryotubes and plunged into LN where they were kept for 24 h.

Control replicates were transferred directly to MS + BA (4.44 µM) and NAA (2.69 µM) and placed in the culture room as described in *in vitro* culture.

Warming, detoxification and regrowth

Rewarming, detoxification and regrowth procedures were performed same as for cardamom. Treated controls were processed in the same way except that they were not stored in liquid nitrogen.

Viability tests using TTC and plant regeneration

Viability tests using TTC (Stponkus and Lamphear, 1967; Harding and Benson, 1995) was performed with cryopreserved shoots for immediate survival assessment and shoot formation was expressed as a percentage of the total number of shoots forming normal shoots 8 weeks after plating. Fifteen shoots were used in each experiment with three replicates.

BLACK PEPPER

Plant material and culture conditions

For cryopreservation experiments, explants consisting of 6-8 mg clumps of globular torpedo and heart shaped secondary embryos were dissected from the embryogenic cultures. All embryogenic cultures were maintained in the dark at 25^0C. At all stages of clone maintenance and experimentation, all cultures were incubated under the standard conditions described above.

Cryopreservation of black pepper somatic embroyos was carried out following the basic protocol of encapsulation dehydration and vitrification techniques.

Encapsulation Dehydration

1. Somatic embryos clumps consisting of the globular, heart and torpedo embryos were pipetted with 4% sodium alginate solution and dispensed drop wise in liquid SH medium containing 0.1 M calcium chloride.

2. After encapsulation beads (each containing one clump) were left for 30 min in the calcium chloride solution to ensure polymerization of calcium alginate.
3. The alginate coated embryos were then pregrown on liquid SH medium containing different sucrose concentrations 0.1M, 0.3M, 0.5M, 0.7M, 0.9M or 1.0M for a pregrowth period of one day.
4. After pregrowth embroyos were dehydrated in the air current of the laminar flow cabinet from 93 % down to 16 % of moisture content (fresh weight basis) in the beads.
5. After desiccation the algimate coated embroyos were rapidly frozen by direct immersion into liquid nitrogen.
6. After 24h of storage at -196^{0}C, samples were thawed in the laminar air flow (2-5 minutes) at 40^{0}C.
7. Thawed beads were further transferred to standard culture medium for recovery.
8. Non frozen control (pregrowth and desiccation) were included in all the experiments.
9. The water control of samples was determined as follows; total fresh weight of 15 embroyos cluster exploits with three replicates was measured after sucrose preculture and during the desiccation period (0-8h). after the final period of desiccation the embroyos were oven dried at 35^{0}C for 24h to determine the dry weight. Embroyo water content on each period was calculated from these values and expressed as the percentage of fresh weight over dry weight.

Vitrification

1. Harvested somatic embryo clumps were precultured for 3 days on SH basal medium containing 0.3M sucrose.
2. Pretreated 15 embryo clumps were then placed to a 2ml cryovial with 1.8 ml of PVS2 vitrification solution consisting of 30% w/v

glycerol 15% w/v DMSO 15% w/v ethylene glycol in basal medium containing 0.4 M sucrose (Sakai *et al* 1990).

3. After 0, 30 60 90 or 120 min at 25^{0}C in PVS 2 solution the embryo clumps were resuspended in 0.6 ml of PVS2 and were then plunged in liquid nitrogen (LN).

Warming, detoxification and regrowth

1. After 24h embryo clumps were removed from LN and rewarmed by 2 min immersion in a 40^{0}C water bath, and the PVS2 solution was drained off.
2. The embryo clumps were then washed with liquid basal medium supplemented with 1.2 M sucrose with two changes of liquid medium, each of 10 min, before being placed on fitter paper discs on basal medium gelled with 0.5% agar in petri dishes.
3. After 24 h they were transferred with out any fitter paper, to fresh basal medium gelled with 0.6% agar in petri dishes (25 ml of medium per petri dish and 15 clumps to a petri dish) where they remained until they were evaluated 6 weeks later.
4. Uncooled samples presultured in medium containing 0.3M sucrose and treated for 0-120 min with PVS2 solution (treated controls) were also included.

Viability tests using TTC and plant regeneration

Viability was estimated by means of a triphehyltetrazolium chloride reduction assy (TTC test, Stemponkus & Lanphear, 1967). 10 embryos were incubated in 3 ml of 0.6% TTC solution in 0.05M Potassium phosphate buffer (pH 7.5) at 28^{0}C for 24h. viability was measured 2 days after post culture and expressed as the percentage reduction in TTC activity over that of control cells

(embryos without any preculture and dehydration). Plant regeneration was expressed as a percentage of the total number of embryos forming normal plantlets 8 weeks after plating. Fifteen embryo clumps were used in each experiment with three replicates.

PIPER BARBERI

Plant material and culture conditions

Apical and axillary shoot tip meristems with 2-3 leaf primordia (about 0.8- 1.5mm in size) of an endangered piper species (*Piper barberi* Gamble.) were used for experiments.

Encapsulation

1. Shoots were grown on WPM amended 13.31µM BAP and 4.65 µM kinetin and 0.8-1mm shoot tips were collected and suspended in WPM supplemented with 4% (w/v) Na-alginate, 2M glycerol and 0.4 M sucrose.
2. The mixture including shoot tips was dropped with a sterile pipette into 0.1M CaCl2 solution containing 2M glycerol and 0.4 M sucrose and left for 20 min to form beads about 4 mm in diameter.
3. Each bead was containing at least one shoot tip.

Preculture

1. A direct and stepwise preculture procedure were tested to determine how the final sucrose concentrations of the preculture medium affected tolerance of shoot tips to dehydration and subsequent freezing.
2. In the direct preculture encapsulated shoot tips were cultured for 3 days on WPM enriched with 0.25, 0.5, 0.75 or 1 M sucrose.

3. In the stepwise procedure, the beads were precultured in an initial sucrose concentration of 0.25 M which was elevated daily by 0.25M until the designated final sucrose concentration of 0.5, 0.75,1.0 or 1.25 M was reached.

Encapsulation Dehydration

1. After encapsulation and stepwise preculture, to desiccate beads were placed in open petridishes and dried in the air flow of a laminar flow bench, for periods of 0-8 h to determine the optimal dehydration time.
2. The water content of the beads was measured by weighing them prior and after drying in an oven at 80^0C for 48h.
3. For cryopreservation experiments, beads were placed in cryovials (10 per vial), frozen in liquid nitrogen and stored for 48 h, after which they processed as described below for EnVi.

Encapsulation vitrification

1. Encapsulation and preculture were the same as for encapsulation dehydration treatment.
2. Following preculture the beads were treated for 0-120 min at room temperature with a loading solution composed of WPM containing 2M Glycerol and one of the following seven sucrose concentrations of 0, 0.2, 0.4, 0.6, 0.8,1.0 or 1.2M.
3. Loading for various durational 0, 30, 60, 90 and 120 min at room temperature was also tested using the loading solution containing WPM supplemented with 2M Glycerol and 0.6 M sucrose.
4. The beads were rapidly surface dried by blotting on sterile tissue paper and dehydrated with plant vitrification solution 2 (PVS2) (Sakai *et al*, 1990), at 25^0C for various lengths of time.

5. Vitrified beads were transferred into a 2ml cryovial (ten beads per vial) containing 1.8 ml PVS2 and directly immersed in liquid nitrogen for 48 h.

Thawing, detoxification and plating

1. After PVS2 treatment at 0^0C and cooling LN beads were thawed by removing the vials from the LN maintaining them for 5-10 s at air temperature and then plunging them for 2-3 minutes in a water bath adjusted 40^0C.
2. After thawing, PVS2 was drained from the cryovials and replaced with a liquid WPM medium containing 1.2M sucrose (Detoxification solution Sakai *et al*, 1991) in which the beads were washed for 20 min.
3. The beads were then post cultured in a petridish (diameter 90mm) containing 25ml semisolid WPM. The cultures were placed at 25^0C in the dark for one week and then transferred to the light conditions described for the stock cultures. Subculture was done after one month

Plant Regeneration

In the EnVi and EnDe procedures surviving shoot tips were identified by green colour following 3 weeks of post culture. Regrowth was defined as the shoot tips that regenerated into sheds in 8 weeks of post culture. Elongated shoots were used for micropropagation and rooting. For rooting shoots of 1-2 cm were transferred to solid WPM devoid of phytohormones. After 12 weeks rooted shoots were placed into plastic pots containing sterile sand

and coir pith, and covered with a plastic bag to maintain high humidity and prevent plantlets from wilting. After one week, the plastic bag was gradually opened to reduce humidity.

GENETIC STABILITY

Molecular characterization

RAPD and ISSR analysis has done to evaluate genetic fidelity of the cryopreserved lines of cardamom, ginger, black pepper, and *P.barberi* conserved in liquid nitrogen for 24-48 hours. In all these species 8 -13 samples were compared with the control (mother plant).

Isolation of DNA

In the present study CTAB method (Ausubel *et al*., 1995) was followed to isolate DNA from the leaf tissue. The procedure is as follows.

1. Grind 2 g of young leaves in liquid nitrogen with a mortar and pestle and add 8 ml of preheated (60^0C) CTAB buffer. Add 0.2% β -Mercaptoethanol prior to use.
2. Incubate at 60 ^{0}C for 1hour

3. Extract with equal volume of chloroform: isoamyl alcohol (24:1) at 10,000 rpm for 10 minutes at room temperature
4. Take the aqueous phase and add 2/3 rd volume of ice cold isopropanol
5. Incubate at –20 ^{0}C for 30 minutes and centrifuge at 10,000 rpm for 15 minutes at 4 ^{0}C
6. Discard the supernatant and invert the tube on tissue paper for a few minutes and dissolve the pellet in 1.5 ml of TE buffer. Store at room temperature over night

Purification of DNA

1. Add 10 µg/ml of RNase A and incubate at 37 ^{0}C for 30 minutes
2. Add equal volume of Tris saturated phenol, mix it well and centrifuge at 10,000 rpm for10 minutes
3. To the aqueous phase add equal volume of phenol: chloroform: isoamyl alcohol
4. (25:24:1), shake and centrifuge at 10,000 rpm for 10 minutes
5. Take the aqueous phase and add equal volume of chloroform:isoamyl alcohol (24:1), shake and centrifuge at 10,000 rpm for 10 minutes
6. To the aqueous phase add one-tenth volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of ethanol and incubate at –20 ^{0}C for one hour or at –70 ^{0}C for 30 minutes
7. Centrifuge at 10,000 rpm for 10 minutes and wash the pellet in 70% ethanol (10,000 rpm for 5 minutes)
8. Air dry the pellet and dissolve in 1.5 ml TE and estimates the yield

Preparation of buffers and stocks

The buffers and stocks required for the study were prepared as per the methods given by Sambrook *et al*. (1989) (Tables 7 & 8)

Table 7 Stock solutions prepared for DNA extraction*

Solution	Method of preparation
1M Tris (pH 8.0)-500ml	Dissolve 60.55 g of Tris base (Sigma) in 300 ml distilled water adjust pH to 8.0 by adding conc. HCl. Make up the volume to 500 ml. dispense to reagent bottles and sterilize by autoclaving
0.5M EDTA (pH 8.0)	Dissolve 93.05 g of EDTA-disodium salt (Sigma) in 300 ml of water. Adjust pH to 8.0 by adding NaOH pellets. Adjust the final volume to 500 ml. Dispense into reagent bottles and autoclave.
5M NaCl - 500 ml	Weigh 146.1 g NaCl (Merck) add 200 ml of water and mix well. When the salts get completely dissolved, adjust the final volume to 500 ml. Dispense into reagent bottles and autoclave.
3M sodium acetate (pH 5.2)- 250 ml	Dissolve 61.523 g of sodium acetate (Qualigens) in 200 ml of distilled water. Adjust the pH to 5.2 and with glacial acetic acid, make up the volume to 250ml. Sterilize by autoclaving
Ethidium bromide (10 mg/ml) - 100ml	Add 1 g of Ethidium Bromide to 100 ml of distilled water. Keep on magnetic stirrer till the dye gets dissolved. Dispense to amber coloured reagent bottle and store at 4^0C.
70% ethanol - 500 ml	Take 360 ml of ethanol, mix with 140 ml of distilled water. Dispense to reagent bottle and store at 4^0C.
Chloroform:isoamyl alcohol (24:1) - 500 ml	Measure 450 ml of chloroform and 20 ml of isoamyl alcohol. Mix and store in reagent bottle at room temperature.
1M $MgCl_2$ - 100 ml	Weigh 20.33 g of $MgCl_2$, dissolve in double distilled water, make up to 100 ml, dispense into reagent bottles and autoclave.

All the stock solutions were dispensed into reagent bottles and autoclaved

Table 8 Buffers and the methods of preparation for genomic DNA extraction.

Buffer	Method of preparation
CTAB extraction buffer-1litre, 100 mM Tris-HCl (pH 8.0) (Sigma) 20 mM EDTA (pH 8.0), (Sigma) 1.4 M NaCl (Sigma) 2% CTAB (w/v) Merck 0.2% β mercaptoethanol (v/v) Merck	Measure 100ml Tris (1 M), 280 ml of NaCl, 40 ml of EDTA (0.5 M). Mix with about 400 ml of hot distilled water, add 20 g of CTAB to this. Adjust final volume to one liter. Dispense to reagent bottles and autoclave. Just before use, add 0.2% β-mercaptoethanol

TE (0.1 mM) buffer -100ml 100 mM Tris HCl (pH 8.0) 0.1 mM EDTA (pH 8.0)	Take 1ml of Tris HCl (1M), 20 ml of EDTA (0.5 M). Mix with 99 ml of sterile distilled water taken in a reagent bottle, mix thoroughly, autoclave.
Gel loading buffer (6x) – 100 ml, 0.25% Bromophenol blue (BPB) (Sigma) 30% glycerol (Merck)	Dissolve 0.25 g of BPB in 99 ml of 30% Glycerol. Keep on magnetic stirrer for several hours to get the dye completely dissolved. Dispense to reagent bottles and store at 4^0C.
RNase A (10 mg/ml)	Make up 10 mg/ml RNase in distilled water. Boil for 10 minutes to destroy DNase. Divide into 1 ml aliquots and store at -20^0C.
50x TAE stock – 1 litre	Tris base 242g, 0.5 M EDTA 100ml glacial acetic acid 57.1 ml. Adjust the volume to 1 litre with distilled water
10x DNA loading dye	Ficoll (Type 400) 25% (9w/v), Bromophenol blue 0.4 % (w/v) Xylene cyanol FF 0.4% (w/v)

Quality and quantity analysis of DNA

Qualitative and quantitative analysis of DNA was performed by electrophoresis on 0.8% agarose gel. Electrophoresis was carried out at 90 – 100 volts for 3 h to allow proper resolution. λ weight marker was used as marker to quantify the genomic DNA. The image was visualized under UV Transilluminator in Biorad Gel Doc 1000 system and stored at – 20^0C for further studies.

RAPD analysis

RAPD profiles were developed as per the method suggested by Williams *et al*., (1990) with minor modifications (Nirmal babu *et al*., 2003; Ravindran *et al*., 2004). The dNTPs, Taq polymerases and other chemicals were procured from Genei, Bangalore, India. Arbitrary primers from Operon Technologies Inc. Alameda, California were used for PCR reactions.

The reaction mixture (Table 9) and the profile for PCR was given below for all crops studied.

Table 9 Reaction mixture for PCR

Component	**Volume (1x)**
Sterile distilled water	11.75 µl
10x PCR buffer	2.50 µl
dNTPs (1mM)	3.75 µl
Primer (5 pmoles/ µl)	2.00 µl
$MgCl_2$ (10 mM)	1.5 0 µl
Taq polymerse (2U/ µl)	0.5 0 µl
Template DNA (10 ng/µl)	3.0 0 µl
Total reaction volume	**25.0 0 µl**

The PCR reaction mix was prepared using all the seven components in 0.2 ml sterile thin walled microfuge tube. The final reaction volume was 25 µl.

The PCR reaction profile is given below

32 cycles:

1st	93 ^{0}C for 3 minutes	1 cycle
IInd	93 ^{0}C for 1 minute	
	37 ^{0}C for 1 minute	31 cycles
	72 ^{0}C for 1 minute	
IIIrd	72 ^{0}C for 15 minutes	1cycle

Primers (Operon Technologies Inc, Alameda, California) showing good polymorphism (Table 10) were used for RAPD analysis.

Table 10 Operon primers used for developing RAPD profiles

Primer	Crop	Sequence
OPE 18	CARDAMOM	GGACTGCAGA
OPD 13		GGGGTGACGA
OPB 13		TTCCCCCGCT
OPA 12		TCGGCGATAG
OPD 12		CACCGTATCC
OPD 11		AGCGCCATTG
OPA 14	GINGER	TCTGTGCTGG
OPF 01		ACGGATCCTG
OPB 06		TGCTCTGCCC
OPB 07		GGTGACGCAG
OPB 17		AGGGAACGAG
OPA 08		GTGACGTAGG
OPC 05	BLACK PEPPER & *P.BARBERI*	GATGACCGCC
OPA 17		GACCGCTTGT
OPD 11		AGCGCCATTG

ISSR analysis

The protocol for ISSR analysis was adapted from that of Wolf and Liston, 1998. Reactions were carried out with dinucleotide repeat primers (synthesized at Genei, Bangalore, Table 11) with two 3’ anchored primers. 30 pico moles of primers were used for

single 25µl reaction. Primer annealing temperature was raised to 50^0C, other PCR conditions were same as for RAPD assay.

Table 11 UBC primers used for developing ISSR profiles

Sequence	Primer Number	Crop
$(CT)_7$ TG	IS 1	CARDAMOM &
$(GA)_6$ GG	IS 8	GINGER
$(CT)_7$ TG	IS 1	BLACK PEPPER
$(CT)_7$ GC	IS 3	& *P.BARBERI*
$(CA)_7$ GT	IS 5	

STATISTICAL EVALUATION

Means and standard errors were calculated and the statistical difference between mean values of post thaw regeneration was assessed using Analysis of Variance (ANOVA) using Duncan's multiple range test (P=0.05).

The present study was aimed to standardize an efficient technology for cryopreservation of genetic resources in cardamom, ginger and black pepper.

Micropropagation forms the backbone of cryopreservation especially in vegetatively propagated and cross pollinated crops. The present study utilized the micropropagation protocols already standardized at Indian Institute of Spices Research, Calicut for multiplication of quality stock materials and reduction of plant size for experimentation. Various approaches like encapsulation dehydration, encapsulation vitrification and vitrification were studied and the best method was selected for cryopreservation of each crop. The genetic fidelity of cryopreserved material was assessed by molecular profiling as and when needed.

CRYOPRESERVATION OF CARDAMOM

Micropropagation for multiplication of stock materials

Initiation and multiplication

The protocols developed at IISR (Nirmal babu *et al.,* 1997) were used to initiate and multiply cardamom stock materials in aseptic conditions. The culture medium used was MS + 0.5 mg/l Kinetin for establishment and MS + BA (1 mg/l) + IBA (0.5 mg/l) for multiplication of plantlets. The various stages are represented in Figure 1. ***Maintenance of cultures***

The initiated cardamom cultures were maintained in growth regulator free medium to minimize the effect of somaclonal variation and to maintain genetic stability. The pathogen free plants were used as sources of explants for cryopreservation

experiments. Micro shoots from multiplying cultures were used as explants.

Encapsulation dehydration

To establish an efficient encapsulation dehydration procedure, shoots were first encapsulated in sodium alginate and to improve survival after cryopreservation, sucrose preculture and desiccation were added.

Encapsulation

The miniature shoots obtained from multiplying cultures (Fig 2 a b) were used for encapsulation with 4% sodium alginate and to develop beads or synthetic seeds for cryopreservation (Fig 2 c). Regeneration of encapsulated shoots were close to 100%. The artificial beads or seeds with high viability were then used for cryopreservation using different methods (Fig 2 & 3).

Dehydration

Aseptically precultured beads on 0.3 M – 1.0 M sucrose concentrations were briefly blotted on filter paper and were dehydrated by air drying on a laminar air flow bench for 10 hours.

The changes in the water content of the precultured beads during dehydration is presented in Table 12.

Table 12 Effect of dehydration duration on the water content, recovery after dehydration alone(LN-), and followed by cryopreservation (LN+)*.

Dehydration time	Water content %	Survival LN-	Survival LN+

0	91± 3 a	100 ± 0 a	0 ± 0 f
1	80 ± 4 b	94 ± 2 b	4 ± 2 e
2	72 ± 3 bc	92 ± 2 b	9 ± 2 d
3	70 ± 4 c	90 ± 2 b	13 ± 2 d
4	68 ± 3 c	89 ± 2 b	27 ± 3 c
5	64 ± 3 c	77 ± 4 c	31 ± 2 c
6	46 ± 2 d	78 ± 1 c	45 ± 3 b
7	30 ± 3 e	77 ± 2 c	51 ± 2 ab
8	20 ± 1 f	71 ± 2 c	60 ± 5 a
9	15 ± 2 fg	47 ± 3 d	20 ± 5 c
10	12 ± 0.58 g	32 ± 3 e	12 ± 2 d

*Shoot clumps of cardamom were encapsulated in 4% (w/v) Na alginate with 2M Glycerol and 0.4 M sucrose prior to pretreatment with 0.3 – 1.0 M sucrose, 24 h each, in a stepwise manner.Means of three observations are represented in the table.

The initial water content was 91% on a fresh weight basis and it decreased rapidly to 72% within first 2 h and then slightly but steadily dropped to 64% after 5 h, followed by a drastic decrease to 46% after 6 h, 30% after 7 h and 20% after 8 h, the water content was then gradually dropped to 15 and 12% after 9 and 10 h of dehydration. The dehydrated shoots are cryopreserved by dipping in liquid nitrogen for 24 h. The cryopreserved shoots were then thawed at 40°C for 2 – 3 minutes and cultured on medium as mentioned in materials and methods for post thaw recovery.

The survival of both non-cryopreserved (LN-) and cryopreserved (LN+) shoots were greatly influenced by the water content of the precultured beads, though in different patterns, the survival of non cryopreserved (LN-) shoots were ranged from 100 – 32 % during dehydration while that of cryopreserved (LN+) shoots

ranged from 0 – 60 %. The maximum survival (60%) of cryopreserved shoots was detected when the water content was at 20% with 8 hrs of dehydration. After which survival percentage has decreased to 20 and 12 % respectively after 9 and 10 hours at 15 and 12 % water content.

Although the encapsulation dehydration technique was proposed as more efficient for rapid cooling (Ashmore, 1997), the results showed that the encapsulation dehydration treatment in the present experiment did not increase survival rate above 60% regardless of the dehydration treatment durations from 9 – 10 h, Therefore an additional vitrification step with PVS2 solution was added to increase the survival of cryopreserved shoots of cardamom.

Encapsulation vitrification

Excised shoots used for cryopreservation must be in a physiologically optimal state to ensure high level of dehydration tolerance to PVS2 and to produce vigorous recovery growth. In encapsulation vitrification treatment, after preculturing with 0.3 M sucrose for 16 h, dehydration tolerance to PVS2 was induced by treating the shoots with sucrose and glycerol. Encapsulated and osmoprotected cardamom shoots were dehydrated with PVS2 and plunged into LN and held for at least 24 h at -196^{0}C. The

cryopreserved shoot was then thawed at 40^0C and cultured on MS + BA (1mg/l) + NAA (0.5mg/l) for post thaw recovery.

As shown in Table 13, the highest percentage of survival was achieved for shoots treated with a mixture of 2 M glycerol and 0.6 M sucrose. 2 Molar glycerol with either 0.4 M, 0.8 M or 1.0 M sucrose gave some what lower survival percentage.

It was also observed that pretreatment with sucrose alone with out dehydration did not produce a substantial increase in osmotolerance of vitrified cardamom shoots (Table 13). In addition use of osmoprotectant treatment actually reduced the germination and growth of control shoot buds.

Table 13 Effect of osmoprotection treatments on shoot formation of encapsulated vitrified shoots of cardamom cooled to -196^0C.

Osmoprotectant(s)	Survival (%) LN- (control)	Survival (%) LN+
None	0 ± 0 f	0 ± 0 g
0.4 M sucrose	16 ± 2 e	6 ± 1 f
0.6 M sucrose	28 ± 2 d	13 ± 2 e
0.8 M sucrose	22 ± 3 de	11 ± 2 e
1.0 M sucrose	37 ± 5 c	18 ± 1d
0.4M sucrose + 2M Glycerol	67 ± 2 b	47 ± 2 b
0.6 M sucrose + 2M glycerol	86 ± 2 a	65 ± 3 a
0.8 M sucrose + 2M Glycerol	66 ± 3 b	47 ± 4 b
1.0 M sucrose + 2M glycerol	63 ± 4 b	38 ± 1 c

In the vitrification protocol, the exposure duration to PVS2 must be optimized with or without encapsulation to ensure high percentages of survival after cryopreservation. In the present experiment exposure to PVS2 produced a time dependent survival of encapsulated, vitrified shoots. The optimal dehydration durations with PVS2 were 2 h at 25^{0}C (Table 14).

Table 14 Effect of exposure period to PVS2 at 25^{0}C on percentage of survival of encapsulated cardamom shoots*.

Dehydration(min) period at 25^{0}C	Survival % LN –	Survival % LN +
0	100 ± 0 a	0 ± 0 g
30	95 ± 3 b	43 ± 3 bc
60	96 ± 2 b	39 ± 0.6 cd
90	83 ± 4 c	51 ± 3 b
120	89 ± 5 bc	62 ± 3 a
150	80 ± 3 c	30 ± 4 d
180	34 ± 3 d	15 ± 2 e
210	16 ± 2 e	8 ± 2 f

*Precultured, encapsulated shoots were osmoprotected with 2 M Glycerol and 0.6 M sucrose at 25^{0}C for 60 min. ten to 15 shoots were treated in each of three replicates.

Apical domes from encapsulated and vitrified shoots were started to develop within 8 days and grew into normal shoots within 4 weeks (Fig 3). Though these plants appeared identical to non-cryopreserved ones, this treatment also did not increase

survival rate above 65 %, Therefore only vitrification, the freeze – avoidance mechanism that enables hydrated cells, tissues and organs to survive at the temperature of liquid nitrogen (Sakai 1960) was tried as the next experiment which eliminates encapsulation.

Vitrification

For successful cryopreservation, it is essential to avoid lethal intracellular freezing which occurs during rapid cooling in liquid nitrogen (Sakai and Yoshida, 1967; Sakai 1985; 1995). Thus specimens to be preserved have to be sufficiently dehydrated to avoid intracellular freezing and thus vitrify upon rapid cooling in liquid nitrogen.

In this experiment, to enhance tolerance to vitrification solution (PVS2), a two step sucrose preculture with 0.3 and 0.75 M sucrose for one day each and an osmo protection step with a loading solution of 2 M glycerol and 0.4 M sucrose were performed prior to PVS2 treatment.

The precultured and osmoprotected shoots were dehydrated with PVS2 at 25^0C for various lengths of time to determine the optimal time of exposure to PVS2 before immersion in LN. The cryopreserved shoot was thawed and put on recovery medium as mentioned before for regeneration. The percentage of shoot formation of vitrified shoot tips increased considerably between 40

and 50 and reached a maximum (about 70%) at 50-60 min. The shoots dehydrated with PVS2 upto 100 min but LN treatment (treated control) retained a high level of shoot formation (70 %) at 60 min incubation (Table 15)

Table 15 Effect of exposure time to PVS2 at 25^{0}C on percentage of shoot formation of shoots of cardamom cooled to – 196^{0}C*.

Time(min)	Survival(%) LN-	Survival(%) LN+
10	91 ± 2 a	5 ± 2 d
20	85 ± 3 a	14 ± 3 d
40	91 ± 3 a	30 ± 4 c
60	92 ± 3 a	70 ± 4 a
80	76 ± 4 b	56 ± 3b
100	67 ± 2 c	38 ± 5c

*Precultured shoots were osmoprotected with 2M Glycerol and 0.4 M sucrose for 20 min. they were then dehydrated with PVS2 for various lengths of time prior to a plunge into liquid nitrogen.

Cryopreserved cardamom shoots were turned light brown upon thawing, but slowly recovered on recovery medium and surviving shoots turned green within 10 days and successfully recovered shoots resumed growth within 2-3 week. The first sign of shoot growth was evident by leaf development (3 weeks after cryopreservation) and was followed by shoot development. No callus formation was observed (Fig 4).

Influence of shoot size

For high post thaw shoot recovery rates, it is essential for the size and developmental stage of the cryostored material to be

appropriate (Takagi, 2000). The survival and shoot regrowth of smaller shoots (1-2 mm) were significantly better than those of larger shoots (3-4 mm). The survival rate was 91 % when explants of 1 – 2 mm was used and this was reduced to 66 % when the explants are 3 – 4 mm and was further reduced to 36 % when the explant size was 4 -5 mm (Table 16) The smaller the meristematic region, the more it tends to consist of a homogeneous population of small actively dividing cells. These characteristics made them more tolerant to dehydration than highly vacuolated and differentiated cells which also form part of larger apices hence shoots sized 1-2 mm were employed in all vitrification experiments.

Table 16 Effect of shoot size on survival of cardamom shoots following cryopreservation*.

Explant Size	Survival %	Shoot recovery %
1-2 mm	91 ± 2 a	70 ± 4 a
3-4 mm	66 ± 4 b	36 ± 3 b
4.5-5 mm	36 ± 3 c	21 ± 3 c

*Apices were dehydrated with PVS2 solution at 25^{0}C for 60 min prior to immersion in liquid nitrogen. Assessment was made 8 weeks after cryopreservation.

Influence of recovery medium

Cardamom shoots could be amenable to oryopreservation provided that the tissue culture protocol for small size cardamom

shoots are sufficiently developed. Hitherto, the shoots used in protocols for cardamom micropropagation have been approximately 1-2 mm in length and have been cultured on medium containing 0.5-1.0mg/l BA and NAA. Successful culture of cardamom shoots of 1-2 mm in length was found optimal for cryostorage in this study. The importance of using recovery media with the appropriate plant growth regulator supplements for the special requirements of cryopreserved material has been emphasized by Touchell *et al.,* 2002.

Table 17 Effect of recovery medium composition on survival and shoot recovery frequencies of meristematic clumps of cardamom following cryopreservation (Assessment made 8 weeks after plating on recovery medium)

Growth regulators in recovery medium (µM)	Survival (%)		Shoot recovery (%)	
	LN-	LN+	LN-	LN+
BA (2.22)	60 ± 5 c	47 ± 4 c		41 ± 2 c
NAA(2.69)		42 ± 3 c		52 ± 2 ab
BA (2.22) + NAA (2.69)	70 ± 5 bc	60 ± 5 b	52 ± 3 d	56 ± 2 ab
BA (2.22) + NAA (5.37)	80 ± 5 ab	62 ± 4 b	63 ± 3 c	58 ± 3 ab
BA (4.44) + NAA (2.69)	80 ± 5 ab	90 ± 1 a	80 ± 2 b	68 ± 5 a
BA (4.44) + NAA (5.37)	90 ± 5 a	60 ± 2 b	79 ± 2 b 89 ± 4 a 78 ± 2 b	56 ± 2 ab
	80 ± 3 ab			

To optimize the medium for the growth of small cryostored shoots six recovery media were compared in cardamom (Table 17). Among cryopreserved apices (LN+), both survival and shoot recovery were significantly better on medium with both 1mg/l BA

and 0.5 mg/l NAA than on medium with BA alone, but the further addition of BA and NAA brought no further significant improvement.

Influence of explant age

The condition of the stock plants from which shoot meristems are taken may have a major bearing on cryopreservation results (Reed, 2000). A major observation made during this study was that ; among shoots of different ages tested, 3 and 5 week shoots exhibited almost identical recovery rates (61 % and 62 % respectively). Reducing the culture period of donor shoots to 3 weeks is advantageous as it saves time in the cryopreservation protocol.

Plant Regeneration

After 8 weeks of culture in recovery medium in petri dishes developing shoots were transferred to proliferation medium for further shoot growth. After two transfers of 15 days each (approximately 3 months after thawing) shoots grew to 2-3 cm tall and were thus suitable for further shoot proliferation and for rooting (Fig 2i,3i & 4g). To induce rooting in the clones, shoots longer than 2 cm were separated and transferred to MS basal medium devoid of phytohormones.

Thus in this study, a two-step vitrification methodology (LS-PVS2 treatments) was moderately successful in cryopreserving cardamom shoots, so that they could be recovered for plant regeneration. Although shoot recovery frequencies need to be improved for a more effective cryopreservation protocol, these results suggest a feasible system since further propagation from a few surviving cyropreserved explants is now possible.

Effect of thawing temperature

Though 40 - 45^{0}C is the normal thawing temperature recommended for cryopreserved material, a small experiment was conducted to find the optimum thawing temperature for recovery of cryopreserved cardamom shoots. After maintaining the cryovials at room temperature for 5s, thawing temperatures ranging from 5 – 55^{0}C were used and their survival rates estimated. The survival rates ranged for 0 % at 5^{0}C to 70 % at 40^{0}C and a thawing temperature of 35 and 45^{0}C resulted in a survival rate of 60 and 61 % respectively (Table 18). This rate was again reduced to 61, 55 and 43 % when the thawing temperature was raised to 45, 50 and 55^{0}C respectively.

Table 18 Effect of thawing temperature on survival of cryopreserved shoots of cardamom by encapsulation dehydration, encapsulation vitrification and vitrification. Means of two independent experiments with three replication are presented.

Temperature^{0}C	Survival (%)

5	0 ± 0 e
25	14 ± 3 d
30	50 ± 5 bc
35	60 ± 5 ab
40	70 ± 5 a
45	61 ± 2 ab
50	55 ± 2 b
55	43 ± 2 c

TTC microscopic test

After the TTC treatment the control shoots (not frozen) were clearly red coloured indicating their viability. Among shoots subjected to cryopreservation, the distinction between viable and non-viable tissues was clear; viable tissues had a bright, red colour (Fig 3d & 4e), while the dead tissues were dark due to oxidation processes.

Genetic stability analysis of cryopreserved material

Genetic fidelity is an important factor in any conservation programme. The conserved plants were multiplied in *in vitro* and observed for differences and deficiencies during recovery and growth after cryopreservation. These *in vitro* multiplied cultures did not exhibit any deficiency symptoms or deformities and morphologically were similar to the control in its appearance and growth.

In the present study the genetic fidelity of conserved genotypes of cardamom were assessed by RAPD and ISSR profiling.

Molecular characterization using RAPD and ISSR

DNA markers can detect even minute differences in nucleic acid sequences which were otherwise difficult to detect in morphological characterization. In the present study an attempt was made to use RAPD and ISSR polymorphism as an index for genetic stability of cryopreserved genotypes.

Genomic DNA was isolated using CTAB method from *in vitro* leaves of 8 – 13 different plantlets of cryopreserved materials and was found to be of reasonably good quality. The extracted samples were further purified by RNase treatment followed by phenol:chloroform extraction and precipitation of DNA with absolute alcohol.

Quantification of DNA

The amount of DNA in the purified samples was calculated by comparing with standard λ DNA marker on agarose gels as shown in the Table 19. The cardamom contained 100 – 200 ng/µl of DNA.

Table 19 Concentration of DNA in cardamom genotypes samples used for RAPDand ISSR analysis.

S.No	Sample	Concentration of DNA(ng/µl)

Cardamom		
1	1 (Control)	150
2	2 (Cry* line)	100
3	3 (Cry line)	100
4	4 (Cry line)	150
5	5 (Cry line)	200
6	6 (Cry line)	125
7	7 (Cry line)	100
8	8 (Cry line)	100
9	9 (Cry line)	100
10	10 (Cry line)	100
11	11 (Cry line)	100
12	12 (Cry line)	100
13	13 (Cry line)	100
14	14 (Cry line)	100

*Cry line – Randomly selected cryopreserved lines

RAPD and ISSR profiles of cryopreserved lines

The genetic fidelity of the cryopreserved and control plants of cardamom, were tested by comparing RAPD and ISSR profiles generated by six arbitrary Operon 10-mers and two ISSR primers (IS 1- $[(CT)_7$ TG] and IS 8 -$[(GA)_6$ GG]). In cardamom the primers used in the present study was selected based on the amount of polymorphism they are showing among different genotypes of each crop.

The 6 selected RAPD-primers (OPD-11, OPA – 12, OPD -12, OPB – 13, OPD – 13, OPE-18) (Fig 5 & 6) and two ISSR primers (IS 1- $[(CT)_7$ TG] and IS 8 -$[(GA)_6$ GG]) (Fig 7) used for the experiment of cardamom yielded a total of 160 clear bands. Both

cryopreserved plants and the mother plant showed an identical banding pattern. The banding pattern obtained are shown in Figs 5, 6 & 7 for RAPD and ISSR markers. No reproducible variation of the RAPD and ISSR profiles within the control and cryopreserved lines of cardamom were observed.

CRYOPRESERVATION OF GINGER

Micropropagation for multiplication of stock materials

Initiation and multiplication

The micropropagation protocols developed at IISR (Geetha 2002) were used to multiply ginger stock materials. The culture medium used was MS + 0.5 mg/l Kinetin for establishment and MS + BA (1mg/l) for multiplication of shoots. The various stages of establishment, growth, multiplication and reduction in size are represented in Fig 8. This results not only in multiplication of sufficient disease free stocks, but also reducing the explant size to be suitable for cryopreservation. Reduction in size is an important prerequisite for efficient dehydration and cryopreservation.

Maintenance of cultures

The initiated and multiplied ginger cultures were maintained in growth regulator free medium to minimize the effect of somaclonal variation and to maintain genetic fidelity. The aseptic and miniatured shoot bud explants obtained from these cultures were used for cryopreservation experiments.

Encapsulation dehydration

Encapsulation

Ginger shoots consisting of the apical dome with 3-4 leaf primordia were encapsulated in 4 % sodium alginate and regrowth of encapsulated shoot tips remain close to 100%.

Preculture and dehydration

Ginger shoots consisting of the apical dome with 3-4 leaf primordia (Fig 10 a b) were encapsulated in 4% sodium alginate (Fig 9 c d) and subsequently precultured in liquid MS medium containing 0.1, 0.3, 0.5, 0.7, 0.9 and 1.0 M sucrose for 24 h each and were dehydrated in laminar flow cabinet (at room temperature and humidity) for periods of 0-10 h (Fig 9 e g) and in each hour a sample of 15 shoots were subjected to cryopreservation by plunging in liquid nitrogen for 24h. The cryopreserved shoots were then thawed at 40^0C for 2 – 3 minutes and cultured on MS medium containing BA (1mg/l) and NAA (0.5mg/l).

The regrowth of both non-cryopreserved (-LN) and cryopreserved (+LN) shoots was greatly influenced by the water content of the precultured beads. The initial water content of the control beads was high (97%), This was reduced to 88% by 6 days of culture on increasing concentrations of sucrose containing medium. The moisture content was then gradually decreased to

78, 68, 57, 46, 39 and 21% at 1, 2,3,4,5 and 6h of dehydration. The best results in terms of cryopreserved shoot recovery (around 41%) were obtained by preculturing in progressive increase of sucrose together with 6 h of dehydration in the laminar air flow chamber (Table 20).

Table 20 Moisture content (% on fresh weight basis) (MC) and recovery of encapsulated ginger shoots (without cryopreservation -LN and after cryopreservation +LN) after encapsulation dehydration treatment*.

Dehydration period	Moisture content	Survival % LN-	Survival % LN+
0	88 ± 2 a	82 ± 2 a	0 ± 0 g
1	78 ± 3 a	76 ± 3 ab	11 ± 2 f
2	68 ± 3 b	75 ± 3 ab	13 ± 2 ef
3	57 ± 4 c	70 ± 2 bc	20 ± 2 d
4	46 ± 3 d	68 ± 3 bc	24 ± 2 d
5	39 ± 2 d	68 ± 2 bc	32 ± 3 bc
6	21 ± 3 e	47 ± 2 d	41 ± 3 a
7	18 ± 3 ef	35 ± 2 e	22 ± 3 d
8	16 ± 3 ef	20 ± 2 f	12 ± 3 ef
9	12 ± 2 f	10 ± 3 gh	4 ± 3 g
10	10 ± 2 f	5 ± 2 h	2 ± 3 g

*subjected to 6 days of preculture on increasing sucrose concentration medium and to various desiccation periods, before and after rapid cooling in liquid nitrogen ($P<0.01$). Assessment was made after 2 months of treatment. Fifteen encapsulated shoots were treated in each of three replicates. Bar indicates standard error.

After 6 h of dehydration there was a slight decrease in moisture contents ranging from 18, 16, 12 and 10% with 7, 8, 9 and 10 h of dehydration with survival rates ranging from 22 – 2% (Table 20) after cryopreservation. The cryopreserved shoot buds resumed growth within 7 days of culturing, without callus formation (Fig 9 i l).

Encapsulation dehydration treatment did not increase survival rate above 40 % regardless of the dehydration treatment durations ranging from 7 – 10 h, therefore inorder to improve post thaw recovery rates, dehydration of encapsulated and naked ginger shoot buds with plant vitrification solution (PVS2) were used in the following experiments.

Encapsulation vitrification

In encapsulation vitrification treatment, ginger shoot buds were encapsulated in 4% sodium alginate and to enhance the osmotolerance of ginger shoots in the vitrification solution, the shoot buds were pre incubated with 0.1 M sucrose for 24 h after encapsulation and precultured with 0.3 M sucrose for 16 h at room temperature. Pre incubation alone and preculture without pre incubation gave very low recovery rates (Table 21). The best result was obtained when both treatments are given, with that 81.4 % of the plantlets survived cryopreservation treatment. When none of them are used there was 100 % mortality.

Table 21 Effect of a 1-day preincubation in 0.1M sucrose for 24h and preculture in 0.3 M sucrose for 16h on percentage of shoot formation of encapsulated vitrified ginger shoots*.

Pre incubation	Preculture	Shoot formation (%) ± SE
+	+	81.4 ± 0.4 a
+	-	23.8 ± 0.5 c
-	+	31.4 ± 0.3 b
-	-	0 ± 0 d

*The encapsulated beads were then osmoprotected with 2M glycerol and 1.6M sucrose for 3h at 25^0C and then dehydrated with PVS2). Fifteen shoots were treated in each of three replicates.

Thus, preincubation followed by preculture was found to be an important step for the successful cryopreservation of ginger by encapsulation vitrification

. In the vitrification method, the direct exposure of less tolerant apices to a highly concentrated vitrification solution may lead to harmful effects due to hyper osmotic stress or chemical toxicity (Ishikawa *et al.,* 1996). Thus the acquisition of osmotolerance to the PVS2 solution is reported to be essential for the successful cryopreservation of shoot buds excised from *in vitro* grown plants (Nishizawa *et al.,* 1993) and a loading treatment was deemed essential for this (Huang *et al.,* 1995). In the present study to determine the best combination of osmoprotectants for ginger shoots, solutions that included various concentrations of sucrose with 2 M glycerol were examined (Table 22).

Table 22 Percentage of shoot formation at 8 weeks for encapsulated-vitrified shoots of ginger treated with different sucrose concentrations*.

2M Glycerol + Sucrose Conc.	Survival (%) (LN-)	Survival (%) (LN+)
0.4	90 ± 4 a	16 ± 2 e
0.6	92 ± 3 a	22 ± 2 e
0.8	85 ± 3 a	32 ± 2 d
1.0	82 ± 2 a	42 ± 3 cd
1.2	85 ± 3 a	47 ± 3 bc
1.4	82 ± 2 a	54 ± 4 b
1.6	90 ± 4 a	66 ± 3 a
1.8	85 ± 3 a	40 ± 4 cd

*Encapsulated, pre-incubated and precultured shoots were treated with 2M glycerol + sucrose at 25 ^{0}C for 3 h before being dehydrated with PVS2 for 40 min at 25ºC; Fifteen shoots were treated in each of three replicates.

As shown in Table 22, the highest percentage of shoot formation (about 66 %) was obtained when precultured and preincubated shoots were treated with 2 M glycerol and 1.6 M sucrose for 3 h at 25^{0}C (Fig 10). Pretreatment with sucrose solution alone produced less than 24 % shoot formation.

Vitrification

It has been reported that modifications of the typical one step vitrification procedure (i.e, treatment with the PVS2 solution, followed by direct plunging into LN) can sometimes be required for a better survival of cryopreserved meristems (Brison *et al.,*1995; Towill 1995). In the present experiment inorder to induce gradual tolerance to vitrification and obtain a satisfactory recovery after

cryopreservation, ginger shoots were first precultured in 0.3 M sucrose for 72 h, cryoprotected with various concentrations of DMSO and Glycerol (5% DMSO, 10% DMSO, 5% glycerol, 10% glycerol, 5% DMSO + 5% glycerol) for 20 min at room temperature and osmoprotected with loading solution (2 M glycerol + 0.4 M sucrose) for 20 min at 25ºC prior to treatment with vitrification solution (PVS2). The vitrified shoots were subjected to rapid freezing in liquid nitrogen for 24 h. The cryopreserved shoots were thawed and put on recovery medium as mentioned before for regeneration.

In the vitrification protocol, an optimum dehydration with vitrification solution (PVS2) is the key step for producing high levels of recovery and growth after cryopreservation. Properly dehydrated shoot tips treated with PVS2 solution showed less injury during dehydration, increased vitrification and thereby avoiding the risk of intracellular freezing upon rapid cooling with LN. To optimize the exposure time to PVS2 solution the excised shoots were treated with PVS2 for 0-60 min at 25^0 C prior to plunging into LN (Table 23).

Table 23 Effect of exposure time to PVS2 solution at 25ºC on ginger shoots cooled to -196^0 C by vitrification*.

Exposure time to PVS2(min)	Survival (%) LN-	Survival (%) LN+
0	90 ± a	0 ± 0 d
10	92 ± a	49 ± 4 c

20	92 ± a	55 ± 3 c
30	90 ± a	70 ± 2 b
40	95 ± a	80 ± 1 a
50	90 ± a	68 ± 4 b
60	92 ± a	51 ± 4 c

*Shoots were dehydrated with PVS2 solution before immersion into LN. Approximately 15 shoots were tested in each of three replicates.

Shoot formation depended upon the length of the exposure to the PVS2 solution. The highest shoot formation (about 95%) was obtained when the shoots were dehydrated with PVS2 for 40 min at 25ºC before immersion into LN.

The maximum post thaw recovery (80 %, Table 24) was resulted when shoots were precultured in 0.3 M sucrose, cryoprotected with a mixture of 5% DMSO + 5% glycerol and osmoprotected with loading solution followed by treatment with vitrification solution (PVS2) for 40 min at 25ºC . Preculture with 5 % DMSO and 5 % glycerol when done alone gave recovery rates of 60 and 70 % respectively. When the concentration of DMSO and glycerol increased to 10 % the survival rate was reduced to 54 and 51 % respectively.

Table 24 Effects of preculture and cryoprotectants on regeneration of shoots after exposure to liquid nitrogen (+LN) and without (-LN) during the vitrification procedure*.

Cryoprotectants	Survival (%) LN-	Survival (%) LN+
5 % DMSO	92 ± 3 a	
10 % DMSO	85 ± 3 a	60 ± 4 bc
5 % Glycerol	90 ± 4 a	54 ± 4 c
10 % Glycerol	82 ± 3 a	70 ± 4

5 % DMSO + 5 % Glycerol	90 ± 3 a	51 ± 6 c 80 ± 5 ab a

*Approximately 15 shoots were tested for each of three replicates. Data taken at 8 weeks.

Cryopreserved ginger shoots turned light brown within one day after warming (Fig 11 c), but upon culture on recovery medium surviving buds turned green within 10 days and successfully recovered buds resumed growth within 2-3 weeks. The first sign of shoot growth was evident by leaf development (4 weeks after cryopreservation) and was followed by shoot development without intermediate callus formation (Fig 11 e-h).

TTC microscopic test

After the completion of TTC test the control shoots buds (not frozen) were clearly red coloured. Among shoot buds subjected to cryopreservation, viable tissues had a bright, red colour(Fig 9 h, 10 g & 11 d) ; while the dead tissues were dark due to oxidation processes.

Genetic stability analysis of cryopreserved material

The conserved plants were multiplied in *in vitro* and observed for differences and deficiencies during recovery and growth after cryopreservation. These *in vitro* multiplied cultures did not exhibit any deficiency symptoms or deformities and

morphologically were similar to the control in its appearance and growth.

In the present study the genetic fidelity of conserved genotypes of ginger were assessed by RAPD and ISSR profiling.

Molecular characterization using RAPD and ISSR

Genomic DNA was successfully isolated using CTAB method from in vitro leaves of 8 – 13 different plantlets of cryopreserved materials. The amount of DNA in the purified samples was calculated by comparing with standard λ DNA marker on agarose gels as shown in the Table 25. The ginger samples contained 100 – 200 ng/µl of DNA.

Table 25 Concentration of DNA in ginger genotypes used for RAPD analysis.

Accession	Sample	Concentration of DNA(ng/µl)
Ginger		
Maran	1 (Control)	150
Maran	3 (Cry line)	200
Maran	4 (Cry line)	100
Maran	5 (Cry line)	125
Maran	6 (Cry line)	100
Maran	7 (Cry line)	100
Maran	8 (Cry line)	100
Maran	9 (Cry line)	100
Maran	10 (Cry line)	150
Maran	11 (Cry line)	100
Maran	12 (Cry line)	100
Maran	13 (Cry line)	100
Maran	14 (Cry line	100

*Cry line – Randomly selected cryopreserved lines

RAPD and ISSR profiles of cryopreserved lines.

The present study was conducted to screen cryopreservation induced genetic variations (if any) in *ginger* plantlets by employing RAPD and ISSR-PCR assay. The samples analyzed represent *in vitro* plants regenerated after cryopreservation at second multiplication passages. The leaf samples were collected at random. Total 12 RAPD and 4 ISSR primers were initially screened and finally 6 RAPD (OPA – 8, OPA – 14, OPB – 6, OPB – 7, OPB – 17, OPF – 1) and 2 ISSR (IS 1- $[(CT)_7$ TG] and IS 8 -$[(GA)_6$ GG]) primers were chosen for the present study. These 8 primers generated 62 PCR amplification products and were scored for the regenerated plantlets (Fig 12, 13 & 14). Identical RAPD (Fig 12 & 13) and ISSR (Fig 14) pattern was obtained from the material from culture prior to cryopreservation and after cryopreservation in all the three techniques applied.

CRYOPRESERVATION OF BLACK PEPPER

Initiation and multiplication of stock materials

Black pepper embryogenic cultures were established following the method described by Nair & Dutta Gupta (2003) (Fig 15 a-h). Since they are from maternal tissues, they are genetically similar to mother plants. Cryopreservation is employed in *in vitro* propagation systems based on somatic embryogenesis to avoid the

somaclonal variation and loss of embryogenic potential that can occur during long term maintenance of actively growing embryogenic cultures. So far the research on cryopreservation of the black pepper is confined to seed conservation only (Chaudhury & Chandel, 1994). Seeds being highly heterogenous and recalcitrant true to type conservation is not possible. Moreover the post storage survival of seeds is only 45%. There are no published reports of cryopreservation of black pepper somatic tissues. The aim of the present study is to develop a cryopreservation protocol for somatic embryos developed from maternal tissues of black pepper based on the encapsulation dehydration and vitrification procedures.

Encapsulation dehydration

To establish an efficient encapsulation dehydration procedure, the somatic embryo system developed by Nair & Gupta (2003;2005) for iduction of development and cyclic somatic embryogenesis was used. The somatic embryos were first encapsulated in sodium alginate and to improve survival after cryopreservation, sucrose preculture and desiccation were added.

Encapsulation and preculture

Somatic embryos clumps consisting of the globular, heart and torpedo embryos (Fig 16 a) were encapsulated in 4% sodium alginate (Fig 16 b) and pregrown on liquid SH medium containing

different sucrose concentrations 0.1 M, 0.3 M, 0.5 M, 0.7 M, 0.9 M or 1.0 M for a pregrowth period of one day.

Dehydration

Encapsulated beads pregrown at the different sucrose concentrations and desiccated from 1 to 8 h in the laminar air flow cabinet(Fig 16 c) indicated that after a 6 hour desiccation period the moisture content ranged from 25 – 20 % (fresh weight basis) regardless of the type of preculture.

Pretreatment with sucrose concentrations ranging from 0.1 M to 1.0 M was treated and this resulted in varying degree of survival after cryopreservation (Table 26). The survival was increased from 37 % to 62 % when the concentration of sucrose pretreatment was increased to 0.7 M. Further increase of sucrose concentration to 1.0 M has reduced the survival indicating that 0.7 M sucrose is optimal for pretreatment of encapsulated somatic embryos of black pepper. Thus the best cryopreservation protocal resulted after 1 day preculture in liquid medium with 0.7 M sucrose, desiccation down to 21 % moisture content in the beads at 6h of dehydration and rapid freezing by direct immersion into liquid nitrogen (Table 26).

Table 26 Effect of sucrose preculture on the survival (%) of encapsulated Black pepper var Karimunda somatic embryos after pregrowth

desiccation down to 20% moisture content in the beads and Rapid freezing

Variety	Pregrowth treatment (Sucrose in M)	Survival(%) at 6h LN-	Survival(%) at 6h LN+
Somatic embryos of BP Var Karimunda	0.1 M	77 ± 4 a	37 ± 4 c
	0.3 M	77 ± 2 a	44 ± 3 bc
	0.5 M	71 ± 2 a	50 ± 5 b
	0.7 M	78 ± 1 a	62 ± 4 a
	1.0 M	67 ± 3 a	52 ± 3 ab

The encapsulated somatic embryos pregrown in 0.7 M sucrose were further dehydrated to various moisture contents. Desiccation of sucrose precultured and encapsulated somatic embryos for 1 – 8 hours has resulted in reduction of moisture content to 93 – 16 % after 8 hours. The corresponding survival rates of desiccated synseeds after cryopreservation has increased for 11 % survival at 93 % moisture content to 62 % survival at 21 % moisture content. Further reduction of moisture level to 18 and 16 % has resulted in decreased survival rates (Table 27).

Table 27 Effect of sucrose preculture and dehydration on the survival (%) and moisture content of encapsulated Black pepper var Karimunda somatic embryos after pregrowth and desiccation down to 16% moisture content in the beads and Rapid freezing

Hours of dehydratio	Moisture content.	Survival (%) LN-	Survival (%) LN+

n			
0	93 ± 2 a	100 ± 0 a	11 ± 1 f
1	81 ± 3 b	92 ± 2 b	14 ± 1 ef
2	73 ± 3 c	92 ± 2 b	21 ± 2 e
3	63 ± 3 d	88 ± 2 b	33 ± 2 d
4	49 ± 3 e	85 ± 2 b	42 ± 3 c
5	38 ± 4 f	76 ± 4 c	53 ± 2 b
6	21 ± 3 g	78 ± 1 c	62 ± 4 a
7	18 ± 2 g	76 ± 2 c	52 ± 3 b
8	16 ± 1 g	74 ± 2 c	39 ± 4 cd

The cryopreserved somatic embryos were thawed at 40^{0}C and cultured on SH basal medium for recovery and regrowth. Cryopreserved embryos first turned brown, but 2 weeks later new proliferation of globular stage embryos was evident from the thawed somatic embryos depending on treatment(Fig 16 e-k). Almost all non desiccated embryos died with moisture content above 93 % regardless of whether they had received sucrose pretreatment or not.

Thus in the present study, cryopreservation of somatic embryo clumps by encapsulation dehydration resulted in the survival rate around 62 %. To further increase the survival rate therefore dehydration of embryo cultures with PVS2 was tried instead of desiccation in the next experiment.

Vitrification

Black pepper somatic embryos were precultured for 3 days on SH basal medium containing 0.3M sucrose and subjected to

vitrfication treatment for various periods (0, 30 60 90 or 120 min at 25^0C) of time before rapidly freezed in liquid nitrogen.

The somatic embryo clumps used in this study (Fig 17 a) showed good tolerance of the vitrification solution PVS2 exposure for up to 120 min causing no significant reduction of their embryogenic levels which generally remained greater than 85 % before cryopreservation.. The PVS2 treatments affording the highest embryogenesis levels among cryopreserved embryos were 60 min for black pepper embryos (Table 28)

Table 28 Embryo recovery levels of somatic embryos of black pepper variety Karimunda after preculture on 0.3M sucrose medium and subsequent exposure to PVS2 solution for various periods with (LN+) or without (LN-) subsequent immersion in liquid nitrogen. Untreated controls received neither sucrose pre sulture no PVS2 treatment. Assessment were made 6 weeks after treatment.

Exposure to PVS2 (min)	Survival (%) LN-	Survival (%) LN+
0	91± 3 a	10 ± 2 b
30	89 ± 3 a	61 ± 4 a
60	87 ± 5 a	71 ± 2 a
90	89 ± 3 a	64 ± 2 a
120	89 ± 5 a	68 ± 5 a

The exposure to PVS2 treatments for 0 – 120 minutes has reduced survival from 91 to 89 % only after 120 minutes of exposure. However after cryopreservation the survival rate was reduced to 10 – 71 %. The exposure of somatic embryos to PVS2

treatment for 30, 60, 90 and 120 minutes has given survival rates of 61 – 71 %. It results there is no significant differences between them. This indicates treatment to PVS2 has significantly increased the survival rate of cryopreserved material. Thus survival rate of black pepper somatic embryos was enhanced from 62 % when encapsulation dehydration treatment was done to 71 % when vitrification method was given. This vitrification after cryopreservation has resulted in 71 % survival of black pepper somatic embryos which is almost double that of the survival rates reported earlier.

Following thawing, cryopreserved PVS2 treated embryos of black pepper variety Karimunda first turned brown, but two weeks later their surviving cells started to proliferate cream globular stage embryos (Fig 17 b j).

In this study germination into plantlets has been obtained from somatic embryos. Coordinated germination (growth of both root and shoot) was observed in 60% of embryos (Fig 17 k & l). No visible differences were apparent in development and morphology of plantlets from cryopreserved material in comparison with those from uncooled explants.

TTC microscopic test

After completion of the TTC test, the control embryos (not frozen) were clearly red coloured. Among Embryos subjected to

cryopreservation, viable tissues had a bright, red colour (Fig 16 d) ; while the dead tissues were dark due to oxidation processes.

Genetic stability analysis of cryopreserved material

The conserved somatic embryos were cultured *in vitro* and observed for differences and deficiencies during recovery and growth after cryopreservation. These *in vitro* cultured embryos did not exhibit any deficiency symptoms or deformities and morphologically were similar to the control in its appearance and growth. They were able to produce secondary embryos during culture and regrowth.

In the present study the genetic fidelity of plants regenerated from conserved somatic embryo clumps of black pepper was assessed by RAPD and ISSR profiling.

Molecular characterization using RAPD and ISSR

DNA markers can detect even minute differences in nucleic acid sequences which were otherwise difficult to detect in morphological characterization. In the present study an attempt was made to use RAPD and ISSR polymorphism as an index for genetic stability of cryoprserved genotypes. Genomic DNA was isolated using CTAB method from *in vitro* leaves of 13 different plantlets of cryopreserved materials and was found to be of reasonably good quality. The extracted samples were further

purified by RNase treatment followed by phenol:chloroform extraction and precipitation of DNA with absolute alcohol.

Quantification of DNA

The amount of DNA in the purified samples was calculated by comparing with standard λ DNA marker on agarose gels as shown in the Table 29. The black pepper contained 100 – 150 ng/µl of DNA.

Table 29 Concentration of DNA in black pepper somatic embryo samples used for RAPD and ISSR analysis.

Accession	Sample	Concentration of DNA(ng/µl)
Black Pepper	1 (Control)	150
Karimunda	2 *(Cry	100
Karimunda	line)	100
Karimunda	3 (Cry line)	100
Karimunda	4 (Cry line)	100
Karimunda	5 (Cry line)	125
Karimunda	6 (Cry line)	150
Karimunda	7 (Cry line)	100
Karimunda	8 (Cry line)	100
Karimunda	9 (Cry line)	100
Karimunda	10 (Cry	100
Karimunda	line)	100
Karimunda	11 (Cry	100
Karimunda	line)	100
Karimunda	12 (Cry line)	
	13 (Cry line)	
	14 (Cry line)	

*Cry line – Randomly selected cryopreserved lines

RAPD and ISSR profiles of cryopreserved lines

The genetic fidelity of the SE cultures of black pepper cv karimunda treated with cryoprotectants with and without cryostorage, was tested by comparing the RAPD profiles generated with 3 arbitrary 10- mers(OPD – 11, OPA – 17, OPC – 5) (Fig 18 a) and three ISSR primers (IS 1- $[(CT)_7$ TG] , IS 3 - $[(CT)_7$ GC], IS 5 - $[(CA)_7$ GT]) (Fig 18 b). As controls, RAPD and ISSR profiles were generated from the samples of untreated and non-frozen SE cultures of the same cell lines. The three tested RAPD and ISSR primers produced a total of 32 strong and reproducible fragments, the number of amplified products varied from 4 to 6 per primer. None of the primers, however, was able to detect any polymorphism between cryopreserved and control lines.

CRYOPRESERVATION OF *PIPER BARBERI*

Initiation, multiplication and maintenance of stock materials

Establishment of cultures from shoot explants taken from field grown plants were done as per the protocol developed by Nirmal babu *et al* (Nirmal babu *et al*., 1992) (Fig 19 a g). The best response was obtained on *WPM* supplemented with 13.31 µM BA and 4.65 µM kinetin for the production of multiple shoots while growth regulator-free medium was adjudged better for rooting. The multiplied cultures were subsequently maintained on growth regulator free WPM to nullify the effect of growth regulators and

these cultures with miniature explants were used for cryopreservation experiments. Two cryopreservation techniques like encapsulation dehydration and encapsulation vitrification with rapid freezing were employed to cryopreserve endangered *P.barberi* shoot tips.

Encapsulation dehydration

Encapsulation

Apical and axillary shoot tip meristems with 2-3 leaf primordia (about 0.8- 1.5 mm in size) were encapsulated in 4 % (w/v) sodium alginate with 2 M glycerol and 0.4 M sucrose and were subjected to direct and stepwise preculture with sucrose concentrations ranging from 0.25 – 1.25 M.

Effect dehydration on survival

The encapsulated and precultured shoot tips were desiccated by dehydrating in the laminar air flow chamber under aseptic conditions. After dehydrating for 8 hours, encapsulated beads were directly plunged to liquid nitrogen for cryopreservation. After two days the cryopreserved shoots were thawed at 40^0C for 2 – 3 minutes and cultured on WPM for post thaw recovery and growth.

Changes in the water content of the precultured, encapsulated and desiccated P.barberi shoots and survival of cryopreserved shoot tips during encapsulation dehydration are presented in the table 30.

Table 30 Survival of cryopreserved shoot tips of *P.barberi* Gamble as a function of changes in the water content of beads by encapsulation, dehydration*.

Hours of desiccation	Moisture content(%)	Survival % LN-	Survival % LN+
0	90 ± 5 a	98 ± 1 a	0 ± 0 f
1	75 ± 6 b	96 ±.6 ab	1 ± 0.3 e
2	65 ± 4 b	96 ± 1 ab	11 ± 2 d
3	40 ± 5 c	94 ±.6 b	17 ± 4 cd
4	30 ± 3 cd	88 ± 1 c	26 ± 3 b
5	27 ± 5 cde	81 ± 2 cd	28 ± 3 b
6	20 ± 5 def	76 ± 3 de	40 ± 5 a
7	14 ± 2 ef	40 ± 1f	24 ± 3 bc
8	12 ± 3 f	18 ± 2 g	8 ± 3 d

*Encapsulated shoot tips were precultured with increasing sucrose concentrations and dehydrated by air on a flow bench for various periods of time after which they were immersed in liquid nitrogen for 48 h.

The initial water content was 90 % on a fresh weight basis and it decreased rapidly to 75 % after one hour of dehydration in the laminar air flow and to 40 % after 3 h, and then slowly but steadly reduced to 20 % after 6h with 30 & 27 % at 4 & 5 h of dehydration. However after cryopreservation the survival rate was negligible or very low (< 28 %) in all treatments except one. Before cryopreservation the highest survival percentage was 98 % at 90 % moisture content. The survival rate was above 94 % upto 40 %

moisture content , after which the survival rate was decreasing less than 40 % at 14 % moisture content and about 18 % survival at 12 % moisture content. The highest survival rate 40 % was obtained when shoot tips were encapsulated in 4 % (w/v) sodium alginate with 2 M glycerol and 0.4 M sucrose and were subjected to stepwise preculture with sucrose concentrations ranging from 0.25 – 0.75M (the final concentration 0.75 M) at 20 % of water content at 6 h of dehydration and futher dehydration in the laminar air flow resulted in survivals less than 25 %. Inorder to increase survival rate further a different cryopreservation procedure like encapsulation vitrification was attempted.

Encapsulation vitrification

To further enhance the survival rate encapsulation and vitrification procedure was also tested.. This was achieved through preculture treatments, loading solution and the duration of vitrification solution treatment at which the shoot tips best withstand cryopreservation.

Effect of preculture and vitrification on survival

In the direct preculture all shoot tips survived following preculture with 0.25M or 0.5M sucrose. The highest survival rate (45 %) of cryopreserved shoot tips was obtained by preculturing in 0.5 M sucrose for 3 days (Table 31), treated with a loading solution of 2 M Glycerol and 0,6 M sucrose for 60 min followed by

dehydration with plant vitrification solution 2 (PVS2) for 3 h at 25^{0}C prior to direct immersion, liquid nitrogen for 48 h.

Table 31 Effect of sucrose concentrations in preculture medium on survival of precultured vitrified and cryopreserved shoot tips of *Piper barberi* Gamble by encapsulation vitrification*.

Sucrose Concentration (M)	Survival (%) Preculture	Survival (%) Vitrified	Survival (%) Cryopreserved
0.25	98 ± 1 a	98 ± 1 a	21 ± 2 b
0.5	98 ± 1a	98 ± 1 a	45 ± 5 a
0.75	78 ± 11b	76 ± 8 b	16 ± 1b
1.0	36 ± 6 c	29 ± 2 c	6 ± 0.6 c

*Encapsulated shoot tips were directly precultured for 3 days on the basic medium enriched with different concentrations of sucrose. Precultures shoot tips were treated with a loading solution of 2M Glycerol and 0,6M sucrose for 60 min followed by dehydration with plant vitrification solution 2 (PVS2) for 3h at 25^{0}C prior to direct immersion, liquid nitrogen for 48 h. Means of two independent experiments with three replications are presented.

To further enhance the survival rate in the stepwise preculture, all shoot tips withstood preculture with sucrose concentrations ranging from 0.25 to 1 M. More or less similar results obtained with survival of vitrified shoot tips. Survival of cryopreserved shoot tips increased with increasing sucrose concentrations and the highest survival rate (70%) was obtained when 0.7 5M sucrose (Table 32) pretreated shoot tips were treated with a loading solution of 2M glycerol and 0.6 M sucrose for 60 min,

followed by dehydration with plant vitrification solution 2 (PVS2) for 3h at 25^{0}C prior to direct immersion in liquid nitrogen for 48 h.

Table 3 Effect of sucrose concentrations in preculture medium on survival of precultured vitrified and cryopreserved shoot tips of *P.barberi* Gamble by encapsulation vitrification*.

Sucrose Concentration (M)up to	Survival (%) Preculture	Survival (%) Vitrified	Survival (%) Cryopreserved
	98 ± 0.7 a	98 ± 0.7 a	
0.25	98 ± 0.7 a	98 ± 0.7 a	15 ± 2 d
0.5	98 ± 0.7 a	98 ± 0.7 a	40 ± 1 c
0.75	95 ± 3 a	94 ± 2 a	70 ± 4 a
1.0			57 ± 3 b

*Encapsulated shoot tips were precultured on basic medium using stepwise increasing sucrose concentrations to reach the different final concentrations. Precultured shoot tips were treated with a loading solution of 2M glycerol and 0.6 M sucrose for 60 min, followed by dehydration with plant vitrification solution 2 (PVS2) for 3h at 25^{0}C prior to direct immersion in liquid nitrogen for 24h. Means of two independent experiments with 3 replications are presented.

Thus slow and gradient exposure to more concentrated vitrification has resulted in high (70 %) survival of encapsulated and pretreated shoot tips of *P.barberi.*

A comparison of two cryogenic procedure with respect to survival and regrowth.

The encapsulation vitrification procedure produced higher survival (70 %) and regrowth (65 %) (Fig 20 a-e) of cryopreserved shoot tips than encapsulation dehydration which gave 40 and 30%

of survival and regrowth, respectively as calculated based on the total number of shoot tips treated.

Genetic stability analysis of cryopreserved material

Genetic fidelity is an important factor in any conservation programme. In the present study the genetic fidelity of conserved genotypes were assessed by molecular characterization.

The conserved plants were observed for differences and deficiencies during recovery and growth after cryopreservation.

Molecular characterization using RAPD and ISSR

In the present study an attempt was made to use RAPD and ISSR polymorphism as an index for genetic stability of cryoprserved *P.barberi*. DNA was isolated and RAPD and ISSR profiles were developed from 8 randomly selected replicates of the plantlets regenerated from shoot buds of *P.barberi*.

Genomic DNA was successfully isolated using CTAB method from in vitro leaves of 8 different plantlets of cryopreserved materials. The isolated DNA samples were dissolved in TE buffer and its quality was tested on 0.8 % agarose gels, and was found to be of reasonably good quality. The extracted samples were further purified by RNase treatment followed by phenol:chloroform extraction and precipitation of DNA with absolute alcohol.

Quantification of DNA

The amount of DNA in the purified samples was calculated by comparing with standard λ DNA marker on agarose gels as

shown in the table 33. The *P.barberi* samples contained 100 – 200 ng/µl of DNA.

Table 33 Concentration of DNA in *P. barberi*, genotypes used for RAPD & ISSR analysis.

Accession	Sample	Concentration of DNA(ng/µl)
P.barberi		
P.barberi	1 (Control)	150
P.barberi	2 (Cry line)	100
P.barberi	3 (Cry line)	125
P.barberi	4 (Cry line)	100
P.barberi	5 (Cry line)	200
P.barberi	6 (Cry line)	100
P.barberi	7 (Cry line)	150
P.barberi	8 (Cry line)	100
P.barberi	9 (Cry line)	125

*Cry line – Randomly selected cryopreserved lines

RAPD and ISSR profiles of cryopreserved lines

To assess the genetic fidelity of plantlets regenerated from cryopreserved shoot tips of *Piper barberi*, the RAPD and ISSR patterns were compared with that of *in vitro* grown control plantlets. Out of 6 RAPD primers screened, 3 primers (OPA 17, OPC 5, OPD 11) produced clear reproducible bands. Altogether the RAPD & ISSR primers yielded 42 scorable bands (Fig 21 a b) and fragment patterns of plantlets from cryopreserved shoot tips were

identical to those of *in vitro* grown control plantlets for all the primers tested.

CONSERVATION OF PLANT GENETIC RESOURCES

Plant genetic resources are crucial in sustaining the world's population. Conservation and sustainable use of genetic resources is essential for food security and secure genetic stocks are a fundamental requirement in plant breeding (Holden & Williams, 1984; Wilkes, 1983). Advances in biotechnology have generated new opportunities for genetic resources conservation and utilization. Techniques like *in vitro* culture and cryopreservation have made it easy to collect and conserve genetic resources, especially of species that are difficult to conserve as seeds.

In vitro culture techniques can be utilized at various stages in the conservation and use of plant germplasm. These techniques are invaluable to complement other plant health and conservation strategies, particularly for vegetatively propagated species and species with recalcitrant seeds. Spice crops such as cardamom, ginger and black pepper belong to this group. The germplasm of these crops are conserved in clonal field repositories because they are propagated vegetatively. Seeds of black pepper and cardamom are considered recalcitrant and in ginger there is no seed set. All these crops are threatened by serious soil borne diseases of fungal, bacterial and viral origin and outbreak of any of these diseases can wipe out the field germplasm repository. Hence it is important to formulate *in vitro* conservation strategy as a safe

additive to the field gene bank. During the past several years, different *in vitro* culture techniques have been developed and applied to conserve plant germplasm. However, the maintenance of large collections in standard *in vitro* storage systems requires laborious and continued maintenances of tissue, as most cultures need subculturing at regular intervals of at least once in a year to prevent loss of viability. In addition, the risks of contamination and somaclonal variation increase with time and increase of subculture cycles (Lambardi & De Carlo, 2003). Even under slow growth conditions, *in vitro* conservation of black pepper, cardamom and ginger requires subculturing at 8-12-months intervals (Geetha *et al*., 1995) and can ensure only medium-term storage. Cryopreservation, where all cellular divisions and metabolic processes of stored cells are stopped is considered a better option for the long term storage of base germplasm. Theoretically, the plant materials can thus be stored without any changes for an indefinite period of time (Engelmann, 1997). Cryopreservation of shoot meristems is a logical choice for the long-term sorage of germplasm of vegetatively propagated plants, conferring genetic stability with minimum space and maintenance requirements (Bajaj, 1978; Withers, 1980; Harding & Benson, 1994; Sakai, 1995).

Most of the experimental systems employed in cryopreservation (cell suspensions, calluses, shoot tips, embryos)

contain high amounts of cellular water and are thus extremely sensitive to freezing injury since most of them are not inherently freezing tolerant. Cells have thus to be dehydrated artificially to protect them from the damages caused by the crystallization of intracellular water into ice (Meryman & Williams, 1985; Mazur, 1984). The techniques employed and the physical mechanisms upon which they are based are different in classical and newer cryopreservation techniques (Withers & Engelmann, 1997). Classical techniques involve freeze induced dehydration, whereas new techniques are based on vitrification (Engelmann, 2000).

Simple and reliable methods for cryopreservation using vitrification (Langis *et al*., 1990; Sakai *et al*., 1990) and encapsulation dehydration (Fabre & dereuddre, 1990) were reported. These new procedures dehydrate a major part of the freezable water from the tissues at non freezing temperatures and enable them to be cryopreserved by being plunged directly into liquid nitrogen (LN). These protocols simplified the cryogenic process and increased the applicability of cryopreservation to a wide range of plant materials.

***In vitro* culture initiation**

Micropropagation (culture initiation, plant regeneration, multiplication and *in vitro* rooting) form the cycle of events that form the backbone of cryopreservation studies. For culture

establishment and multiplication of cardamom, ginger, black pepper and *Piper barberi*, protocols developed at Indian Institute of Spices Research, by Nirmal Babu, 1997 & Geetha, 2002 were utilized. These protocols are proficient and use low levels of or no growth regulators which result in genetic stability, essential for cryopreservation of plant genetic resources. In addition they also supply disease free propagules for cryopreservation.

Cryopreservation

The present cryopreservation methods simplified the cryogenic process and increased the applicability of cryopreservation to a wide range of plant materials. Encapsulation dehydration procedure comprises the gradual osmotic and evaporative dehydration of plant cells prior to LN exposure. It has been utilised for the cryopreservation of somatic embryos and shoot tips in a range of plant species (Hatanaka *et al*.,1994; Gonzalez-Arnao *et al*.,1996). Vitrification allows tissue survival by avoiding ice formation and can be achieved by elevating cell viscosity with exposure to highly concentrated cryoprotectants and dehydration followed by rapid cooling (Dumet *et al*.,1996) and this has proved to be relatively more effective in producing a higher percentage of survival and faster recovery growth than other cryogenic procedures (Matsumoto *et al*., 1995a;1995b; Hirai & Sakai, 1999a;1999b). Over the past decade, the vitrification

method has become the preferred method for the cryopreservation with more than 160 species and cultivars being successfully cryopreserved (Sakai, 2000; Sakai *et al*., 2002; Sakai and Engelmann, 2007).

In the present study cryopreservation protocols like encapsulation dehydration, encapsulation vitrification and vitrification were standardized for long term preservation of spice crops like cardamom, ginger, black pepper and *Piper barberi*.

CRYOPRESERVATION OF CARDAMOM

Encapsulation Dehydration

Shoot apices represent the material of choice for cryopreservation of germplasm of vegetatively propagated species. At first, apices were cryopreserved using classical procedures including pretreatment with cryoprotectants in liquid medium and controlled slow freezing (Engelmann, 1997). However, survival was generally low, often including a transitory callusing phase and results were not always reproducible, because only limited areas of cells in the meristematic region remained viable after freezing (Bagniol *et al*.,1992) due to intracellular ice formation.

In 1990, a new method termed encapsulation-dehydration was developed by Fabre & Dereuddre (Fabre & Dereuddre, 1990) for cryopreservation of shoot-tips and somatic embryos. This

method is based on the technology developed for producing synthetic seeds, *i.e.* the encapsulation of explants in calcium alginate beads (Redenbaugh *et al*., 1986). Encapsulated explants are then precultured in liquid medium with a high sucrose concentration and partially desiccated before freezing. Encapsulating the explants allows to submit them to very drastic treatments including preculture with high sucrose concentrations and desiccation to low moisture contents (MCs) which would be higly damaging or lethal to non-encapsulated samples. Due to the drastic desiccation of explants, most or all freezable water is removed from cells and vitrification of internal solutes takes place during rapid exposure to liquid nitrogen, thus avoiding lethal intracellular ice crystallyzation (Engelmann, 1997). As a consequence, the whole or a large part of the frozen explant is kept intact after thawing, which allows to obtain high survival, rapid and direct regrowth and reproducible results after cryopreservation (Engelmann, 2000).

For some plant species that are sensitive to higher sugar concentrations the preculture of explants on a medium containing sucrose whose concentration is increased in a stepwise manner was found to be necessary inorder to enchance the survival at cryopreserved explants by encapsulation dehydration (Plessis *et al*., 1991, Gonzalez Arnao *et al*., 1998) Progressive increases in

sucrose concentration made it possible to overcome the sensitivity of explants to direct exposure to high sucrose concentrations and it was demonstrated that sucrose reduced the water content of explants, thus enhancing freeze tolerance (Plessis *et al*., 1991; Gonzalez – Arnao *et al*., 1998; Hitmi *et al*., 1999). A high concentration of sucrose inside the plant cells was observed to be beneficial in establishing a vitrification state during freezing and preventing damage caused by dehydration and freezing (Koster, 1991). Sucrose was also reported to maintain plasma membrane integrity by replacing water on the membrane surface and thus stabilizing the protein under dry and freezing conditions (Crowe *et al*., 1987).

To establish an efficient encapsulation dehydration procedure for cardamom, shoots were first encapsulated in 4 % sodium alginate and to improve survival after cryopreservation, 0.3 M – 1.0 M sucrose preculture and desiccation in the laminar air flow chamber for 10 h were added. The maximum survival (60 %) of cryopreserved cardamom shoots by encapsulation dehydration was detected when the water content was at 20 % with 8 hrs of dehydration in the laminar air flow chamber. After which survival percentage was decreased to 20 and 12 % respectively after 9 and 10 hours at 15 and 12 % water content.

High level accumulation of sugars in the explants is able to prevent damage caused by dehydration and freezing and thus increase survival as has been reported for the cryopreservation of sugar beet shoot tips using a cold hardening treatment (Vanden Bussche *et al*., 1999). In the present study it was observed that a high concentration of sugar which was obtained by preculturing shoots in a gradient of sucrose concentrations might have resulted in the induction of high resistance to damage caused by dehydration and freezing. In addition the presence of glycerol and sucrose in the encapsulation solution should also have contributed to the survival of cryopreserved shoot. It is known that glycerol works as a stabilizer of the membranes and sucrose protects cells during desiccation (Takagi, 2000). Moreover, an increased glycerol concentration reduces the desiccation rate and suppresses the formation of intracellular ice by reducing the amount of unbound water (Tsuruta *et al*., 1998). Cryoprotectants in high concentration though prevent intracellular ice formation; an excessive concentration of cryoprotectants will reduce survival chances of explants (Wolfe & Bryant, 2001). In cardamom shoots cryopreservation by encapsulation dehydration, preculture was performed after encapsulation and glycerol could diffuse out of the beads during preculture. In addition, preculture after encapsulation reduced and slowed down the (direct) contact of the plant material

with the preculture solution. This result appeared to agree with those of cryopreservation of *Robinia pseudoacacia* by encapsulation dehydration (Verleysen *et al*.,2005).

For high post thaw shoot recovery rates, it is essential for the size and developmental stage of the cryostored material to be appropriate (Takagi, 2000). In this experiment, the survival shoot regrowth and shoot length of smaller shoots (1-2 mm) were significantly better than those of larger shoots (3-4 mm). The smaller the meristematic region, the more it tends to consist of a homogeneous population of small actively dividing cells with few vacuoles (Nieves Vidal *et al*., 2005). These characteristics made them more tolerant to dehydration than highly vacuolated and differentiated cells which also form part of larger apices hence shoots sized 1-2 mm were employed in all experiments. Similar results have been reported for cassava where Escobar *et al*., (1997) found that shoot growth after cryopreservation also increased significantly by reducing shoot length to 1 – 2 mm.

On the basis of the results obtained in the present study it is found that the preculturing of cardamom shoots with a stepwise sucrose preculture allows an adaptation to the cryoprotectants; while constant preculture can cause an osmotic shock because of the direct contact with high sucrose concentrations (Wolfe & Bryant, 2001). Though the encapsulation dehydration technique

was proposed as more efficient for rapid cooling (Ashmore, 1997), the results showed that the encapsulation dehydration treatment for cardamom shoots did not increase survival rate above 60% regardless of the dehydration treatment durations from 9 – 10 h, therefore an additional vitrification step with PVS2 solution was added to increase the survival of cryopreserved shoots of cardamom.

Encapsulation vitrification

Vitrification refers to the physical process by which a highly concentrated cryoprotective solution supercools to very low temperatures and finally solidifies into a metastable glass, without undergoing crystallization at a practical cooling rate. Samples are thus cryopreserved without detrimental intracellular ice formation (Engelmann, 1997). In the encapsulation-vitrification technique, the explants are encapsulated in alginate beads, loaded and dehydrated with a vitrification solution before rapid immersion in liquid nitrogen.

Successful cryopreservation of plant species by encapsulation vitrification, requires careful control of the various steps vitrified for optimal dehydration of specimens. Properly

dehydrated tissues vitrify during ultra rapid cooling in LN and avoid the risks of osmotic injuries. Hence, though cryoprotection is the key step, pre-treatment strategies have repeatedly been shown to enhance explant survival following cryopreservation (Reed BM, 1996). Explant preculture in a sucrose containing medium is a common treatment for the induction of osmotolerance to the vitrification solution. In the present inestigation 16 h exposure to 0.3 M sucrose gave significantly better survival after vitrification, and is consistent with observations made in wasabi (Matsumoto *et al*, 1994) and in lily (Matsumoto *et al*., 1995).

In encapsulation vitrification treatment, after preculturing with 0.3 M sucrose for 16 h, dehydration tolerance to PVS2 was induced by treating the shoots with sucrose and glycerol (2 M glycerol and 0.6 M sucrose). Encapsulated and osmoprotected cardamom shoots were dehydrated with PVS2 for 2 h at 25^0C and plunged into LN and held for at least 24 h at -196^0C. The cryopreserved shoot was then thawed at 40^0C and cultured on MS + BA (1 mg/l) + NAA (0.5 mg/l) for post thaw recovery. The encapsulation vitrification treatment resulted in 65 % survival of cryopreserved cardamom shoots.

The exposure of shoot tips to PVS2 without an osmoprotection step, causes harmful effects due to osmotic stress. These detrimental effects are best counter balanced by treating

shoot tips with loading solution (the mixture of 2 M glycerol and 0.4 M sucrose) for 20 min at 25^0C after pre-culture with 0.3 M sucrose, as generally noted in successful cryopreservation protocols for many species of both tropical and temperate origins (Sakai *et al*, 2003). In encapsulation vitrification treatment of cardamom shoots, to determine the most effective osmoprotectant, precultured and encapsulated shoots were treated with different osmoprotectants (various concentrations of sucrose with or without 2 M glycerol). The protective effect of osmoprotection might be due to osmotic dehydration, resulting in the concentration of cylosolic stress responsive solutes which were accumulated during pre-culture with sucrose or sorbitol enriched medium (Reinhoud, 1996). Although the significant positive effects attained with LS solution have been demonstrated in many species, a higher concentration of sucrose (0.6 M) than LS solution in combination with 2 M glycerol was necessary for increased osmotolerance of cardamom shoots. A much higher concentration of sucrose (1.6 M) with 2 M glycerol was reported to be effective as osmoprotectants for sweet potato shoot tips (Fabre & Dereuddre, 1990) although the actual protective action mechanism was poorly understood. Further histological and physiological studies of explants at different stages are necessary to understand the induction mechanism of tolerance to PVS2.

Cardamom shoots dehydrated at 25^{0}C with PVS2 for 120 min after preculturing, encapsulation and osmoprotection produced a higher percentage of survival. A similar positive effect of dehydration with PVS2 at 25^{0}C has been reported for shoot tips of wasabi and banana (Matsumoto *et al*., 1994, Thinh *et al*., 1999). In this experiment, the rapid cooling in LN and subsequent warming caused a survival loss of shoots from 89 – 62 % during the dehydration process with PVS2 at 25^{0}C. These results clearly demonstrate that the acquisition of dehydration tolerance to PVS2 is sufficient for shoots to withstand freezing with a vitrification protocol. The same trend has been reported in apical meristems of many species (Sakai *et al*., 2002)

It is particularly important to show that cryopreserved shoots produce plants phenotypically identical to non-treated control plants. A callus phase before shoot formation is not desirable because callus formation might increase the frequency of genetic variants. Cardamom shoots cryopreserved by the encapsulation vitrification technique established in this study formed shoots directly without intermediary callus formation.

The encapsulation vitrification method has many advantages when compared to the vitrification method. The latter requires the handling of many small explants floating or suspended in a solution. Thus it is difficult to handle many small explants in the

solution at the same time. Encapsulated explants are much easier to handle and permit greater flexibility in handling large amounts of material because the time scale at all steps is much longer than with the vitrification method. Encapsulated shoot tips undergo slower dehydration during exposure to PVS2. Thus the current encapsulation vitrification method is found simple and with easier to handle techniques for conservation of cardamom genotypes with reasonable success. However compared to the vitrification procedure it resulted in less post cryopreservation survival.

Vitrification

Vitrification refers to the physical process by which a highly concentrated cryoprotective solution supercools to very low temperatures and finally solidifies into a metastable glass, without undergoing crystallization at a practical cooling rate (Fahy *et al*.,1984). Thus, vitrification is an effective freeze-avoidance mechanism. As a glass is exceedingly viscous and stops all chemical reactions that require molecular diffusion, its formation leads to metabolic inactivity and stability over time (Burke, 1986).

In the vitrification experiment, to enhance tolerance to vitrification solution (PVS2), a two step sucrose preculture with 0.3 and 0.75 M sucrose for one day each and an osmo protection step with a loading solution of 2 M glycerol and 0.4 M sucrose were

performed prior to PVS2 treatment. The shoots dehydrated with PVS2 for 60 min retained a high level of shoot formation (70 %). This suggests that the mortality observed following cryoprotection and LN exposure was not a result of intracellular ice. More likely, shoots were damaged by influx of water following LN exposure (Volk *et al*., 2006). Survival rates of cryoprotected cardamom shoots that were not exposed to LN were 92%, demonstrating some injury in cardamom by the LN exposure procedures. According to Volk & Walters (Volk & Walters, 2006) PVS2 does not cause massive cell shrinkage that is damaging to cells. However, PVS2 dries samples below the unfrozen water content, a moisture level believed to be critical to survival of desiccation sensitive organs (Farrant & Walters1998; Vertucci & Stushno, 1992; Smith *et al*., 2001).

When treated only with loading solution no shoots developed on cryopreserved shoots, but its omission from the pretreatment of apices treated for 60 min with PVS2 resulted in a significant lowering of post cryostorage recovery rates. The osmopretection produced by the loading solution may be explained by the mitigation of injurious effects during the dehydration process with PVS2 by decreasing osmostress and stabilizing membrances (Jitsuyama *et al*, 1997). The optimal exposure time to PVS2 was 60

min and this was the treatment used in all vitrification experiments.

Three mechanisms by which PVS2 aids cryoprotection of shoot tips are : (1)replaces cellular water,(2) changes freezing behaviour of water remaining in cells, and (3)impedes water loss during air drying. Because solution components permeate into cells, PVS2 treatment can broaden, allowable water contents in cryopreserved shoot tips by lessening the damage from excessive cell shrinkage and limiting the risk of ice formation and growth (Volk & Walters, 2006).These effects were observed in many shoot tips, despite the differences in the shoot tip size of the species(Volk & Walters, 2006). The consistency of the vital effects of PVS2 in diverse explant sources suggested its applicability across a broad range of species.

Penetration of some components of PVS2 into the cytoplasm is key to its cryoprotective function. While sometimes considered impermeable (Sakai, 2000), plant cell walls and membranes appear to be permeable to dimethyl sulfoxide and ethylene glycol (Baudot *et al*., 2000; Baudot & Odagescu, 2004 ; Langis & Steponkus 1991; Myers & Steponkus, 1986). Toxicity studies, which show glycerol- induced damage exacerbated by dimethyl sulfoxide and ethylene glycol (Steponkus *et al*., 1992;Volk *et al*., 2006),also point to the permeability of cells to components of PVS2. The mass

of cryoprotectant entering shoot tips is commensurate with the mass of water lost from the tissues (Volk & Walters, 2006). Replacement of water by non-aqueous components occurs (e.g.,dry matter accumulation during plant embryogenesis), and is likely essential for preventing damage from loss of critical cell volume (Meryman, 1974) or surface area (Steponkus *et al*., 1995).The mechanisms which allow cell dehydration without cell shrinkage remain unclear. current concepts of vitrification in biology. Generally,“good glass formers ” are expected to be better protectants because they have higher glass transition temperatures (*T* g),and *T* g is expected to increase with dehydration as a result of the plasticizing effectect of water (Franks, 1985). PVS2 imparts its effect in ‘pre-vitrified ’solutions. These solutions have sufficient mobility to permeate cells and allow water to be displaced. At lower temperatures, these cryoprotective solutions restrict molecular reorganization necessary to nucleate water and allow ice crystals to grow.

Cryoprotection and recovery of plant shoot tips after LN exposure is associated with a reversible loss and readjustment of water within cells without major fluctuations in shoot tip fresh mass or cell size. When shoot tips are fully protected using PVS2, there is no evidence of water freezing transitions. However, evidence that PVS2 prevented ice formation by forming a glass is weak, and

according to Volk & Walters (Volk & Walters, 2006) the protective mechanism is based on a partial restriction of molecular mobility and/or a disorganization of ice crystal structure.

Cardamom shoots could be amenable to cryopreservation provided that the tissue culture protocol for small size cardamom shoots are sufficiently developed. Hitherto, the shoots used in protocols for cardamom micropropagation have been approximately 1-2 mm in length and have been cultured on medium containing 0.5-1.0mg/l BA and NAA. Successful culture of cardamom shoots of 1-2 mm in length was found optimal for cryostorage in this study. The importance of using recovery media with the appropriate plant growth regulator supplements for the special requirements of cryopreserved material has been emphasized (Touchell *et al*., 2002)

Among cryopreserved apices (LN+) of cardamom, both survival and shoot recovery were significant better on medium with both 1mg/l BA and 0.5 mg/l NAA than on medium with BA alone, but the further addition of BA and NAA brought no further significant improvement.

Data regarding the effects of post culture medium on survival and regeneration have been quite limited. In *Populus alba* (Lambardi *et al*., 2000) suggested that the presence of cytokinins in recovery medium is essential for inducing shoot tip regrowth,

whereas the addition of zeatin has been reported to ensure shoot apex survival and growth in *Grevillea scapigera* (Touchell *et al*., 2002). In contrast Wang *et al*., 2000 found that post culture medium containing different concentrations of BA and 2-naphthaleneacetic and (NAA) did not significantly influence the survival of cryopreserved grapevine shoot tips although the higher concentrations of BA or NAA promoted callus formation and depressed shoot elongation. But in contrast to that in the present study the addition of NAA at 0.5 mg/l in conjunction with 1.0 mg/l BA in the recovery medium seems to promote the shoot regrowth of cryopreserved shoots.

The results of the present study reveals that BA on combination of NAA was required for the successful regeneration of cryopreserved cardamom shoots. This again confirmed that the presence of cytokinins in the post culture medium following cryopreservation is essential for the induction and acceleration of shoot recovery, however, the BA concentration has to be carefully adjusted inorder to avoid callus formation.

The condition of the stock plants from which shoot meristems are taken may have a major bearing on cryopreservation results (Reed, 2000). Having determined the optimal size for cryopreservation to be 1-2 mm shoots a general observation was carried out to evaluate their optimal age i.e., the

time following the last subculture of stock cultures when shoots must be excised for cryostorage. In this study, the two ages observed, 3 and 5 week afforded almost identical recovery rates (61% and 62% respectively). Reducing the culture period of donor shoots to 3 week is advantageous as it saves time in the cryopreservation protocol.

After 8 weeks of culture in recovery medium in Petri dishes, developing shoots were transferred to proliferation medium for further shoot growth. After two transfers of 15 days each (approximately 3 months after thawing) shoots achieved 2-3 cm and were thus suitable for both shoot proliferation and for rooting. To induce rooting in the clones of juvenile origin, shoots longer than 2 cm were excised and transferred to MS basal medium devoid of phytohormones.

Like in any cryogenic protocol, the physiological state of the cells and tissues to be cryopreserved must be optimal for the acquisition of maximum possible dehydration tolerance and for producing vigorous recovery growth (Dereuddre *et al*.,1988). Thinh (Thinh, 1997) clearly demonstrated the importance of the structure of shoot tips used as explants for cryopreservation of several tropical monocotyledonous species using vitrification. According to him freezing shoot tips with the apical dome partially covered, usually with one or two shoot leaf primordial, was the key for

attaining high post-LN regrowth (Thinh, 1997). The results of the present study also obeys the same.

In this study, a two-step vitrification methodology (LS-PVS2 treatments) was moderately successful in cryopreserving cardamom shoots, so that they could be recovered for plant regeneration. Although shoot recovery frequencies need to be improved for a more effective cryopreservation protocol, these results suggest a feasible system since further propagation from a few surviving cyropreserved explants is now possible.

Genetic stability of cryopreseserved cardamom

One important aspect of cryopreservation is the genetic fidelity of plants recovered from cryogenic storage. This is particularly true in the case of perennial crops , because the effects of occasional mutations or genetic rearrangements may not be readily observed in young plants but expressed later on in mature plants (Aronen *et al* .,1999). Most of the works consulted dealing with genetic stability after cryopreservation did not find variations in the recovered material. When some differences were found, the cause of this variation was usually attributed to the *in vitro* proliferation or regeneration process instead of the cryopreservation itself (Harding, 1997).

The genetically stability of shoot apices was confirmed by molecular profiling. The RAPD and ISSR assays performed

suggested that no genetic aberrations originated in cardamom plants during culture and cryopreservation. In conclusion, these results show that the two step vitrification procedure can be successfully applied for cryopreservation and recovery of genetically stable cardamom shoots and can be used for conservation of cardamom germplasm.

Tissue and environmental independent expression of DNA-based markers have made them more reliable than morphological and isozymic markers. Random amplified polymorphic DNA (RAPD) is simpler and has proven to be quite efficient in detecting genetic variations, even in closely related organisms (Martin *et al.,* 1991). With inter simple sequence repeats (ISSRs), primers are not proprietary as in SSRs and can be synthesized by anyone and also allow production of a high number of reproducible polymorphic bands. ISSR is a very simple, quick, cost-effective, highly discriminative and reliable method that combines most of the advantages of SSRs and AFLP with the universality of RAPD (Pradeep *et al*. , 2002). They are found to be more useful and reproducible than isozymes and RAPD, and less cumbersome and cost effective for routine application than RFLP (Fang *et al* ., 1997). In addition, the ISSRs are also found to give more polymorphism than any other assay procedure (Virk *et al*., 2000). Since, simple sequence repeat based primers target the fast evolving,

hypervariable sequences (Tautz, 1989), ISSR markers are considered suitable to detect variations among tissue culture produced plants (Chowdari *et al*. ,1998; Leroy *et al*., 2001; Rahman & Rajora, 2001). At present, RAPD and ISSR markers have been widely utilized to detect the genetic similarities and dissimilarities in micropropagated material in various plants (Devarumath *et al*.,2002; Palombi & Damiano, 2002; Martins,*et al* .,2004; Venkatachalam *et al* ., 2007; Sreedhar *et al* .,2007).

Thus in the present study shoots excised from *in vitro* cultures of cardamom were cryopreserved by three different techniques based on encapsulation dehydration, encapsulation vitrification and vitrification. Pregrowth and serial preculture were needed to obtain the best regrowth for all techniques. The vitrification procedure resulted in higher regrowth (70 %) 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.

CRYOPRESERVATION OF GINGER

Cryopreservation is a strategy for long-term conservation of germplasm (Withers, 1980) more so in ginger where there is no seed set. No reports are available on cryopreservation of ginger

except one preliminary report from Indian Institute of Spices Research laboratory in which a success rate of 40-50 % was reported through encapsulation dehydration of ginger shoots (Geetha, 2002). In the present study an efficient cryopreservation technique for *in vitro* grown shoots of ginger was developed with 80 % success based on vitrification procedure. The other two procedures like encapsulation dehydration, encapsulation vitrification gave lower percentage of success. Pregrowth and serial preculture were needed to obtain the best regrowth for all the techniques.

Encapsulation dehydration

In the encapsulation dehydration method, ginger shoots consisting of the apical dome with 3-4 leaf primordia were encapsulated in 4% sodium alginate and subsequently precultured in liquid MS medium containing 0.1, 0.3, 0.5, 0.7, 0.9 and 1.0 M sucrose for 24 h each and were dehydrated in laminar flow cabinet (at room temperature and humidity) for periods of 0-10 h before plunging to liquid nitrogen. The best results in terms of cryopreserved ginger shoot recovery (around 41 %) were obtained by preculturing in progressive increase of sucrose together with 6 h of dehydration in the laminar air flow chamber.

Encapsulation – Dehydration methods were first used in cryopreservation by Fabre and Dereuddre (Fabre & Dereuddre,

1990) and later it has been applied for cryopreservation of many plant species such as grape (Plessis *et al*., 1991), citrus (Gonzalez – Arnao, 2003), kiwifruit (Suzuki *et al*., 1994), apple, pear, mulberry (Niino & Sakai, 1992), rape (Uragami *et al*., 1993), and sweet potato (Bhatti *et al*., 1997).

For successful cryopreservation by encapsulation dehydration, to induce an osmotolerance to shoot buds, preculturing in a gradient of sucrose can be applied. (Hitmi *et al*.,1999). Besides its osmotic and colligative effects,it is known that sugars are competitive protein and membrane stabilizers (Crowe *et al*.,1984). In addition to physical changes, sugar treatments can also result in different physiological and metabolical changes leading to cryoprotection. These include alterations in proteins (Jitsuyama *et al*.,2002; Thierry *et al*.,1999), membrane fatty acids (Vandenbussche *et al*.,1999), and amino acids (Delvalee *et al*.,1989). In ginger no survival was observed when non-desiccated, precultured explants were frozen in liquid nitrogen. On the other hand adding a desiccation step to the protocol, removed a significant amount of crystallisable water from the shoot tissue and this facilitated survival. It has been generally observed that bead moisture contents between 15 - 25 % is required for optimal survival after cryopreservation (Engelmann, 1997), which was also the case in this study. The best survival of

encapsulated ginger shoot buds was obtained at 21% moisture content. Alginate with adequate preconditioning protects shoot tips from injury during dehydration and cooling as well as during warming (Dereuddre *et al*.,1990; Grospietsch *et al*., 1999). In ginger also the results indicated that preculture duration and progressive increase of sucrose concentration and optimal bead moisture content are critical for cryopreservation of ginger shoots. Dehydration longer than 10 h resulted in reduction of bead moisture content to less than 10 %. Very low or no recovery was observed in treatments where the moisture contents were below 10 %. Thus the encapsulation and dehydration method, though easier to use, resulted in low post thaw recovery rates compared to other two cryopreservation methods.

Encapsulation vitrification

In encapsulation vitrification treatment, ginger shoot buds were encapsulated in 4 % sodium alginate and to enhance the osmotolerance of ginger shoots in the vitrification solution, the shoot buds were preincubated with 0.1 M sucrose for 24 h after encapsulation and precultured with 0.3 M sucrose for 16 h at room temperature. The precultured and preincubated shoots were then treated with 2 M glycerol and 1.6 M sucrose for 3 h at 25^{0}C before being dehydrated with PVS2 for 40 min at 25ºC. Here, the recovery rate of ginger shoots (66 %) was higher than that of the earlier

reported success rate. A higher concentration of sucrose (1.6 M in 2 M Glycerol) and a longer period of incubation (3 h) were necessary to increase the osmotolerance of ginger shoot buds to vitrification solution.

During incubation in LS solution for 3 h meristematic cells become plasmolyzed, producing concentrated spherical protoplasts (Sakai *et al*., 2002). As reported earlier plasmolysis might deteriorate the mechanical stress incurred during severe dehydration (Jitsuyama *et al*.,1997). In addition to this, sucrose employed during preculture has an osmotic effect by dehydrating encapsulated samples (Dumet *et al*.,1993; Pennycooke & Towill, 2001;2000; Scottez *et al*.,1992,). Sucrose is also absorbed by tissues during preculture, resulting in an increase in cytosolic stress responsive intracellular solutes (Dumet *et al*.,1993; Scottez *et al*.,1992). The combination of all these effects reduces, or even suppresses, intracellular ice crystallization during freezing, which is correlated with an increase in survival of cryopreserved samples (Dumet *et al*.,1993).

Vitrification

In the present study a three step vitrification methodology (cryoprotective loading – LS -PVS2 treatments) was successful in cryopreserving ginger shoots so that they could be recovered as plants. The preconditioned and cryoprotected shoots were exposed

to 2 M glycerol and 0.4 M sucrose for 20 min at 25ºC before being dehydrated with PVS2 for 40 min at 25ºC. Regrowth occurred after vitrification in all treatments used and it ranged from 51 % to 80 %. In the vitrification method, shoot tips were exposed to PVS2. Several studies have shown that exposure of less tolerant cells and shoot tips directly to the vitrification solution results in harmful effects due to osmotic stress and or chemical toxicity (Agrawal *et al*.,2004; Lambardi, 2002; Matsumoto *et al*.,1994; Panis & Thinh , 2001; Thinh *et al*.,1999). These harmful effects can be alleviated by adequate preconditioning such as cryoprotective loading (Langis & Steponkus,1990) and modification of the typical one step vitrification procedure.(i.e treatment with the PVS2 solution followed by direct plunging into LN). Here shoots cryoprotected with a mixture of 5 % DMSO and 5 % glycerol gave the highest regrowth of 80 % after vitrification. This is probably due to the penetration of cryoprotective compounds effectively due to the additional use of cryoprotectant mix which leads to a more extensive dehydration and penetration of cryoprotective substances in the cells which inturn avoided the risks of osmotic injuries during vitrification.

When cryopreserved shoots were treated only with loading solution, no shoots developed, but its omission from the pretreatment of shoot apices treated for 40 min with PVS2 resulted

in a significant lowering of post-cryostorage recovery rate. The osmoprotection produced by the LS may be explained by the additional properties of glycerol and sucrose like good protection of glycerol for both biological (Hincha *et al*., 1985 Santarius, 1992; Crowe *et al*., 1990) and model membranes (Crowe *et al*., 1990) during freezing and thawing and in addition to purely osmotic effects sucrose have been shown to be extremely efficient in stabilizing soluble proteins (Santoro *et al*., 1992) and peripherally bound membrane proteins (Hincha, 1998) under stress conditions and this may also contribute to cellular cold tolerance.

The PVS2 application time, affording optimal trade off between its toxic and cryoprotective effects on shoot tips, varies widely among different species. Maximum shoot formation rates have been reported to be 20 min for black currant (Benson *et al*.,1996) and persimmon (Matsumoto *et al*.,2001), 60 min for poplar (Lambardi *et al*.,2000), 90 min for mulberry (Niino *et al*.,2000) and tea (kuranuki & Sakai, 1995). In the present study maximum post thaw recovery was obtained when the preconditioned shoots were treated with PVS2 at 25ºC for 40 min.

In the present study a three step vitrification methodology (cryoprotective loading – LS - PVS2 treatments) was the most successful in cryopreservation of ginger shoots and recovery of plants .

Genetic stability of cryopreseserved ginger

The genetic 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. In conclusion, these results show that the three step vitrification procedure can be successfully applied for cryopreservation and recovery of genetically stable ginger shoots and can be used for conservation of ginger germplasm.

In conclusion, an efficient cryopreservation technique for *in vitro* grown shoots of ginger *(Zingiber officinale* Rosc) was developed based on encapsulation dehydration, encapsulation vitrification and vitrification procedures. Pregrowth and serial preculture were needed to obtain the best regrowth for all techniques. The vitrification procedure resulted in higher regrowth (80 %) when compared to encapsulation vitrification (66 %) and encapsulation dehydration (41%). In the vitrification procedure shoots were: precultured in liquid Murashige-Skoog medium containing 0.3 M sucrose for 3 days; cryoprotected with a mixture of 5 % DMSO and 5 % glycerol for 20 min at room temperature; osmoprotected with a mixture of 2 M glycerol and 0.4 M sucrose for 20 min at 25ºC; before being dehydrated with a highly concentrated vitrification solution (PVS2) for 40 min at 25ºC. The

dehydrated shoots were transferred to 2 ml cryotubes, suspended in 1 ml PVS2 and plunged directly into liquid nitrogen. In all the three cryopreservation procedures tested, shoots grew from cryopreserved shoot tips without intermediary callus formation. The genetic stability of cryopreserved ginger shoot buds were confirmed using ISSR and RAPD profiling.

CRYOPRESERVATION OF BLACK PEPPER

For long-term conservation of the germplasm of species that are vegetatively propagated (like black pepper) and have seeds that are recalcitrant, cryopreservation is an efficient method conferring genetic stability with minimum space and maintenance requirements (Engelmann, 1997). In the case of black pepper the few published studies in this area have used seeds (Chaudhury and Chandel, 1995) which are highly heterogenous and considered to be recalcitrant. Cryopreservation of embryogenic cultures could be useful for reliable long term maintenance of genetic resources, and therefore for alleviating the effects of tissue culture induced somaclonal variation, for enabling efficient recovery of propagules, and for reducing labour and supply costs (Cyr, 2000). The present study describes the successful cryopreservation of somatic embryos of black pepper through encapsulation dehydration and vitrification procedures.

Encapsulation dehydration

In encapsulation dehydration treatment, somatic embryos of black pepper allowed the best survival rates (62 %) 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. As demonstrated in other cryopreservation studies (Engelmann *et al.,* 1994a) the water content in pregrown and desiccated beads of around 20-25 % moisture content (Fresh weight basis) appears to be enough to avoid the occurrence of ice crystallization during freezing, several authors have also reported that this level of dehydration was optimal for the cryopreservation of different plant species using the encapsulation dehydration technique (Withers & Engelmann, 1997).

The suitability of the encapsulation dehydration technique seems to be associated to a well protection of structural integrity of cells by stabilizing proteins and phospho lipid bilayers (Dereuddre, 1992). Due to this most cells were not severely damaged, recovery after cryopreservation occurred rapidly and by a direct regrowth of embryos.

High sucrose concentration may increase tolerant to desiccation and it is possible that increased sugar content is involved not only in the effects of sucrose itself but also in those of desiccation which may stimulate the accumulation of certain sugars. Two mechanisms have been postulated for the beneficial

effects of increased sugar content on cell integrity decrease in cell volume due to osmotic processes and direct stabilization of membrances (Percy *et al*., 2001)

Vitrification

Black pepper somatic embryos were precultured for 3 days on SH basal medium containing 0.3 M sucrose and subjected to vitrfication treatment for 60 minutes at 25^0C resulted in 71 % survival after cryopreservation.

Vitrification based protocols have mainly been used to cryostore shoot apices (Sakai, 1997), but recently encouraging results have also been obtained with embryogenic tissues of several plant species (Corredoira *et al.,* 2004; Lambardi *et al.,* 1999; 2002; Touchell *et al.,* 2002). Cryopreservation of black pepper somatic embryos with vitrification procedure is attempted here.

Preculture on a medium containing sugars or sugar alcohols appears to be an essential step in vitrification procedure for successful cryostorage, and in this case preculturing in 0.3 M sucrose for 3 days was in itself sufficient to provide a remarkable degree of protection to PVS2 solution without going for a loading step. The somatic embryos are already at a low moisture content compared to the shoot tips so it takes less dehydration to allow them to vitrify. In addition lower levels of vacuolization and

differentiation in comparison to shoot tips can also account for this. The accumulation of sugars is thought to increase the stability of membrances under conditions of severe dehydration (Crowe *et al.,* 1989) and it also has other effects that may afford protection; sucrose preculture of proliferating meristems of banana caused fragmentation of vacuoles, among other ultrastructural changes (Helloit *et al.,* 2003), and sugar treated somatic embryos of *Daucus Carota* exhibited an altered protein pattern (Thierry *et al.,* 1999). In addition, an indirect effect of sucrose treatment may be the accumulation of water stress protective compounds such as praline (Thierry *et al.,* 1999).

For black pepper embryos the embryogenesis percentages achieved by isolated or clumped globular embryos were significantly higher than those achieved by early torpedo or cotyledonary stage embryos. The fact that cotyledonary embryos had lower post cryostorage embryogenesis levels than globular clumps inspite of their similar size, 1.5 – 2.5 mm, may be a consequence of their more advanced histodifferentiation in comparison to globular or early torpedo stages (Puigderrajols *et al*., 1996).

The embryogenic cultures of black pepper recovered from cryopreservation often showed an enhancement of morphogenetic potential compared with that from the original non-frozen

embryogenic line. The induction of higher productivity by cryopreservation, may possibly be due to the stress placed upon cryostored embryos (PVS2 application exposure to LN, thawing) resulting in preferential destruction on non-embryogenic cells thus leading to a solution of embryogenic material. Similar observations have been reported in *Hevea brasiliensis* (Engelmann & Etienne, 2000) and Chestnut (Corredoira *et al*, 2004, Holliday & Merkle, 2000) embryogenic cultures. The stress originated by PVS2 appliccation could also explain the higher embryo productivity of uncooled PVS2 treated explants in comparison to cryostored explants stressed by both PVS2 and LN treatments.

In this study germination into plantlets has been obtained from somatic embryos. Coordinated germination (growth of both root and shoot) was observed in 60 % of embryos. No visible differences were apparent in development and morphology of plantlets from cryopreserved material in comparison with those from uncooled explants.

As during ultra –rapid freezing in liquid nitrogen the water contained in the cell cytosol can undergo the transition from a vitreous to a crystalline phase during sample thawing due to recrystallization (Grout 1995), causing considerable damage to the tissues and compromising specimen regrowth after plating. Rapid warming in a waterbath can avoid recrystallization and ensure a

proper recovery of vitrified material. Reports dealing with the cryopreservation of embryogenic line always report 40^0C as the thawing temperature (Correidoira *et al*., 2004, Jekkel *et al*.,1998, Lambardi *et al*.,2002, Martinez *et al*., 2003, Tonon *et al*., 2001, Valladares *et al*., 2004), which is same as the case with the present study.

Genetic stability of cryopreserved black pepper

The RAPD and ISSR assays performed with the present black pepper somatic embryo material showed no genetic variation in the treated samples and also in the control samples. The effect of cryopreservation on the genetic fidelity of the somatic embryo cultures has previously been studied with molecular markers in the PGD treated Scots pine material (Haggman *et al*., 1998), in which no evidence of genetic changes was found using RAPDs. It is necessary to consider that cryopreservation procedures may cause stress and damage of different kind (physical, chemical, and physiological)to the plant tissues. Although the effects of those events on the genome are often unknown, the possible variations may not be due to cryopreservation but the result of the whole culture - cryoprotection - regeneration process (Harding, 2004).

Using a combination of two types of markers that amplify different regions of the genome, a better analysis of genetic stability of plantlets can be made (Martins *et al* .,2004). It is

suggested (Palombi & Damiano, 2002) that the use of more than one DNA amplification technique is advantageous in evaluating somaclonal variation while screening the micropropagated plants of kiwi fruit for any genetic variation.

In conclusion, this study shows that the embryogenic lines of *Piper nigrum* cultivar karimunda can be successfully cyopreserved following an encapsulation desiccation (62 % success) and a simple procedure of vitrification/one step freezing in liquid nitrogen (71 % success) which allows both the maintenance of the proliferative characteristics of the line, and the possibility of completing the process of somatic embryo maturation. This finding opens the way to the possibility of long term storage in liquid nitrogen of valuable embryogenic lines of black pepper avoiding the risks of contamination and embryogenic decline resulting from repeated subculturing.

CRYOPRESERVATION OF *PIPER BARBERI*

Piper barberi is reported to be almost extinct and recorded in the red data book of Indian plants (Nayar & Sastry, 1988) and Geetha *et al* (1995) reported medium storage with its *in vitro* shoot buds. In the present study cryopreservation of *P.barberi* shoot tips was attempted through encapsulation dehydration and encapsulation vitrification procedures.

Encapsulation dehydration

In the encapsulation dehydration procedure, apical and axillary shoot tip meristems with 2-3 leaf primordia (about 0.8- 1.5 mm in size) were encapsulated in 4 % (w/v) sodium alginate with 2 M glycerol and 0.4 M sucrose and were subjected to direct and stepwise preculture with sucrose concentrations ranging from 0.25 – 1.25 M. The highest survival rate 40 % was obtained when shoot tips were encapsulated in 4 % (w/v) sodium alginate with 2 M glycerol and 0.4 M sucrose and were subjected to stepwise preculture with sucrose concentrations ranging from 0.25 – 0.75 M (the final concentration 0.75 M) at 20 % of water content at 6 h of dehydration and futher dehydration in the laminar air flow resulted in survivals less than 25 %.

Preculture is used to increase tolerance of shoot tips to dehydration and subsequent freezing. Preculture with high sucrose concentration (0.75 M) increases total soluble protein and sugar contents in treated tissues (Wang *et al*., 2004), resulting in a two fold increase in the survival of cryopreserves cells as compared to preculture with a low sucrose concentration (0.25M). The increase in protein level is considered as one of the early physiological responses of osmotically stressed cells, which may be related to the improvement of freezing tolerance (Jitsuyama *et al*., 2002; Wang *et al*., 2004). Sugar accumulation in cells could maintain plasma membrance integrity by substituting for water on the

membrane surface thus stabilizing proteins under dry freezing conditions (Crowe *et al*., 1987).

Encapsulation vitrification

In the encapsulation vitrification method, survival of cryopreserved *P.barberi* shoot tips increased with increasing sucrose concentrations and the highest survival rate (70%) was obtained when encapsulated shoot tips were precultured on basic medium using stepwise increasing sucrose concentrations to reach the final concentration 0.7 5 M which were then were treated with a loading solution of 2 M glycerol and 0.6 M sucrose for 60 min, followed by dehydration with plant vitrification solution 2 (PVS2) for 3h at 25^0C prior to direct immersion in liquid nitrogen for 48 h.

Here shoot tips are sufficiently dehydrated osmotically by exposure to a highly concentrated vitrification solution (PVS2 solution). But, the direct exposure of shoot tips to PVS2 solution causes harmful effects due to osmotic stress. Thus the key to successful cryopreservation by vitrification is to induce the osmotolerance to PVS2 solution by preconditioning. In *P.barberi* shoot tips treatment with a mixture of 2 M glycerol plus 0.6 M sucrose (LS solution) following preculturing with sucrose was very effective in increasing the osmotolerance of shoot tips to PVS2 solution. As explained in earlier discussions during the treatment with loading solution the shoot tips were dehydrated osmotically

and plasmolysed. These cells were further dehydrated with PVS2 solution and were capable of vitrifying upon rapid cooling into LN.

Genetic stability of cryopreserved *Piper barberi*

For cryopreservation of germplasm it is particularly important that cryopreserved meristems directly produce plants that are identical to the non treated phenotypes (Haskins & Kartha, 1980; Kartha *et al*., 1980; Towill, 1984). No morphological abnormalities and callus formation were observed during the development of plants from encapsulated, dehydrated and vitrified shoot tips. Thus, these methods appears to be promising technology for the cryopreservation of *Piper barberi*. RAPD and ISSR profiling indicates genetic uniformity in cryopreserved shoots of *P.barberi* after regeneration.

In conclusion, the first cryopreservation procedure for *in vitro* grown shoot tips of *P.barberi* has been developed based on encapsulation vitrification and encapsulation dehydration. Encapsulation vitrification resulted in higher survival (70 %) of cryopreserved shoot tips than encapsulation dehydration (40 %). In both cryogenic procedures, shoots regenerated from cryopreserved shoot tips without intermediary callus formation. Genetic fidelity studies showed that the regenerated plants were similar to the controls. Results here indicate encapsulation

vitrification as a simple and efficient method for long term preservation of *P.barberi* plants.

On the basis of the results obtained in the present study, the cryopreservation protocols developed based on encapsulation dehydration, encapsulation vitrification and vitrification procedures, for the long term storage of spice germplasm like cardamom, ginger, black pepper are simple and efficient and we tried to extend the technology to an endangered Piper species *Piper barberi*. Pregrowth and preculture were needed to obtain the best regrowth for all techniques. Vitrification and encapsulation-dehydration are valuable procedures for cryopreserving various materials, including *in vitr*o-grown shoot tips and somatic embryos. In the present study under optimized conditions, vitrification often produces higher levels of post-liquid nitrogen (LN) recovery growth and greatly reduces the time required for dehydration of samples, compared with the encapsulation-dehydration technique.

The physiological state of the cells and tissues to be cryopreserved must be optimal for the acquisition of maximum possible dehydration tolerance and for producing vigorous recovery growth (Dereuddre *et al*.,1988; Withers, 1979). In the present study cryopreservation of shoot tips of monocotyledonous species like cardamom and ginger by vitrification used shoot tips with the apical dome partially covered, usually with one or two

shoot leaf primordial, might be the key for attaining high post-LN regrowth. The various procedures attempted and the strategies developed for cryopreservation of cardamom, ginger and black pepper germplasm is given in the flow chart.

Cryopreservation employed in the present study can also be extended other than germplasm conservation. Many studies have shown that cryopreservation led to more elimination of viruses than by meristem culture (Engelmann, 2003; Helliot *et al*.,2002; Wang *et al*.,2003). The hypothesis is that cryotherapy is based on selective cell destruction by cryopreservation. The differentiated cells of apices which contain viruses also have high water content; they are killed by the formation of ice crystals during freezing. By contrast, virus-free meristematic cells, the multiplication of which leads to growth of apices, have a more concentrated cytoplasm and survive after freezing, this can be applicable to spices germplasm also.

Thus in the present study cryopreservation protocols developed based on encapsulation dehydration, encapsulation vitrification and vitrification procedures, for the long term storage of spice germplasm like cardamom, ginger, black pepper and *P.barberi* are simple and efficient. In all the three cryopreservation procedures tested, plants grew from cryopreserved explants without intermediary callus formation. The genetic stability of

cryopreserved plants were confirmed using ISSR and RAPD profiling and the present study of evaluating different primers (RAPD and ISSR) for amplification of genomic DNA in these spices can also form a basis for the use of these primers for other genetic studies like DNA fingerprinting.

India is blessed not only with rich biological diversity but also with the associated indigenous knowledge. The country is one of the twelve mega diversity centres and harbors 3 of the 28 global hotspots (Chauhan, 1996). It is estimated that up to 100,000 plants, representing more than one third of all the world's plant species, are currently threatened or face extinction (BGCI, 2005). Preservation of the plant biodiversity is essential for plant improvement and provides various compounds to the pharmaceutical, food and crop protection industries. India is considered as the land of spices, over 53 major spices are grown. Spices like black pepper, chilli, ginger, turmeric, cardamom, fennel, fenugreek, coriander and cumin form the economic backbone of large number of people in India.

Cardamom, *Elettaria cardamomum* Maton, considered the “Queen of Spices”, is a large, herbaceous, rhizomatous perennial, belonging to the family *Zingiberaceae.* Cardamom is native to the moist evergreen forests of the Western Ghats of Southern India and is propagated both through seeds and clonally through suckers. Being a cross-pollinated, seeds are heterozygous. Ginger, *Zingiber officinale* Rosc. also belongs to the family *Zingiberaceae* is not known to occur in the truly wild state but is under cultivation since ancient times. Ginger is believed to have originated in Southeast Asia and is propagated only through vegetative means.

India is the largest producer of ginger with rich cultivar diversity (Lawrence, 1984). India, is also the center of origin and diversity for black pepper (*Piper nigrum* L, family *Piperaceae*) considered the 'king of spices,' one of the most widely used spice in the world. Pepper is predominantly propagated using stem cuttings as seeds are heterozygous and seed progenies are not true to type. *Piper barberi*, a closely related species, is very rare and is reported to be almost extinct (Subramanyam and Henry, 1970).

Conservation of germplasm in seed genebanks by storage of desiccated seeds at low temperature is the most efficient, economical and preferred. But this method is not applicable to crops that do not produce seed (e.g., ginger) or with recalcitrant seed (cardamom & black pepper) as well as to plant species that are propagated vegetatively to preserve the unique genomic constitution of cultivars. Conservation in clonal field repositories is the cheapest alternative. But the field collections are exposed to risks of pests, diseases and adverse weather conditions in addition to being labour-intensive. These risks can be mitigated using *in vitro* conservation strategy which is also labour-intensive. Somaclonal variation is another factor to be considered and at best *in vitro* conservation can be used only for medium term conservation. Hence, cryopreservation or freeze-preservation at ultra-low temperature (-196°C, i.e., the temperature of liquid

nitrogen) is a sound additive to conventional *ex situ* approaches for the long-term conservation of base collections of plant genetic resources, since under these conditions, biochemical and most physical processes are completely arrested and the plant material can be stored for unlimited periods (Withers & Engelmann, 1997).

Efficient technologies for cryopreservation of germplasm in cardamom, ginger and black pepper are not yet optimized. Only one report each are available on cryo conservation of black pepper and cardamom, using seeds (Chaudhury & Chandel, 1994; 1995). Heterozygous nature of seeds in these crops makes these conservation techniques not suitable for germplasm conservation. In the present study cryopreservation were standardized using shoot buds/ somatic embryos for optimizing the true to type long term conservation of cardamom, ginger & black pepper germplasm. The genetic integrity of cryopreserved plants was confirmed using molecular markers - RAPD and ISSR.

Cryopreservation of cardamom

Aseptic shoot buds from *in vitro* cultures of cardamom were cryopreserved by three different techniques based on encapsulation dehydration, encapsulation vitrification and vitrification.

In encapsulation dehydration procedure, shoots were first encapsulated in 4 % sodium alginate and to improve survival after

cryopreservation, 0.3 M – 1.0 M sucrose preculture and desiccation in the laminar air flow chamber for 10 h were added. The maximum survival (60 %) was detected when the water content was at 20 % with 8 hrs of dehydration in the laminar air flow chamber.

In encapsulation vitrification treatment, after preculturing with 0.3 M sucrose for 16 h, dehydration tolerance to PVS2 was induced by treating the shoots with 2 M glycerol and 0.6 M sucrose. Encapsulated and osmoprotected cardamom shoots were dehydrated with PVS2 for 2 h at 25^{0}C and plunged into LN and held for at least 24 h at -196^{0}C. The cryopreserved shoot was then thawed at 40^{0}C and cultured on MS + BA (1mg/l) + NAA (0.5mg/l) for post thaw recovery. The encapsulation vitrification treatment resulted in 62 % survival of cryopreserved cardamom shoots.

In the vitrification experiment, 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 dehydrated with PVS2 for 60 min retained a high level of shoot formation (70 %). The vitrification procedure resulted in higher regrowth (70 %) 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.

Thus vitrification procedure was the best for cryopreservation of cardamom shoot buds with 70 % success. This is 20 % higher than earlier reported success of seed conservation. Moreover using clonal material ensures genetic fidelity of conserved material.

Cryopreservation of ginger

An efficient cryopreservation technique for *in vitro* grown shoots of ginger *(Zingiber officinale* Rosc) was developed based on encapsulation dehydration, encapsulation vitrification and vitrification procedures. In the encapsulation dehydration method, ginger shoots consisting of the apical dome with 3-4 leaf primordia were encapsulated in 4 % sodium alginate and subsequently precultured in liquid MS medium containing 0.1, 0.3, 0.5, 0.7, 0.9 and 1.0 M sucrose for 24 h each and were dehydrated in laminar flow cabinet (at room temperature and humidity) for periods of 0-10 h before plunging to liquid nitrogen. The best results in terms of cryopreserved ginger shoot recovery (41 %) were obtained by preculturing in progressive increase of sucrose together with 6 h of dehydration in the laminar air flow chamber.

In encapsulation vitrification treatment, ginger shoot buds were encapsulated in 4 % sodium alginate and to enhance the osmotolerance of ginger shoots in the vitrification solution, the shoot buds were preincubated with 0.1 M sucrose for 24 h after encapsulation and precultured with 0.3 M sucrose for 16 h at room temperature. The precultured and preincubated shoots were then treated with 2 M glycerol and 1.6 M sucrose for 3 h at 25^{0}C before being dehydrated with PVS2 for 40 min at 25ºC. Here, the survival rate of ginger shoots (66 %) was higher than with the encapsulated and dehydrated method.

In the vitrification procedure shoots were: precultured in liquid Murashige-Skoog medium containing 0.3 M sucrose for 3 days; cryoprotected with a mixture of 5% DMSO and 5% glycerol for 20 min at room temperature; osmoprotected with a mixture of 2 M glycerol and 0.4 M sucrose for 20 min at 25ºC; before being dehydrated with a highly concentrated vitrification solution (PVS2) for 40 min at 25ºC. The dehydrated shoots were transferred to 2 ml cryotubes, suspended in 1 ml PVS2 and plunged directly into liquid nitrogen. In all the three cryopreservation procedures tested, shoots grew from cryopreserved shoot tips without intermediary callus formation. The vitrification procedure resulted in higher regrowth (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. In conclusion, these results show that the three step vitrification procedure can be successfully applied for cryopreservation and recovery of genetically stable ginger shoots and thus for the conservation of ginger germplasm.

Thus vitrification procedure was the best for cryopreservation of ginger shoot buds with 80 % success. This is more than 30 % higher than earlier reported success of ginger shoot buds using encapsulation dehydration method. Moreover using clonal material ensures genetic fidelity of conserved material.

Cryopreservation of black pepper

In black pepper the published studies in this area have used seeds for cryopreservation (Chaudhury and Chandel, 1995) which are highly heterogenous and considered to be recalcitrant. Cryopreservation of somatic embryos could be useful for reliable long term maintenance of genetic resources and this also reduce somaclonal variation (Cyr, 2000). The present study describes the successful cryopreservation of somatic embryos of black pepper through encapsulation dehydration and vitrification procedures.

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 vitrfication treatment for 60 minutes at 25^0C resulted in 71 % survival after cryopreservation.

In conclusion, this study shows that the embryogenic lines of *Piper nigrum* cultivar karimunda can be successfully cyopreserved following an encapsulation desiccation procedure (62 % success). This success rate can be enhanced to 71 % using a vitrification/one step freezing in liquid nitrogen. 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 *Piper barberi*

Developing *in vitro* and cryopreservation protocol will help in conserving this endangered species. Procedures for micropropagation and *in vitro* conservation were already available

(Nirmal Babu *et al*., 1992). In the present study cryopreservation technologies were developed for conservation of *P.barberi* lines

In the encapsulation dehydration procedure, apical and axillary shoot meristems with 2-3 leaf primordia of *P.barberi* (about 0.8 - 1.5 mm in size) were encapsulated in 4 % (w/v) sodium alginate with 2 M glycerol and 0.4 M sucrose and were subjected to direct and stepwise preculture with sucrose concentrations ranging from 0.25 – 1.25 M. The highest survival rate 40 % was obtained when shoot tips were encapsulated in 4 % (w/v) sodium alginate with 2 M glycerol and 0.4 M sucrose and were subjected to stepwise preculture with sucrose concentrations ranging from 0.25 – 0.75 M (the final concentration 0.75 M) at 20 % of water content at 6 h of dehydration.

In the encapsulation vitrification method, survival of cryopreserved *P.barberi* shoot tips increased with increasing sucrose concentrations and the highest survival rate (70 %) was obtained when encapsulated shoot tips were precultured on basic medium using stepwise increasing sucrose concentrations to reach the final concentration 0.75 M which were then were treated with a loading solution of 2 M glycerol and 0.6 M sucrose for 60 min, followed by dehydration with plant vitrification solution 2 (PVS2) for 3 h at 25^0C prior to direct immersion in liquid nitrogen for 48 h.

In conclusion with the cryopreservation of *P.barberi* shoot tips, the encapsulation vitrification procedure produced higher survival (70 %) of cryopreserved shoot tips compared to encapsulation dehydration which gave 40 % survival as calculated based on the total number of shoot tips treated. Genetic fidelity studies showed that the regenerated plants were similar to the controls. Results here indicate encapsulation vitrification as a simple and efficient method for long term preservation of *P.barberi* plants.

Thus in the present study cryopreservation protocols developed based on encapsulation dehydration, encapsulation vitrification and vitrification procedures, for the long term storage of spice germplasm like cardamom, ginger, black pepper and *P.barberi* are simple and efficient. In all the three cryopreservation procedures tested, plants grew from cryopreserved explants without intermediary callus formation. The genetic stability of cryopreserved plants were confirmed using ISSR and RAPD profiling and the present study of evaluating different primers (RAPD and ISSR) for amplification of genomic DNA in these spices can also form a basis for the use of these primers for other genetic studies like DNA fingerprinting.

Abraham P & Thulasidaas G 1958 South Indian cardamom and their agricultural value. Tech. Bull., 79, ICAR, New Delhi. : pp. 1-27.

Adaniya S 2001 Optimal pollination environment of tetraploid ginger (*Zingiber officinale* Rosc.) evaluated by in vitro pollen germination and pollen growth in styles. Sci. Hort. 90 : 219-226.

Agrawal A, Swennen R & Panis B 2004 A comparison of four methods for cryopreservation of meristems in banana (Musa spp.) CryoLetters 25 : 101-110.

Ahuja S, Mandal B B, Dixit S & Srivastava P S 2002 Molecular, phenotypic and biosynthetic stability in *Dioscorea flooribunda* plants derived from cryopreserved shoot tips. Plant Sci.163: 971-977.

Ai P F & Luo Z R 2003 Cryopreservation of dormant shoot tips of persimmon by vitrification and plant regeneration Scientia Agricultura Sinica. 36 : 553-556.

Ai P F & Luo Z R 2004 Cryopreservation of *in vitro* shoot-tips of persimmon and date plum by vitrification and genetic stability of regenerated plantlets. Scientia Agricultura Sinica. 37 : 2023-2027.

AICRPS 2001 All India Coordinated Research Project on Spices, Annual Report for 2000-01 Indian Institute of Spices Research, Calicut, Kerala India.

AICRPS 2006 All India Coordinated Research Project on Spices, Annual Report for 2005-06 Indian Institute of Spices Research, Calicut, Kerala India.

Ainsworth C C & Sharp P J 1989 The potential role of DNA probes in plant variety protection. Plant Varieties &. Seeds. 2 : 27-34.

Al-Ababneh S S, Karam N S & Shibli R A 2002 Cryopreservation of sour orange (*Citrus aurantium* L.) shoot tips In Vitro Cell Develop Biol – Plant. 38 : 602-607.

Al-Ababneh S S, Shibli R A, Karam N S & Shatnawi M A 2003 Cryopreservation of bitter almond (*Amygdalus communis* L.) shoot tips by encapsulation dehydration and vitrification. Adv Horticult Sci. 17 : 15-20.

Anonymous 2004 Conservation of spices genetic resources in in vitro gene banks Project Report submitted to the Department of Biotechnology, Govt Of India, Indian Institute of Spices Research, Calicut, Kerala, India.

Aronen T S, Krajnakova J, Häggman H M & Ryynänen L A 1999 Genetic fidelity of cryopreserved embryogenic cultures of open-pollinated *Abies cephalonic*a. Plant Sci. 142 : 163–172.

Arumuganathan & Earle 1991 Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol. Biol. Rep. 9 : 221-231.

Ashmore S E 1997 *Status Report on the Development and Application of In Vitro Techniques fir the Conservation and Use of Plant Genetic Resources*. *ISBN 92-9043 339-6.* International Plant Genetic Resources Institute. Rome. Italy.

Assy-Bah B & Engelmann F 1992b Cryopreservation of mature embryos of coconut (Cocos *nucifera* L.) and subsequent regeneration of plantlets. Cryo-Letters 13: 117-126

Ausubel F M, Brent R, Kingston R E, Moore D D, Seidman, J G, Smith J A & Struhl K 1995 Short Protocols in Molecular Biology, John Wiley & Sons, New York, Ch. 2.4.

Ayad W G, Hodgkin T, Jaradat A & Rao V R 1997 *Molecular Genetic Techniques for Plant Genetic Resources, Report of an IPGRI Worslnp,* ISBN *92-9043-315-9.* International Plant Genetic Resources Institute, Rome, Italy.

Bachiri Y, Song G Q, Plessis P, Shoar–Ghaffari A, Rekab T & Morisset C 2001 Routine cryopreservation of kiwifruit (*Actinidia* spp.) germplasm by encapsulation–dehydration: Importance of plant growth regulators. CryoLetters. 22 : 61–74.

Baek H J, Kim H H, Cho E G, Chae Y A & Engelmann F 2003 Importance of explant size and origin and of preconditioning treatments for cryopreservation of garlic shoot apices by vitrification CryoLetters. 24 : 381-388.

Bagniol S, Engelmann F & Michaux-Ferrière N 1992 Histo-cytological study of apices from *in vitro* plantlets of date palm (*Phoenix*

dactylifera L.) during a cryopreservation process. CryoLetters. 13, 405-412.

Bajaj Y P S 1978 Tuberization in potato plants regenerated from freeze-preserved. meristems. Crop Improvement. 5: 137-141.

Bajaj Y P S 1983 Cassava plants from meristem cultures freeze-preserved for 3 years. Field Crop Research. 7 : 161-167.

Bajaj Y P S 1986 in *Somaclonal Variation and Crop Improvement* (ed) J Semal, Dordrecht, Martinus Nijhoff Publishers. pp : 244-250.

Bajaj Y P S 1995 in *Biotechnology in Agriculture and Forestry, 32. Crynpresenntion of Plant Germplasm I, YPS* Bajaj (ed), Springer-Verlag, Berlin. pp : 398-416.

Balachandran S M, Bhat S R & Chandel K P S 1990. *In vitro* clonal multiplication of turmeric (*Curcuma longa*) and ginger (*Zingiber officinale* Rosc.). Plant Cell Rep. 3 : 521 - 524.

Ball S G & Seilleur P 1986 in *Somaclonal Variation and Crop Improvement* (ed) J. Semal, Dordrecht, Martinus Nijhoff Publishers. pp : 229-235.

Baudot A & Odagescu V 2004 Thermal properties of ethylene glycol aqueous solutions.Cryobiology. 48 : 283 –294.

Baudot A, Alger L & Boutron P 2000 Glass forming tendency in the system water –dimethyl sulfoxide.Cryobiology. 40 : 151 –158.

Benelli C, De Carlo A, Lambardi M & Lynch P T 2001 Vitrification of shoot tips, nodal segments and embryogenic tissue of olive *(Olea europaea* L.) for germplasm cryopreservation. Acta Horticulturae 560:137-140.

Benson E E, Reed B M, Brenann, Clacher K A & Ross D A 1996 Use of thermal analysis in the evaluation of cryopreservation protocols for *Ribes nigrum* L germplasm. Cryoletters. 17 : 347-362.

BGCI (2005). In: *http://www.bgci.org/*

Bhatti M H, Percival T, Davet C D M, Henshaw G G & Blakesley D 1997

Cryopreservation of embryogenic tissue of a range of genotypes of sweet potato (*Ipomoea batatas* L Lam.) using an encapsulation protocol. Plant Cell Reports. 16:802-806.

Blakesley D & Kiernan R J 2001 Cryopreservation of axillary buds of a *Eucalyptus grandis* X *Eucalyptus camaldulensis* Hybrid. CryoLetters. 22 : 13-18.

Bouman H, Tiekstra A, Petutschnig E, Homan M & Schreurs R 2003 Cryopreservation of lilium species and cultivars Acta Horticulturae. 612 : 147- 154.

Brison M, de Boucaud M T & Dosba F 1995 Cryopreservation of in vitro grown shoot tips of two interspecific Prunus root stocks. Plant Sci. 105: 235-242.

Buderim Ginger Co.(Corporate author) 2002 “Buderim Gold”. Plant varieties J. 15:85.

Burke M J 1986 The glassy state and survival of anhydrous biological systems. In AC Leopold, ed, Membranes, Metabolism,and Dry Organisms. Cornell University Press, Ithaca,NY, pp 358-364.

Chang Y & Reed B M 1999 Extended cold acclimation and recovery medium alteration improve regrowth of *Rubus* shoot tips following cryopreservation CryoLetters. 20 : 371–376.

Chang Y, Barker R & Reed B M 2000 Cold acclimation improves recovery of cryopreserved grass (*Zoysia* and *Lolium* sp.). CryoLetters. 21 : 107–116.

Channuntapipat C, Collins G & Sedgley M 2001 Conservation of almond germplasm by cryopreservation. *Cahiers Options Méditerranéennes.* 56 : 101-106.

Charlwood B V & Rhodes M J C 1990 *Secondary Products from Plant Tissue Culture, Proceedings of the Phytochemical Society of Europe (30),* Clarendon Press, Oxford.

Charoensub R, Phansiri S, Sakai A & Yongmenitchai W 1999

Cryopreservation of cassava in vitro-grown shoot tips cooled to -196c by vitrification. CryoLetters. 20 : 89-94.

Charoensub R, Phansiri S, Yongmenitchai W & Sakai A 2003 Routine cryopreservation of in vitro-grown axillary apices of cassava (*Manihot esculenta* Crantz) by vitrification: importance of a simple mononodal culture". Scientia Horticulturae. 98 : 485-492.

Chaterjee G &Prakash J 1996 Genetic stability in commercial tissue culture.In:Prakash J &Pierik RIM (eds)Plant Biotechnology:Commercial Prospects and Problems (pp 11 – 121).Oxford IBH Publishing Co,New Delhi,India.

Chaudhury R & Chandel K P S 1994 Germination studies and cryopreservation of seeds of black pepper (*Piper nigrum* L.) – a recalcitrant species. CryoLetters. 15 : 145 – 150.

Chaudhury R & Chandel K P S 1995 Studies on germination and cryopreservation of cardamom (*Elettaria cardamomum* Maton.) seeds. Seed Sci. & Technol. 23 : 235 –240.

Chauhan K P S 1996 Legal requirements for fulfilling obligations under the convention on biological diversity in India. Indian J. Plant Genetic Resources. 9(2): 183–191.

Chauhan, K P S 1998 International treaties on biological resources: Implications for the Asian region. Indian J. Plant Genetic Resources 11 (1): 1–12.

Chen T H H, Kartha K K, Leung N L, Kurz G W G Chatson, K B & Constabel F C 1984 Cryopreservation of alkaloid producing cell cultures of periwinkle (*Catharanthus roseus*). Plant Physiol. 75 : 726–731.

Cho E G, Hor Y L, Kim H H, Ramanatha Rao V & Engelmann F 2002 Cryopreservation of *Citrus madurensis* embryonic axes by encapsulation-dehydration CryoLetters. 23 : 325-332.

Chowdari K V, Ramakrishna W, Tamhankar S A, Hendre R R, Gupta V S, Sahasrabudhe N A & Ranjekar P K 1998 Identification of minor DNA variations in rice somaclonal variants. Plant Cell Reports. 18 : 55-58.

Clavero-Ramirez I, Galvez-Farfan J, Lopez-Aranda J M & Gonzalez-Benito M E 2005 apex cryopreservation of several strawberry genotypes by two encapsulation-dehydration methods. CryoLetters. 26 : 17-24.

Cooke R J 1989 The use of electrophoresis for the distinctness. testing of varieties of autogamous species. Plant Varieties & Seeds. 2: 3-13.

Correidoira E, San José M C, Ballester A & Vieitez A M 2004 Cryopreservation of zygotic embryo axes and somatic embryos of European chestnut. CryoLetters. 25: 33-42.

Criel B, Panta A, Carpentier S, Renaut J, Swennen R, Panis B & Hausman J F 2005 Cryopreservation and abiotic stress tolerance in potato: a proteomic approach. Commun Agricult Appl Biol Sci. 70 : 83-86.

Cronquist A 1981 An integrated system of classification of flowering plants. Columbia University Press, New York.

Crowe J H, Carpenter J F, Crowe L M & Anchordoguy T J 1990 Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules. Cryobiology. 27:219–231.

Crowe J H, Hoekstra F A, Crowe L M, Anchordoguy T J & Drobnis E 1989 Lipid Phase Transitions measured in Intact Cells with Fourier Transform Integrated Spectroscopy. Cryobiology. 26 : 76 – 84.

Crowe J H, Crowe L M, Carpenter J F & Aurell- Wistrom C 1984 Stabilization of dry phospholipid bilayers and proteins by sugars. Plant Cell Reports. 12 : 89-94.

Crowe J H, Crowe L M, Carpenter J F & Wistrom C 1987 Stabilization of dry phospholipid bilayers and proteins by sugars. Biochem. J. 242 :1/- 10. CryoLetters. 22 : 277-284.

Custodio L, Fernandes N, Carneiro MF & Romano A 2004 Cryopreservation of microspores of carob (*Ceratonia siliqua* L.). Revista de Biologia Lisboa 22, 7-19.

Cyr D R 2000 Cryopreservation: roles in clonal propagation and germplasm conservation of conifers, in Engelmann F & Takagi H (eds) Cryopreservation of tropical plant germplasm. Current Research Progress and Application, JIRCAS, IPGRI, Rome, Italy, pp.261-268.

D'Amato F 1985 Cytogenetics of plant cell and tissue cultures and their regenerates in *Critical Reviews in Plant Sciences,* (ed) BV Conger, CRC Press, Inc. Boca Raton. Florida. pp :73-112.

Dandin S B, Madhusoodanan K J & George K V 1981 Cardamom descriptor. Proc.1Vth symp.Plant.Crops

De Carlo A, Benelli C & Lambardi M 2000 Development of a shoot-tip vitrification protocol and comparison with encapsulation-based procedures for plum (*prunus domestica* l.) cryopreservation CryoLetters. 21: 215-222.

Dean C & Schmidt R. 1995 Plant genomes: a current molecular description. Ann. Rev. Plant Physiol. Plant Mol. Biol. 46: 395-418.

Deb C R 2002 J Plant Biol. 29 : 71-76.

Debabrata S & Naik P S 1998 Cryopreservation of shoot tips of tetraploid potato (*Solanum tuberosum*L.) clones by vitrification. Annals of Botany. 82 : 455-461.

Debabrata S & Naik P S 2000 *Potato, Global Research and Development. Proceedings of the Global Conference on Potat*o, New Delhi, India, 6-11 December 1999, (eds) SMP Khurana, GS Shekhawat, BP Singh & SK Pandey, Volume 1. pp : 256-260.

Decruse S W, Seeni S & Pushpangadan P 1999 Cryopreservation of alginate coated shoot tips of in vitro grown *Holostemma annulare* (roxb.) k. schum, an endangered medicinal plant : influence of pr eculture and dmso treatment on survival and regeneration CryoLetters. 20 : 243-250.

Dekkers A J, Rao A N & Ghosh C J 1991 *In vitro* storage of multiple shoot culture of gingers at ambient temperatures of 24-29°C . Scientia Horticulturae. 47 (1-2) : 157-167.

Delvalee I, Guillaud J, Beckert M & Dumas C 1989 Cryopreservation of

Immature Maize Embryos After Freeze-Hardening in the Ear and In Vitro. Plant Science. 60: 129-136.

Demon I R, Westcott R J & Ford-Lloyd B V 1977 Phenotypic variation of *Solanum tuberosum* L. cv. Dr McIntosh regenerated directly from shoot-tip culture.Potato Res 20, 131-136.

Dereuddre J 1992 Cryopreservation of *in vitro* cultures of plant cells and organs by vitrification and dehydration. In Reproductive Biology and Plant Breeding. Dattée, Y., Dumas, C. and Gallais, A. (eds.), Springer Verlag, Berlin. pp : 291–300.

Dereuddre J, Blandin S & Hassen N 1991 Resistance of alginate-. coated somatic embryos of carrot. (Daucus carom. L.) to desiccation and cryopreservation CryoLetters.12:125-134.

Dereuddre J, Fabre J & Bassaglia C 1988 Resistance to freezing in liquid nitrogen of carnation (*Dianthus caryophyllus* L. var Eolo) apical and axillary shoot tips excised from different aged in vitro plantlets. Plant Cell Reports.**7:** 170-173.

Dereuddre J, Scottez C, Arnaud Y & Duron M 1990 Cryopreservation of some mediterranean small fruit plants *Comptes Rendus de l'Académie des Sciences Paris*, t. 310, Série III. : 317-323.

Devarumath R. M, Nandy S, Rani V & Marimuthu S 2002 RAPD, ISSR and RFLP fingerprints as useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* (China type) and *C. as-samica* ssp. assamica (Assam-India type). Plant Cell Reports. *2*1 : 166–173.

DeVerno L L, Park Y S, Bonga J M, & Barrett J D 1999 Somaclonal variation in cryopreserved embryogenic clones of white spruce (*Picea glauca* (Moench)Voss.), Plant Cell Rep. 18 : 948 –953.

Donets N V, Musin S M & Popov A S 1991 Stability of composition of polymorphic proteins of potato plants produced from meristems after cryogenic storage,Sel 'skokhosyastvennaya Biologiya 3 :76 –83.

Douglas A. Steinmacher, Cleber W, Saldanha, Charles R. Clement & Miguel P Guerra 2007 Cryopreservation of peach palm zygotic embryos. CryoLetters. 28(1) : 13-22.

Drew R, Azimi M & Ashmore S 2005 Development of advanced technologies for germplasm conservation of tropical species. Acta Horticulturae. 694 : 97-100.

Dumet D 1994 Cryoconservation des massifs d'embryons somatiques de palmier à huile (*Elaeis guineensis* Jacq.) par déshydratation–vitrification. Etude du rôle du saccharose pendant le prétraitement. Thèse d'Université, Univ. Paris. 6 : 125

Dumet D D & Benson E E 2000 Biochemical and physical studies of cryopreserved plant germplasm. IPGRI, Rome. Invited presentation for the JIRCAS/IPGRI Workshop Oct. 1998 "Cryopreservation of Tropical Plant Germplasm: Current Progress and applications pp 43-56. Published by Japanese International Research Centre for Agricultural Sciences and IPGRI. F. Engelmann and H. Takagi (eds.) IPGRI, Rome.

Dumet D, Engelmann F, Chabrillange N, Duvall Y 1994 Effect of desiccation and storage temperature on the conservation of cultures of oil palm somatic embryos. CryoLetters. 15 : 85-90.

Dumet D, Berjak P & Engelmann F 1996 Cryopreservation of zygotic and somatic embryos of tropical species producing recalcitrant or intermediate seeds. In Razdan M. K. and Cocking E. C. (Eds.) *Conservation of plant genetic resources in vitro. Vol. 1*. M/S Science, USA.

Dumet D, Berjak P & Engelmann F 1997 Cryopreservation of zygotic and somatic embryos of tropical species producing recalcitrant or intermediate seeds. In. Razdan M.K. and Cocking E.C. (eds.), *Conservation of Plant Genetic Resources In vitro Vol. 1: General Aspects.* Science Publishers Inc., USA. p : 153–174.

Dumet D, Engelmann F, Chabrillange N & Duvall Y 1993 Cryopreservation of oil palm *(Elaeis guinensis* Jacq.) somatic embryos involving a desiccation step. Plant Cell Rep.: 12: 352-355.

Dussert S & Engelmann F 2006 New determinants for tolerance of coffee (*Coffea arabica* l.) seeds to liquid nitrogen exposure. CryoLetters . 27 (3): 169-178.

Eksomtramage T , Paulet F, Guiderdoni E, Glaszmann J C & Engelmann F 1992 Delvelopment of a cryopreservation process for embryogenic calluses of a commercial hybrid of sugarcane (*Saccharum sp* .) and application to different varieties.CryoLetters. 13: 239 - 252.

Eksomtramage T, Paulet F, Noyer J L, Feldmann P & Glaszmann J C 1992 Utility of isozymes in sugarcane breeding. Sugar Cane. 3 : 14 - 21.

Ellis D, Skogerboe D, Hellier B & Volk G 2006 Implementation of garlic cryopreservation techniques in the national plant germplasm system. CryoLetters. 27 : 99-106.

Engelmann F 2000 in *Cryopreservation of Tropical Plant Germplasm - Current Research Progress and Application*s, (eds) F Engelmann & H Takagi, JIRCAS, Tsukuba/IPGRI, Rome, pp.8-20.

Engelmann F 2003 in *Proceedings of the International Workshop on Cryopreservation of Bio-genetic Resource*s. International Technical Cooperation Center, RDA, Suwon, Korea, 3-5 June 2003, pp. 19-40.

Engelmann F & Etienne H 2000 Cryopreservation of embryogenic calli of Hevea brasiliensis In : Somatic embryogenesis in woody plants. - Dordrecht : Kluwer Academic Publishers. p : 729-746.

Engelmann F 1991 *In vitro* conservation of tropical plant germplasm -review. Euphytica. 57 : 227-243.

Engelmann F 1992 Cryopreservation of embryos. In *Reproductive Biology and Plant Breeding, Dattée, Y., Dumas, C. and Gallais, A.* (eds.), Springer Verlag, Berlin. p : 281–290.

Engelmann F 1997 *In vitro* conservation methods,in:B.V. Ford-Lloyd, J.H.Newbury,J.A.Callow (Eds.), Biotech- nology and Plant Genetic Resources:Conservation and Use,CABI,Wellingford., pp :119 – 162.

Engelmann F 2004 Plant cryopreservation: Progress and prospects. In vitro Cell.& Dev. Biol.-Plant. 40: 427-433.

Engelmann F, Dambier D & Ollitrault P 1994a Cryopreservation of embryogenic cell suspensions and calluses of citrus using a simplified freezing process. *Cryo–Letters* 15: 53–58.

Engelmann F, Dumet D, Chabrillange N, Abdelnour–Esquivel A, Assy–Bah B, Dereuddre J & Duval Y 1995 Cryopreservation of zygotic and somatic embryos from recalcitrant and intermediate–seed species. Plant Genet. Resources Newsletter. 103 : 27–31.

Escobar R H, Mafla G & Roca W W 1997 A methodology for recovering cassava plants from shoot tips maintained in liquid nitrogen. Plant Cell Reports. 16:474-478.

Fabre J & Dereuddre J 1990 Encapsulation-dehydration: a new approach to cryo-. preservation of Solanum shoot-tips. CryoLetters. 11 : 413-426.

Fahy G M, MacFarlane D R, Angell C A & Meryman H T 1984 Vitrification as an approach to cryopreservation. Cryobiology. **2**1: 407-426.

Fang D Q, Roose M. L, Krueger R. R. & Federici C T 1997 Fingerprinting trifoliate orange germplasm accessions with isozymes, RFLPs and inter-simple sequence repeat markers. Theor. Appl. Genet. *9*5 : 211–219.

Farrant J M & Walters C 1998 Ultrastructural and biophysical changes in developing embryos of *Aesculus hippocastanum* L. in relation to the acquisition of tolerance to drying. Physiol. Plant. 104 : 513 –524.

Finkle B J & Ulrich J M 1979 Effect of cryoprotectants in combination on the survival of frozen sugarcane cells. Plant Physiol. 63 : 598–604.

Finkle BJ, Zavala M E & Ulrich J M 1985 Cryoprotective compounds in the viable freezing of plant tissues. In: *Cryopreservation of Plant Cells and Organs*. K.K. Kartha ed., CRC press, Boca Raton, Florida. pp : 75–113.

Flachsland E, Terada G, Scocchi A, Rey H, Mroginski L & Engelmann F 2006 cryopreservation of seeds and *in vitro*-cultured protocorms of oncidium bifolium sims. (orchidaceae) by encapsulation-dehydration CryoLetters. 27: 235-242

Franks F 1985 Biophysics and Biochemistry at Low Temperatures,Cambridge University Press,Cambridge,UK. : 465 –470.

Gagliardi R F, Pacheco G P, Cameiro L A, Valls J F M, Vieira M L C & Mansur E 2003 Cryopreservation of *arachis* species by vitrification of *in vitro*-grown shoot apices and genetic stability of recovered plants. CryoLetters. 24 : 103-110.

Gagliardi R F, Pacheco G P, Valls J F M & Mansur E 2002 Cryopreservation of cultivated and wild *arachis* species embryonic axes using desiccation and vitrification methods. CryoLetters. 23 : 61-68.

Gamble J S 1925 Flora of the presidency of Madras Vol.2 (Reprint 1967), BSI, Calcutta.

Gamez-Pastrana R, Martinez-Ocampo Y, Beristain C I & Gonzalez-Arnao M T 2004 An improved cryopreservation protocol for pineapple apices using encapsulation-vitrification. CryoLetters. 25 : 405-414.

Geetha S P 2002 Unpublished Ph.D. thesis, University of Calicut, India.

Geetha S P, Manjula C & Sajina A 1995 *In vitro* conservation of genetic resources of spices. In *Proceedings Seventh Kerala Science Congress* January 27-29 Palakkad Kerala. pp : 12-16.

George E F 1996 Plant Propagation by Tissue Culture. Part 2 – In Practice. 2nd edn. Exergetics, Edington, UK.

Gilliland T J 1989 Electrophoresis of sexually and vegetatively propagated cultivars of allogamous species. Plant Varieties & Seeds. 2 : 15-25.

Golmirzaie & Panta 2000 Structural observation on Potato shoot-tips after thawing from liquid nitrogen. IPGRI. : 388-389.

Golmirzaie A M & Panta A 2000 Advances in potato cryopreservation at CIP in *Cryopreservation of Tropical Plant Germplasm - Current Research Progress and Application*s, (eds) F Engelmann & H Takagi, JIRCAS, Tsukuba and IPGRI, Rome. pp : 250-254.

Gonzalez-Arnao M T 2003 Cryopreservation of ovules and somatic embryos of citrus using the encapsulation-dehydration technique. Cryoletters 10:85-94.

González-Arnao M T, Engelmann F, URRA C, Morenza M & RIOS A, 1998 Cryopreservation of citrus apices using the encapsulation-dehydration technique. CryoLetters. 19 : 177-182.

Gonzalez-Arnao M T, Engelmann F, Huet C & Urra C 1993 Cryopreservation of encapsulated apices of sugarcane: effect of freezing procedure and histology CryoLetters. 14 : 303-308.

Gonzalez-Arnao M T, Juarez J, Ortega C, Navarro J & Duran-Vila N 2003 Cryopreservation of ovules and somatic embryos of citrus using the encapsulation-dehydration technique CryoLetters . 24 : 85-94.

González-Arnao M T, Moreira T & URRA C 1996 Importance of pregrowth with sucrose and vitrification for the cryopreservation of sugarcane apices using en-capsulation- dehydration. CryoLetters. 17 : 141-148.

Gonzalez-Arnao M T, Ravelo M M, Villavicencio C U, Montero M M & Engelmann F 1998 Cryopreservation of pineapple (*Ananas comosus*) apices by vitrification. CryoLetters. 19 : 375-382.

Gonzalez-Benito E M, Martin C, Iriondo J M & Perez C 1999 in *Plant Conservation*
Biotechnology, (ed) EE Benson, Taylor and Francis, London. pp : 251-264.

Gonzalez-Benito M E & Perez C 1994 Cryopreservation of embryonic axex of two cultivars of hazelnut (*Corylus avellana* L) *Botanic Gardens Micropropagation Newsletter.* 1 : 82-84.

González-Benito M E, Mendoza-Condoriand V H & Molina-García A D 2007 cryopreservation of *in vitro* shoot apices of *oxalis tuberosa* mol. CryoLetters. 28 (1) : 23-32.

Gonzalez-Benito M E, Nuñez-Moreno Y & Martin C 1998 A protocol to cryopreserve nodal explants of antirrhinum microphyllum by encapsulation-dehydration CryoLetters. 19 : 225-230.

Graham J A 1936 Methods of ginger cultivation in Jamaica. Jamaica Agric. Soc. 40 : 231-232.

Grospietch M, Stodulkova E & Zamecnik J 1999 Effect of osmotic stress on the dehydration tolerance of Solanum tuberosum shoot tips. CryoLetters. 20 : 339-346.

Grout B W W 1995 Introduction to the in vitro preservation of plant cells, tissues and organs. In: Grout, B.W.W. (Ed.), Genetic Preservation of Plant Cells in Vitro. Springer–Verlag, Berlin. pp : 1–20.

Guo Y X & Liu Y J 2006 *Acta Botanica Boreali Occidentalia Sinica.* 26 : 1605-1611.

Haberlandt G 1902 Kultinversuche mit Isolierten Pflanzellen Sber Akad Wiss Wien. 111 : 69-92.

Haggman H M, Ryynanen L A, Aronen T S & Krajnakova J 1998 Cryopreservation of embryogenic cultures of Scots pine Plant Cell Tiss & Org Cult. 54 : 45-53.

Hallden C, Hansen M, Nilsson N O & Hjerdin A 1996 Competition as a source of errors in RAPD analysis.Theor Appl Genet. 93 : 1185-1192.

Halmagyi A & Pinker I 2006 Plant regeneration from Rosa shoot tips cryopreserved by a combined droplet vitrification method. Plant Cell Tiss & Org Cult. 84 : 145-153.

Hanada H, Miyamoto Y & Fujioka T 2002 *Bulletin of the Wakayama Research Center of Agriculture, Forestry and Fisheries.* 3 : 43-38.

Hao Y J, Cheng Y J & Deng X X 2003 *GUS* Gene Remains Stable in Transgenic Citrus Callus Recovered from Cryopreservation. CryoLetters. 24 : 375-380.

Hao Y J, You C X & Deng X X 2002 Effects of cryopreservation on developmental competency, cytological and molecular stability of citrus callus CryoLetters. 23 : 27-35.

Hao Y, You C & Deng X 2002 Analysis of ploidy and the patterns of amplified fragment length polymorphism and methylation sensitive amplified polymorphism in strawberry plants recovered from cryopreservation.CryoLetters. 23 :37- 46.

Hao Yu J, You C X & Deng X X 2002b Analysis of ploidy and the patterns of amplified fragment length polymorphism and methylation sensitive amplified polymorphism in strawberry plants recovered from cryopreservation CryoLetters. 23 : 37-46.

Hao Yu-J, Lui Q L & Deng X X 2001 Effect of Cryopreservation on Apple Genetic Resources at Morphological, Chromosomal, and Molecular Levels. Cryobiology 43**,** 46-53.

Harding K & Benson E E 1995 Methods for the Biochemical and Molecular Analysis of cryopreserved plant tissue cultures. In: Genetic Preservation of Plant Cells *In Vitro*. (Ed B.W.W. Grout) Ch. 8, 113-169 Springer Verlag Press.

Harding K & Benson E E 2001 The use of microsatellite analysis in Solanum tuberosum L. in vitro plantlets derived from cryopreserved germplasm. CryoLetters. 22: 199 -208.

Harding K & Benson E E 1994 A study of growth, flowering and tuberisation in plants derived from cryopreserved potato shoot-tips. CryoLetters. 15:59-66.

Harding K & Benson E E 2000 Analysis of chloroplast DNA in *Solanum tuberosum* plantlets derived from cryopreservation. CryoLetters. 21 (5) : 279-288.

Harding K & Millam S 1999 Analysis of ribosomal RNA genes in somatic hybrids between wild and cultivated *Solanum* species. Molecular Breeding. 5(1) : 11-20.

Harding K 1991 Molecular stability of the ribosomal RNA genes in Solanum tuberosum plants recovered from slow growth and cryopreservation. Euphytica. 55: 141- 146.

Harding K 1991 Restriction enzyme mapping of the ribosomal RNA genes in *Solanum tuberosum*: Potato rDNA restriction enzyme map. Euphytica. 54 : 245-250.

Harding K 1992 Detection of ribosomal RNA genes by chemiluminescence in *Solanum tuberosum* L.: a rapid and non-radioactive procedure for the characterisation of potato germplasm. Potato Research. 35 : 199-204.

Harding K 1996 Approaches to assess the genetic stability of plants recovered from *in vitro* culture. In: Proceedings of the International Workshop on *In Vitro* Conservation of Plant Genetic Resources. M.N.Normah, M.K.Narimah and M.M.Clyde (Eds.). Plant Biotechnology Laboratory, University Kebangsaan Malaysia, Kuala Lumpur, Malaysia. pp : 137-170.

Harding K 1997 Stability of the ribosomal RNA genes in *Solanum tuberosum* L. plants recovered from cryopreservation. Cryo-Letters. 18 : 217-230.

Harding K 1999 Stability assessments of conserved plant germplasm. In: Plant Conservation and Biotechnology, E.E.Benson (Ed.). Taylor & Francis Ltd., London. pp : 97-107.

Harding K 2004 Genetic integrity of cryopreserved plant cells: a review. CryoLetter. 25: 3 –22.

Harding K, Benson E E & Clacher K 1997 Plant Conservation Biotechnology: Applications in Agriculture: An Overview. Agro-Food-Industry, Hi-tech. 8(3) : 24-29.

Harding K, Marzalina M, Krishnapillay B, Nashatul Z, Normah M N & Benson E E 2000 Molecular stability assessments of trees regenerated from cryopreserved Mahogany seed germplasm using non-radioactive techniques to examine chromatin structure and DNA methylation status of the ribosomal genes. Journal of Tropical Forestry Science. 12 (1) : 149-163.

Haskins R H & Kartha K K 1980 Freeze-preservation of pea meristems: cell survival. Can. J. Bot. 58: 833-840.

Hatanaka T, Yasuda T, Yamaguchi T, Sakai A 1994 Direct regrowth of encapsulated somatic embryos of coffee (*Coffea Canephora*) after cooling in liquid nitrogen. CryoLetters. 15: 47-52.

Heiken A 1960 Spontaneous and X-ray induced somatic aberrations in Solanum tuberosum L. Almqvisy and Wiksell, Stockholm.

Helliot B, Panis B, PouMay Y, Swennen R, Lepoivre P & Frison E 2002 Cryopreservation for the elimination of cucumber mosaic and banana streak viruses from banana *(Musa* spp.). Plant Cell Rep. 20: 1117-1122.

Helliot B, Swennen R, Poumay Y, Frison E, Lepoivre P & Panis B 2003 Ultrastructural changes associated with cryopreservation of banana *(Musa* spp.) highly proliferating meristems. Plant Cell Reports. 21:690-698.

Hincha D K 1998 Release of two peripheral proteins from chloroplast thylakoid membranes in the presence of a Hofmeister series of chaotropic anions. Arch. Biochem. Biophys. 358:385–390.

Hincha D K, Heber U & Schmitt J M 1985 Antibodies against individual thylakoid membrane proteins as molecular probes to study chemical and mechanical freezing damage *in vitro*. Biochim. Biophys. Acta.;809:337–344.

Hirai D & Sakai A 1999 Cryopreservation of *in vitro*-grown axillary shoot-tip meristems of mint *(Mentha spicata* L.) by encapsulation vitrification. Plant Cell Reports. 19: 150-155.

Hirai D & Sakai A 2001 Recovery growth of plants cryopreserved by encapsulation - vitrification *Bull Hokkaido Prefectural Agricultural Experiment Station.* 80 : 55-64.

Hirai D & Sakai A 2003 Simplified cryopreservation of sweet potato (Ipomoea batatas (L.) Lam.) by optimizing conditions for osmoprotection Plant Cell Reports. 21 : 961-966.

Hirai D 2001 Studies on cryopreservation of vegetatively propagated crops by encapsulation vitrification method in *Rep Hokkaido Prefectural Agricultural Experiment Stations.* 99 : 1- 58.

Hirano T, Godo T, Mii M & Ishikawa K 2005 Cryopreservation of immature seeds of Bletilla striata by vitrification. Plant Cell Reports. 23 : 534-539.

Hirano T, Ishikawa K & Mii M 2005 Cryopreservation of immature seeds of *Ponerorchis graminifolia* var. *suzukiana* by vitrification . CryoLetters. 26 : 139-146.

Hirata K, Phunchindawan M, Kanemoto M, Miyamoto K & Sakai A 1995 CryoLetters. 16 : 122-127.

Hirata K, Phunchindawan M, Tukamoto J, Goda S & Miyamoto K 1996 Cryopreservation of microalgae using encapsulation-dehydration CryoLetters. 17 : 321-328.

Hitmi A, Barthomeuf C & Sallanon H 1999a Cryopreservation of Chrysanthemum cinerariaefolium shoot tips Effects of pretreatment conditions and retention of biosynthetic capacity. CryoLetters. 20:109–20.

Hitmi A, Barthomeuf C & Sallanon H. 1999b Rapid mass propagation of Chrysanthemum cinerariaefolium Vis by callus culture and ability to synthesize pyrethrins. Plant Cell Reports. 19:156–60.

Hitmi A, Coudret A, Barthomeuf C & Sallanon H 1999c The role of sucrose in freezing tolerance in Chrysanthemum cinerariaefolium L cell cultures. CryoLetters. 20:45 – 54.

Holden J H W & Williams J T 1984 Crop Genetic Resources: (eds) Conservation and Evaluation, published by Allen and Unwin, London.

Holliday C P & Merkle S A 2000 Preservation of American chestnut germplasm by cryostorage of embryogenic cultures. J . Am. Chestnut Foundation 14: 46-52.

Holttum, R. E 1950 The Zingiberaceae of the Malay peninsula. Gardens Bull., (Singapore). 13 : 1-50.

Hornung R, Holland A, Taylor H F & Lynch P T 2001. Cryopreservation of *Auricula* shoot tips using the encapsulation/dehydration technique. CryoLetters. 22 : 27–34.

Huang C N, Wang J H, Yan Q S, Zhang W Q & Yan Q F 1995 Plant regeneration from rice (Oryza sativa L.) embryogenic suspension cells cryopreserved by vitrification. Plant Cell Reports. 14 : 730-734.

IBPGR 1977 *Descriptors for the Cultivated Potato, (eds)* 7 Huaman. JT Williams. W Salhuana & Vincent L, Rome, Italy. AGPE : BPGR/77,'32.

IBPGR 1983 Practical constraints affecting the collection and exchange of wild species and primitive cultivars. Report on Secretariat Consultation 14–15 March 1983. AGPG: IBPGR/83/49, FAO Rome.

IISR 2002 Indian Institute of Spices Research, Ann. Rep. for 2001-02, IISR, Calicut.

IPGRI / CIAT 1994 Establishment and operation of a pilot *in vitro* active genebank. Report of a CIAT–IPGRI Collaborative Project using Cassava (*Manihot esculenta* Crantz.) as a model. IPGRI, Rome.IPGRl Technical Bulletin 2, Int Plant Gen Resources Inst, Rome, Italy.

Ishikawa K, Harada K, Mii M, Sakai A, Yoshimatsu K & Shimomura K 1997 Cryopreservation of zygotic embryos of Japanese terrestrial orchid (Bletilla striata) by vitrification Plant Cell Reports. 16, 754-757.

Ishikawa M, Suzuki M, Nakamura T, Kishimoto T, Robertson AJ & Gusta L V 2006 Effect of Growth Phase on Survival of Bromegrass Suspension Cells Following Cryopreservation and Abiotic Stresses. Annals of Botany. 97 : 453-459.

Ishikawa M, Tandon P, Suzuki M & Yamaguishi Ciampi A 1996 Cryopreservation of bromegrass (Bromus inermis Leyss) suspension cultured cells using slow prefreezing and vitrification procedures. Plant Sci. 120:81-88.

Iwananga M 1994 Role of International organisations in global genetic resource management. In Plant Genetic Resource Management in the Tropics. JIRCAS International Symposium Series. No. 2. P. 1–6. Proc. 27th International Symposium on Tropical Agriculture Research, Japan International Research Centre for Tropical Agricultural Sciences, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan, August. : 25 – 26 1993.

Jekkel Zs, Gyulay G, Kiss J, Kiss E & Heszky L E 1998 Cryopreservation of horse chestnut (*Aesculus hippocastanum* L.) somatic

embryos using three different freezing methods. Plant Cell Tiss. Org. Cult.52: 193-197.

Jitsuyama Y, Suzuki T, Harada T & Fujikawa S 1997 Ultrastructural study on mechanism of increased freezing tolerance due toextracelluar glucose in cabbage leaf cells. CryoLetters 18, 33-44.

Jitsuyama Y, Suzuki T, Harada T & Fujikawa S 2002 Sucrose incubation increases freezing tolerance of asparagus (*Asparagus officinalis* L.) embryogenic cell suspensions. CryoLetters 23, 103-112.

Jokipii S, Ryynanen L, Kallio P T, Aronen T & Haggman H 2004 A cryopreservation method maintaining the genetic fidelity of a model forest tree, *Populus tremula* L. × *Populus tremuloides* Michx. Plant Sci. 166 : 799-806.

Jung D W, Sung C K, Touno K, Yoshimatsu K & Shimomura K 2001 Cryopreservation of Hyoscyamus niger adventitious roots by vitrification. J Plant Physiol. 158 : 801-805.

Kamiya T, Sasaki K, Yoshimatsu K & Shimomura K 1995 in *Proceedings of the Japanese Society of Plant Tissue Culture, 14th Annual Meeting Repor*t. p : 112.

Karp A 1993 Are your plants normal? – Genetic instability in regenerated and transgenic plants" Agro-Food-Industry Hi-Tech May/June. : 7-12.

Karp A, Kresovich S, Boat K V, Ayad W G & Hodgkin T 1997 Molecular *Tools in Plant Genetic Resources Conversation: a Guide to Technologies,* ISBN 92-9043-323-X. IPGRl Technical Bulletin 2, Int Plant Gen Resources Inst, Rome, Italy.

Kartha K K & Engelmann F 1994 Cryopreservation and germplasm storage. In *Plant Cell and Tissue Culture*. Vasil, I. K. and Thorpe, T. A. (eds.), Kluwer Academic Publishers, Dordrecht/Boston/London. pp : 195–230.

Kartha K K, Leung N L & Pahl K 1980 Cryopreservation of straw-berry meristems and mass propagation of plantlets, J. Am. Soc. Hort. Sci. 105 : 481–484.

Kartha K. K 1985 In. *Cryopreservation of Plant Cells and Organs*.

Kartha, K. K. (ed.) CRC Press, Boca Raton, Florida. pp : 243–267.

Kartha K.K, Leung N L & Mronginski L A 1982. *In vitro* growth responses and plant regeneration from cryopreservation meristems of cassava (*Manihot esculenta* Crantz.). Z. Pflanzenphysiol. 107: 133 – 140.

Kim H H, Cho E G, Baek Y J, Kim C Y & Engelmann F 2004 Cryopreservation of garlic shoot tips by vitrification: Effects of dehydration, Rewarming, unloading and regrowth conditions. CryoLetters. 25 : 59-70.

Kobayashi S & Sakai A 1997 Cryopreservation of *Citrus sinensis* cultured cells. *In* "Conservation of Plant Ge-netic Resources *in Vitro*" (M. K. Razdan and E. C. Cocking, Eds.) Vol. 1. pp : 201–223.

Kohmura H, Ikeda Y & Sakai A 1994 CryoLetters. 15 : 289-298.

Kohmura H, Sakai A, Chokyu S & Yakuwa T 1992 Cryopreservation of in vitro-cultured multiple bud clusters of asparagus (*Asparagus officinalis* L. cv Hiroshimagreen (2n=30) by the techniques of vitrification. Plant Cell Reports. 11 : 433-437.

Koster K L 1991 Glass formation and desiccation tolerance in seeds. Plant Physiology. 96 : 302–304.

Kryszszuk A, Keller J, Grube M & Zimnoch-Guzowska E 2006 Cryopreservation of potato (*Solanum tuberosum* L.) shoot tips using vitrification and droplet method Journal of Food, Agriculture and the Environment. 4 : 196-200.

Kuranuki Y & Sakai A 1995 Cryopreservation of *in vitro* –grown shoot tips of tea (*Camellia sinensis*)by vitrification.CryoLetters. 16 : 345 –352.

Lambardi M & De Carlo A 2003 Application of tissue culture to the germplasm conservation of temperate broad-leaf trees. In: S.M. Jain and K. Ishii (eds) *Micropropagation of Woody Trees and Fruit*s, Kluwer Academic Publishers, Dordrecht. pp : 815-840.

Lambardi M, Capuana M, De Carlo A & Tonon G 1999 Cryopreservation

of forest species by vitrification of organs and tissues from *in vitro* culture. Proceedings of the International Congress “Applications of biotechnology to forest genetics”. Vitoria-Gasteiz, Spagna, 22-25/9/1999, pp. 313-320.

Lambardi M, de Carlo A & Capuana M 2005 cryopreservation of embryogenic callus of *aesculus hippocastanum* l. by vitrification/one-step freezing. CryoLetters. 26 : 185-192.

Lambardi M, de Carlo A, Benelli C & Bartolini G 2001 Cryopreservation of woody species by vitrification of shoot tips and embryogenic tissue.Acta Horticulturae. 560 : 125-128.

Lambardi M, Fabbri A & Caccavale A 2000 Cryopreservation of white poplar *(Populus alba* L.) by vitrification of *in vitr*o-grown shoot tips. Plant Cell Reports. 19 : 213-218.

Lambardi M, Lynch P.T, Benelli C., Mehra A., Siddika A. 2002 Towards the cryopreservation of olive germplasm. Adv. Hort. Sci. 16(3-4) : 165-174.

Lambardi M. 2002 Cryopreservation of Germplasm of Populus (Poplar) Species. In: L. Towill and Y.P.S. Bajaj (eds), Cryopreservation of Plant Germplasm II. Biotechnology in Agriculture and Forestry, Vol. 50. Springer, Berlin Heidelberg. pp : 269-286.

Lambardi M., Benelli C., De Carlo A 2005 cryopreservation as a tool for the long-term conservation of woody plant germplasm: development of the technology at the cnr/ivalsa institute of florence in the role of biotechnology Villa Gualino, Turin, Italy. : 5-7. March.

Landsmann J & Uhrig H 1985 Somaclonal variation in *Solanum tuberosum*. detected at the molecular level. Theor Appl Gen. 71 *:* 500-505.

Langis R & Steponkus P L 1990 **Cryopreservation of Rye Protoplasts by Vitrification.** Plant Physiol. 92 :666-671.

Langis R & Steponkus P L 1991 Vitrification of isolated rye protoplasts:protection against dehydration injury by ethylene glycol. CryoLetters. 12: 107 –112.

Langis R, Schnabel B, Earle E D & Steponkus P L 1989 Cryopreservation of *Brassica campestris* L. cell suspensions by

vitrification.CryoLetters. 10 : 421-428.

Langis R, Schnabel Preikstas B, Earle B J & Stepokus P L 1990 Cryopreservation of carnation shoot tips by vitrification. Cryobiology 276: 658-659.

Larkin P J, Scowcroft W R 1981 Somaclonal variation - a novel. source of variability from celt cultures for plant improvement. Theor Appl Genet. 60 : 197-214.

Lawrence B.M 1984 Major tropical spices: Ginger (Zingiber officinale Rose.). Perfumer & Flav. 9 : 1-40.

Leroy X .J, Leon K, Hily J M, Chaumeil P & Branchard M 2001 Detection of *in vitro* culture-induced instability through inter-simple sequence repeat analysis. - Theor. appl. Genet. 102 : 885-891.

Liu H, Yu W, Dai J, Gong Q, Yang K & Lu X 2004 Cryopreservation of protoplasts of the alga *Porphyra yezoensis* by vitrification Plant Science. 166 : 97-102.

Liu Y, Nasrallah M & Gao RF 1999 *Forestry Studies in China.* 1 : 11-18.

Liu YG, Wang Y & Liu LX 2004 Analysis of genetic variation in surviving apple shoots following cryopreservation by vitrification Plant Science. 166 : 677-685.

Lowe A J, Hanotte O & Guarino L 1996 Standardization of molecular genetic techniques for the characterization of germplasm collections:the case of random amplified polymorphic DNA (RAPD). Plant Gen.Res.News Lett. 107 : 50 –54.

Mabanza J, Otabo F R & Moussouami C 2001 Conservation *in vitro* du germplasme de cultivars Africans de manioc (*Manihot esculenta* Crantz). Plant Genetic Resources Newsletter. 125 : 29–32.

Mabberley D J 1987 The Plant Book, Cambridge University Press, Cambridge.

Madhusoodanan K J, Kuruvilla K M, Radhakrishnan V V & Potty S N 1998 Cardamom hybrids for higher yield and better quality capsule. Spice India. 11(3) : 6-7.

Madhusoodanan K. J, Radhakrishnan V V & Kuruvila K M 1999. Genetic resources and diversity in cardamom. In Sasikumar, B., Krishnamoorthy, B., Rema, J., Ravindran, P. N. and Peter, K. V. (eds.). *Biodiversity, Conservation and Utilization of Spices, Medicinal and Aromatic Plants.* Indian Institute of Spices Research, Calicut. Pp : 68–72.

Makowska Z, Keller J & Engelmann F 1999 Cryopreservation of apices isolated from garlic (*Allium sativum* L.) bulbils and cloves. CryoLetters. 20: 175-182.

Malaurie B, Trouslot M F, Engelmann F & Chabrillange N 1998 Effect of pretreatment conditions on the cryopreservation of in vitro-cultured yam (*Dioscorea alata* "brazo fuerte" and *D. Bulbifera* "nouméa imboro") shoot apices by encapsulation-dehydration. CryoLetters. 19 : 15-26.

Mandal B B, Chandel K P S & Dwivedi S 1996 Cryopreservation of yam (dioscorea spp.) shoot apices by encapsulation-dehydration. CryoLetters. 17 : 165-174.

Mandal B B, Malik S K & Chandel K P S 1996 Cryopreservation of encapsulated apices of yams (Dioscorea spp.): ultra structural studies on recovery growth, Cryo-Letters. 88 : 65/76 (Selected Abstracts 73).

Mari S, Engelman F, Chabrillange N, Huet C & Michaux–Ferriere N 1995 Histo–cytological study of apices of coffee (*Coffea racemosa* and *C.sessiliflora) in vitro* plantlets during their cryopreservation using the encapsulation–dehydration technique. CryoLetters. 16 : 289–298.

Marin M L, Gogorcena Y, Ortiz J & Duran-Vila N 1993 Recovery of whole plants of sweet orange from somatic embryos subjected to freezing thawing treatments. Plant Cell, Tiss & Org Cult. 34 *:* 27-33.

Martin G B, Williams J G K & Tanksley S D 1991 Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines. *Proc. Natl. Acad. Sci. USA. 8*8 : 2336–2340.

Martinet Montero M E, Gonzalez Arnao M T, Borroto Nordelo C, Puentes

Diaz C & Engelmann F 1998 CryoLetters 19: 171-176.

Martinez M T, Ballester A & Vieitez A M 2003 Cryopreservation of embryogenic cultures of *Quercus robur* using desiccation and vitrification procedures. Cryobiology. 46: 182-189.

Martins M, Sarmento D & Oliveira M M 2004 Genetic stability of micropropagated almond plantlets as assessed by RAPD and ISSR markers. Plant Cell Reports. 23 : 492–496.

Mathew P J & Mathew P M 1992 Cytological studies on Piper barberi,a rare species endemic to Western Ghats.Journal of Spices and aromatic crops. 1 : 81-83.

Matsumoto T, Nako Y, Takahashi C & Sakai A 1997 Induction of in vitro cultured masses of shoot primordia of hybrid statice and its cryopreservation by vitrification. HortScience. 32 : 309-311.

Matsumoto T & Sakai A 1995 An approach to enhance dehydration tolerance of alginate-coated dried meristems cooled to -196 CryoLetters. 16 : 299-306.

Matsumoto T & Sakai A 2000 Cryopreservation of in vitro-cultured axillary shoot tips of Vitis by vitrification. Acta Horticulturae. 538 : 173-175.

Matsumoto T & Sakai A 2003 Cryopreservation of axillary shoot tips of in vitro-grown grape (Vitis) by a two-step vitrification. Euphytica. 131 : 299-304.

Matsumoto T, Sakai A, & Yamada K 1994 Cryopreservation of *in vitro*-grown apical meristems of wasabi *(Wasabia jabonic*a) by vitri-fication and subsequent high plant regeneration, Plant Cell Reports. 13 : 442–446.

Matsumoto T, Mochida K, Itamura H & Sakai A 2001 Cryopreservation of persimmon (*Diospyros kaki* Thunb.) by vitrification of dormant shoot tips. Plant Cell Reports. 20 : 398-402.

Matsumoto T, Sakai A & Nako Y 1998 a novel preculturing for enhancing the survival of in vitro-grown meristems of wasabi (wasabia japonica) cooled to -196 c by vitrification. CryoLetters. 19 : 27-36.

Matsumoto T, Sakai A & Yamada K 1995 Cryopreservation of in vitro-grown apical meristems of lily by vitrification. Plant Cell Tiss and Org Cult. 41 : 237-241.

Matsumoto T, Sakai A, Takahashi C & Yamada K 1995a Cryopreservation of *in vitr*o-grown apical me-ristems of wasabi *(Wasabia japonic*a) by encapsulation-vitrification method. CryoLetters. 16 : 189-196.

Matsumoto T, Takahashi C, Sakai A & Nako Y 1998 Cryopreservation of in vitro-grown apical meristems of hybrid statice by three different procedures. Scientia Horticulturae. 76 : 105-114.

Mayard-Smith J 1998 *Evolutionary Genetics* (2nd Ed.)., Oxford University Press, Oxford.

Mayne W W 1951 Report on cardamom cultivation in South India ICAR. Tech. Bull. p : 50.

Mazur P 1984 Freezing of living cells: mechanisms and applications. American Journal of Physiology 247, Cell Physiology 16, 125-142.

McCown B H & Amos R 1979 Initial trials of commercial propagation with birch. Proc.Inter.Plant Prop.Soc.29:387-393.

McMichael A C, Harris M & Camlin M S 1989 Plant varieties & seeds.

2:45-51

Meryman H T & Williams R J 1985 Basic principles of freezing injury to plant cells: Natural tolerance and approaches to Cryopreservation. In Kartha K K (edn) *Cryopreservation of Plant Cells and Organs*. CRC Press, Boca Raton, Florida. Pp. 13-47.

Meryman H T 1974 Freezing injury and its prevention in living cells,Annu.Rev.Biophys.Bioeng. 3: 341 –363.

Miaja M L, Gambino G, Vallania R & Gribaudo I 2004 Cryopreservation of *Vitis vinifera* L. somatic embryos by vitrification or encapsulation-dehydration. Acta Horticulturae. 663 : 599-603.

Mikula A 2006 Comparison of three techniques for cryopreservation and reestablishment of long-term Gentiana Tibetica suspension cultures. CryoLetters. 27 : 269-282.

Moges A D, Shibli R A & Karam N S 2004 Cryopreservation of African violet (*Saintpaulia ionantha* Wendl.) shoot tips. In Vitro Cellular and Developmental Biology-Plant. 40 : 389-395.

Moukadiri O, O'Connor J E & Cornejo M J 2002 Effects Of The Cryopreservation Procedures On Recovered Rice Cell Populations. CryoLetters. 23 *:* 11-20.

Murashige T & Skoog F 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures.Physiol.Plant.15:473-497.

Myers S P & Steponkus P L 1986 Permeation of dimethyl sulfox- ide into rye protoplasts during freezing of the suspending medium. Cryo Letters. 7 : 41 –54.

Nair R R & Dutta Gupta S 2005 Effect of explants and genotypes on primary somatic embryogenesis in black pepper (Piper nigrum L.). Cytologia, 70: 195-202.

Nair RR & Dutta Gupta 2003 Somatic embryogenesis and plant regeneration in black pepper (Piper nigrum L.): Direct somatic embryogenesis from tissues of germinating seeds and ontogeny of somatic embryos. J. Hortic. Sci. Biotechnol, 78 : 416-421.

Nash T 1966 Chemical constitution and physical properties of compounds able to protect living cells against damage due to freezing and thawing. In. Cryobiology, Meryman, H. Y. (ed.), Academic Press, New York. pp : 197–211.

Nayar M P & Sastry A R K 1988 *Red Data Book of Indian Plant*s, Botanical Survey of India, Kolkata, , 2 : 181-182.

Nieves Vidal, Sanches C, Jorquera L, Ballester A & Viettez A M 2005 Cryopreservation of chestnut by vitrification of in vitro grown shoot tips. In Vitro Cell.Dev. Biol. 41: 63-68.

Niino T, Seguel I, & Murayama T 2000 Cryopreservation of vegetatively propagated species (mainly mulberry). *In* Cryopre-servation of tropical plant germplasm. Current research progress and application. *Edited by* F. Engelmann. Japan International Research Center for Agricultural Sciences/International Plant Genetic Resources Institutes, Rome. pp. 194–199.

Niino T & Sakai A 1992 Cryopreservation of alginate coated in vitro grown shoot tips of apple, pear and mulberry. Plant Science 87: 199-206.

Niino T, Sakai A, Enomoto S & Kato S 1992b Cryopreservation of in vitro grown shoot tips of mulberry by vitrification. Cryo-Letters. 13 : 303-312.

Niino T, Hettiarachchi A, Takahashi J & Samarajeewa P K 2000 cryopreservation of lateral buds of *in vitro*-grown innala plants (*solemostemon rotundifolius*) by vitrification CryoLetters. 21 : 349-356.

Niino T, Sakai A & Yakuwa H 1992 Cryopreservation of shoot tips of garlic by vitrification. CryoLetters. 13 :51-58.

Niino T, Sakai A, Enomoto S, Magosi J & Kato K 1992c Cryopreservation of *in vitro* grown shoot tips of mulberry by vitrification. Cryoletters. 13 : 379–388.

Niino T, Sakai A, Yakuwa H & Nojiri K 1992 Cryopreservation of in vitro-grown shoot tips of apple and pear by vitrification. Plant Cell Tiss and Org Cult. 28 : 261-266.

Niino T, Takano J, Saga T & Kobayashi M 2003 Horticultural Research Japan. 2 : 241-245.

Niino T, Tanaka D, Ichikawa S, Takano J, Ivette S, Shirata K & Uemura M 2003 Cryopreservation of in vitro-grown apical shoot tips of strawberry by vitrification. Plant Biotechnology. 20 : 75-80.

Niino T, Tashiro K, Suzuku M, Ohuchi S, Magoshi J & Akihama T 1997 Cryopreservation of in vitro grown shoot tips of cherry and sweet cherry by one-step vitrification. Scientia Horticulturae. 70 : 155-163.

Niino T., Sakai, A., Yakuwa, H. and Nojori, K. 1992a. Cryopreservation of *in vitro* grown shoot tips of apple and pear by vitrification. Plant Cell Tiss. Org. Cult. 28: 261–266.

Niino T, Tanaka D, Tantely R R, Fukui K & Shirata K 2007 Cryopreservation of basal stem buds of in vitro grown mat rush (Juncus spp) by vitrification. Cryoletters. 28:197-206.

Nirmal Babu K, Ravindran P N & Peter K V 1997 Protocols for micropropagation of Spices and Aromatic Crops. Indian Institute of Spices Research, Calicut, India.

Nirmal Babu K 1997 *In vitro studies in ginger, Zingiber officinale* Rose. Unpublished Ph.D. Thesis, University of Calicut, Kerala, India.

Nirmal Babu K, Geetha S P, Manjula C, Ravindran P N & Peter K V 1994 Medium term conservation of cardamom germplasm - an *in vitro* approach. In *Abstract Second Asia-Pacific Conference on Agricultural Biotechnology*, Madras, India. p :57.

Nirmal Babu K, Geetha S P, Minoo D, Ravindran, P N & Peter K V 1999a *In vitro* conservation of cardamom (*Elettaria cardamomum* Maton.) germplasm. Plant Genetic Resources newsletter. 119 : 41-45.

Nirmal Babu K, Geetha, S P, Minoo D, Ravindran P N & Peter K V 1999b *In vitro conservation of germplasm*. In: Ghosh, S.P (Ed.) Biotechnology and Its Application in Horticulture. Narosa Publishing House, New Delhi. pp : 106-129.

Nirmal Babu k, Minoo D, Geetha S P, Ravindran P N & Peter K V 2005 Advances in Biotechnology of herbs. Indian J.Bot.Res. 1(2):155-214.

Nirmal Babu K, Nair R R, Johnson George K & Ravindran P N 1992 *Piper barberi* Gamble – a redescription of the species with a note on the karyotype.J.Spicea and Aromatic Crops 1(1):88-93.

Nirmal Babu K, Ravindran P N & Peter K V 2000 *Biotechnology of Spices*. In: Chadha K.L., P.N. Ravindran, and L. Sahijram (Eds.) *Biotechnology of Horticulture and Plantation Crops*, Malhotra Publishing House, New Delhi. pp : 487-527.

Nirmal Babu k, Ravindran P N & Sasikumar B 2003 Field evaluation of tissue cultured plants of spices & assessment of their genetic stability using molecular markers. Final report submitted to

Department of Biotechnology, Govt of India. Pp.94.

Nirmal Babu K, Samsudeen K & Ratnambal M J 1992 In vitro plant regeneration from leaf derived callus in ginger, Zingiber officinale Rosc. Plant Cell Tiss.Org.Cult.29:71-74.

Nirmal Babu K, Samsudeen K, Minoo D, Geetha S P & Ravindran P N 2005 in *Ginge*r, (eds) PN Ravindran & K Nirmal Babu , CRC Press, Boca Raton, USA. pp. 181- 210.

Nishizawa S, Sakai A, Amano Y & Matsuzawa T 1993 Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification. Plant Sci. 91: 67-73.

Niwata E 1995 Cryopreservation of apical meristems of garlic (*Allium sativum* L.) and high subsequent plant regeneration. CryoLetters. 16 : 102-107.

Oh S D 1997 The effect of prefreezing treatment and cryoprotectants on the survival of cryopreserved somatic embryos and plant regenration in Korean native citrus species. Acta Horticulturae. No. 447 : 499-505.

Palombi M A & Damiano C 2002 Comparison between RAPD and SSR molecular markers in detecting genetic variation in kiwi fruit *(Ac-tinidia delicosa* A. Chev). Plant Cell Reports. *2*0 : 1061–1066.

Panis B & Thinh N T 2001 Cryopreservation of *Musa* germplasm. INIBAP Technical Guideline 5 (J. V. Escalant and Sharrock, S. (eds.). International Network for the Improvement of Banana and Plantain, Montpellier, France. pp : 44.

Panis B 1995a Cryopreservation of banana germplasm. *Ph. D. Thesis No. 207*. Catholic University, Leuven, Belgium.

Panis B 1995b Cryopreservation of *Musa* germplasm. Info*Musa*. 4 : 17–20.

Paul H, Daigny G & Sangwan-Noreel BS 2000 Cryopreservation of apple (Malus×domestica Borkh.) shoot tips following encapsulation-dehydration or encapsulation-vitrification. Plant Cell Reports. 19 : 768-774.

Paulet F, Eksomtramage T, Lu YH, D' Hont A & Glaszmann J C 1994 *Proceedings of ISSCT Sugar breeding workshop,* CIRAD/ISSCT, Montpellier, France.

Paulet F, Engelmann F & Glaszmann J C 1993 Cryopreservation of apices of in vitro plantlets of sugarcane. Plant Cell Reports. 12 : 525-529.

Paulus V, de Boucaud M T & Dosba F 1993 *Comptes Rendus de l'Académie d'Agriculture de France.* 79 : 93-102.

Pennycooke J C & Towill L E 2001 medium alterations improve regrowth of sweet potato (*ipomoea batatas* {l.} lam.) shoot tips cryopreserved by vitrification and encapsulation-dehydration. CryoLetters. 22 : 381-389.

Pennycooke J C & Towill L E 2000 Cryopreservation of shoot tips from in vitro plants of sweet potato (Ipomoea batatas (L.) Lam.) by vitrifica-tion, Plant Cell Reports. 19 : 733–737.

Percy R E L, Livingston N J, Moran J A & Aderkas von P 2001 Desiccation, cryopreservation and water relations parameters of white spruce (Picea glauca) and interior spruce (Picea glauca x engelmannii complex) somatic embryos, Tree Physiol. 21: 1303-1310

Peter KV, Ravindran P N, Nirmal Babu K, Sasikumar B, Minoo D, Geetha S P & Rajalakshmi K 2002 Establishing in vitro conservatory of spices germplasm. *ICAR Project Report*, Indian Institute of Spices Research, Calicut, Kerala, India. p : 131.

Phillips R L, Kaeppler S M & Olhoft P 1994 Genetic instability of plant tissue cultures: breakdown of normal controls. Proc Nat Acad Sci, USA. 91 : 5222-5226.

Phunchindawan M, Hirata K, Miyamoto K & Sakai A 1997 Cryopreservation of encapsulated shoot primordia induced in horseradish (*Armoracia rusticana*) hairy root cultures. Plant Cell Reports. 16 : 469-473.

Plessis P, Leddet C & Dereuddre J 1991 Resistance to dehydratation and to freezing in liquid nitrogen of alginate-coated shoot-tips of

grape vine (*Vitis vinifera* L. cv. Chardonnay). *Comptes Rendus de l'Académie des Sciences Paris*, t. 313, Série III. : 373-381.

Popova E V 2007 Personal communication in Sakai A and Engelmann Florent (2007) Vitrification, encapsulation vitrification and droplet vitrification: a review. CryoLetters. 28(3) : 151-172.

Potter R H & Jones M G K 1991 Molecular analysis of genetic stability in In vitro *Methods for Conservation of Plant Genetic Resources,* (ed) JH Dodds, Chapman and Hall, London. pp : 71-89.

Powell W, Morgante R, Andre C, Hanafey M, Vogel J, Tingey S & Rafalsky A 1996 The comparison of RFLP, RAPD,AFLP and SSR (microsatellite) markers for germplasm analysis. - Mol. Breed. 2 : 225-238.

Pradeep Reddy M, Saral N & Siddiq E A 2002 Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica. 128 : 9–17.

Premkumar J & Madhusoodanan K J 1995 Immature fruit fall in small cardamom. *Spice India* **8**(7): 5–7.

Previati A, da Re F, Lambardi M, de Carlo A & Schiavon L 2002 in *Atti VI Giornate Scientifiche SOI Worksho*p, Spoleto, Perugia, 23-25 Apr. pp : 99-100.

Puigderrajols P, Fernandez Guijarro B, Toribio M & Molinas M 1996 Origin and early development of secondary embryos in Quercus suber L. Int J Plant Sci 157: 674-684.

Rahman M & Rajora O 2001 Microsatellite DNA somaclonal variation in micropropagated trembling aspen *(Populus tremuloide*s). - Plant Cell Reports.20 : 531-536.

Ramana K V & Eapen S J 1992 Plant parasitic nematodes of black pepper and cardamom and their management. In Proc. Of National Seminar on Black pepper and Cardamom, 17- 18th May, 1992, Calicut, Kerala, pp. 43-47

Rao N S K, Narayana Swamy S, Chacko E K & Doreswamy M E 1982 Regeneration of plantlets from callus of *Elettaria cardamomom*

Maton, Proc. Indian Acad. Sci., 91(B): 37-41.

Ravindran P N & Nirmal Babu K 1994 Genetic Resources of Black Pepper,In K.L.Chandha and P.Rethinam(Eds)*Adv.in Horticulture* Vol.9(1),Malhotra Pub.Co.,New Delhi. pp: 99-120.

Ravindran P N, Nirmal Babu K & Shiva K N 2005 in *Ginge*r, (eds) PN Ravindran, K Nirmal Babu, CRC Press Washington, DC, pp 15-87.

Ravindran P N, Nirmal Babu K, Peter K V, Abraham C Z & Tyagi R K 2004 Genetic Resources of Spices- the Indian scenario. In. Dhillon B et al.,(eds) Crop Genetic Resources: An Indian Perspective,Indian Society for Plant Genetic Resources, New Delhi.

Ravindran P N, Sasikumar B, George J K, Ratnambal M J, Nirmal Babu K & Zachariah T J 1994 Genetic resources of ginger (*Zingiber officinale* Rosc.) and its conservation in India. Plant Genetic Resources Newsletter, No. 98. pp : 1-4.

Redenbaugh K, Paasch B D, Nichol J W, Kossler M E, Viss P R & Walker K A 1986 Somatic seeds: encapsulation of asexual plant embryos. Biotechnology. 4: 797-801.

Reed B M 1992 Cryopreservation of Ribes apical meristems. Cryobiology. 29: 740.

Reed B M 1996 in Techniques in In vitro Conservation. Proceedings International Workshop In Vitro Conservation Plant Genetic Resources, (eds) MN Normah, MK Narimah & MM Clyde, Univ. Kebangsaan Malaysia Publ.,Kuala Lumpur, pp 73-87.

Reed B M 2000 Genotype considerations in temperate fruit crop cryopreservation. In: Engelmann F, Takagi H, (eds) Cryopreservation of tropical plant germplasm. Tsukuba: JIRCAS; Rome: IPGRI; 246-249.

Reed B M, DeNoma J & Chang Y 2000 Application of cryopreservation protocols at a clonal genebank in Engelmann F & Takagi H (eds) Cryopreservation of tropical plant germplasm. Tsukuba : JIRCAS; Rome: IPGRI;246-249.

Reed B M, DeNoma J, Luo J, Chang Y & Towill L E 1998a Cryopreservation and long–term storage of pear (*Pyrus* L.) genetic resources. *In Vitro* Cell. Dev. Biol. – Plant. 34 : 256–260.

Reed B M, Dumet D, Denoma J M & Benson E E 2001 Validation of cryopreservation protocols for plant germplasm conservation: A pilot study using *Ribes* L. Biodiversity and Conservation. 10 : 939-949.

Reed B M, Paynter C L, DeNoma J & Chang Y 1998b Techniques for medium and long term storage of pear (*Pyrus* sp.) genetic resources. Plant Genetic Resources News Letter. 15 : 1–5.

Reed BM & Yu X 1995 Cryopreservation of in vitro-grown gooseberry and currant meristems. CryoLetters. 16 : 131-136.

Reinhoud P J 1996 *Cryopreservation of Tobacco Suspension Cells by Vitrificatio*n. Doctoral Paper, Rijks University, Leiden, The Netherlands pp 1-95.

Reinhoud P J, Schrijnemakers E W M, van Iren F & Kijne J W 1995 Vitrification and a heat-shock treatment improve cryopreservation of tobacco cell suspensions compared of two-step freezing. Plant Cell Tiss and Org Cult. 42 : 261-267.

Ridley H N 1912 Spices, Mc Millian & Co. Ltd, London.

Rosales P B 1938 An agronomic study of the native and Hawaiian gingers. Philipp. Agric. 26 : 807-822.

Ryynanen L 1998 Effect of abscisic acid, cold hardening and photoperiod on recovery of silver birch. Cryobiology. 36 : 32-39.

Sakai A & Yoshida S 1967 Survival of plant tissue at super-low temperature. VI. Effects of cooling and rewarming rates on survival. Plant Physiol. 42:1695-1701.

Sakai A 1985 Cryopreservation of shoot-tips of fruit trees and herbaceous plants, pp. 135-158. In Cryopreservation of plant cells and organs (Ed., K.K. Kartha). CRC Press, Boca Raton, Florida.

Sakai A & Engelmann 2007 Vitrification, encapsulation vitrification and

droplet vitrification: a review. Cryo Letters 28: 151-172

Sakai A 1960 Survival of the twigs of woody plants at -196°C. Nature 185: 392-394.

Sakai A 1995 Cryopreservation for germplasm collection in woody plants. *In* Somatic embryogenesis in woody plants, Vol. 1. History, molecular and biochemical aspects, and applications. ed. Jain, S. M. et al., 293-315.

Sakai A 1995 Cryopreservation of germplasm of woody plants, in Y.P.S.Bajaj (Ed.), Biotechnology in Agriculture and Forestry, vol.32. Cryopreservation of Plant Germplasm I, Springer, Berlin, Heidelberg : pp.53 –69.

Sakai A 1997 Potentially valuable cryogenic procedures for cryopreservation of cultured plant meristems. Pp. 53–66 in *Conservation of Plant Genetic Resources* In Vitr*o. Volume 1: General Aspects.* M.K. Razdan and E.C. Cocking (eds.). Science Publishers Inc., Enfield, USA.

Sakai A 2000 Development of cryopreservation techniques,in:F. Engelmann,H.Takagi, (Eds.), Cryopreservation of Tropical Plant Germplasm.Current research progress and applica- tion.Japan International Research Center for Agricultural Sciences,Tsukuba,Japan/International Plant Genetic Resources Institute,Rome,Italy. pp : 1 –7.

Sakai A, Hirai D & Charoensub R 2003 in Proceedings International Workshop on Cryopreservation of Bio genetic Resources, (eds) Kim CY et al., NIAB, RDA, Suwon, Republic of Korea.pp 3-18.

Sakai A, Kobayashi S & Oiyama I 1990 Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. Var.brasiliensis Tanaka) by vitrification. Plant Cell Rep.9:30-33.

Sakai A, Kobayashi S & Oiyama I 1991 Cryopreservation of nucellar cells of navel orange (Citrus sinensis Osb.) by a simple freezing method. Plant Sci. 74 : 243-- 248.

Sakai A, Kobayashi S & Oiyama I 1991 Survival by vitrification of nucellar cells of navel orange (*Citrus sinensis* Osb. Var.brasiliensis Tanaka) cooled to -1960C. Journal of Plant Physiology 137: 465-470.

Sakai A, Matsumoto T, Hirai D & Charoensub R 2002 in Plant Cold Hardiness, (eds) Li PH & Palva ET. Kluwer Academic/Plenum Publishers, New York. pp 109-119.

Sakai A, Matsumoto T, Hirai D & Niino T I 2000 Newly developed encapsulation-. dehydration protocol for cryopreservation. CryoLetters. 21 : 53-62.

Sakai A. 1958 Survival of plant tissues at super low temperatures, II. Low Temp. *Sci. Ser. B.* 16 : 41–53.

Sakai S & Nagamasu H 2000 Systematic studies of Bornean Zingiberaceae III. Tamijia: a new genus. Edinburgh Journal of Botany. 57 : 245-255.

Sam Y Y & Hor Y L 1999 in *Proceedings of the IUFRO Seed Symposium 1998 "Recalcitrant Seeds"*, Kuala Lumpur, Malaysia, 12-15 October 1998, (eds) M Marzalina, KC Khoo, N Jayanthi, FY Tsan & B Krishnapillay, Forest Research Institute Malaysia. pp : 146-152.

Sambrook J, Fritsch E F & Maniatis T 1989 Molecular Cloning – a laboratory manual. Vol.3. Cold Spring Harbor Laboratory Press, New York.

San Jose M C, Jorquera L, Vidal N, Corredoira E & Sanchez C 2005 Cryopreservation of european chestnut germplasm. Acta Horticulturae. 693 : 225-231.

Sandhya Gupta & Barbara M Reed 2006 Cryopreservation of shoot tips of blackberry and raspberry by encapsulation-dehydration and vitrification. CryoLetters. 27 (1) : 29-42

Sangeeta A, Mandal B B, Sonali-Dixit S & Srivastava P S 2002 Molecular, phenotypic and biosynthetic stability in *Dioscorea floribunda* plants derived from cryopreserved shoot tips. Plant Science. 163 : 971-977.

Sant R, Taylor M & Tyagi A 2006 Cryopreservation of *in vitro*-grown shoot-tips of tropical taro (*colocasia esculenta* var. *esculenta*) by vitrification *CryoLetters.* 27 : 133-142.

Santarius K A 1992 Freezing of isolated thylakoid membranes in complex media. VIII. Differential cryoprotection by sucrose, proline and glycerol. Physiol. Plant. 84:87–93.

Santoro M M, Liu Y, Khan S M A, Hou L X & Bolen D W 1992 Increased thermal stability of proteins in the presence of naturally occurring osmolytes. Biochemistry. 31:5278–5283.

Sastri B N 1952 *The wealth of India – Raw Materials*. Vol 3, D–E. New Delhi, Council of Scientific and Indl Res. pp : 150–160.

Schafer-Menuhar A, Schumacher H & Wangner G 1996 Dynamics of capping protein and actin assembly in vitro: Uncapping barbed ends by polyphosphoinositides. J. Cell Biol. 135:169-179.

Schafer–Menuhr A 1996 Storage of old potato varieties in liquid nitrogen. IPGRI Newsl. for Europe. 7 : 8.

Schenk R U & Hildebrandt A C 1972 Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Botany, 50:199-204.

Schnabel-Preikstas B, Earle E D & Steponkus P L 1992 Cryopreservation of chrysanthemum shoot tips by vitrification. Cryobiology. 29 :738 -739.

Schoenweiss K, Meier-Dinkel A & Grotha R 2005 Comparison of cryopreservation techniques for long-term storage of ash (*fraxinus excelsior* l.). CryoLetters. 26 : 201-212.

Scottez C, Chevreau E, Godard N, Arnaud Y, Duron M & Dereuddre J 1992 Cryopreservation of cold-acclimated shoot tips of pear *in vitro* cultures after encapsulation-dehydration. Cryobiology. 29 : 691-700.

Scowcroft W R 1984 *Genetic Variability in Tissue Culture: Impact on Germplasm Conservation and Utilisation,* International Board for Plant Genetic Resources, Report (AGPG: IBPGR/84/152). Rome.

Scowcroft W R 1985 Somaclonal variation: the myth of clonal. uniformity.:in *Genetic Flux in Plants,* (eds) B Hohn & ES Dennis, SpringerVerlag, New York. pp : 217-243.

Scowcroft W R, Ryan S A, Brettel R I S & Larkin P J 1984 in *Crop Genetic Resources: Conservation and Evaluation,* (eds) JHW Holders & JT Williams, George Allen & Unwin Ltd., London. pp :

258-267.

Semal J 1986 *Somaclonal Variations and Crop Improvement,* Martinus Nijhoff Publishers, Dordrechl.

Sharma N & Sharma B 2003 cryopreservation of shoot tips of *picrorhiza kurroa* royle ex benth., an indigenous endangered medicinal plant, through vitrification. CryoLetters. 24 : 181-190.

Shatnawi M, Engelmann F, Frattarelli A, Damiano C & Withers L A 1999 Cryopreservation of apices from *in vitro* plantlets of almond (*Prunus dulcis* Mill.). CryoLetters. 20 : 13- 20.

Shibli R A & Al-Juboory K H 2000 Cryopreservation of 'nabali' olive (*Olea europea* l.) somatic embryos by encapsulation-dehydration and encapsulation-vitrification. CryoLetters 21: 357-366.

Shibli R A 2000 Cryopreservation of black iris (*Iris nigricans*). somatic embryos by encapsulation – dehydration. CryoLetters: 21 : 39-46.

Simmonds N W 1979 Principles of crop improvement. Longman Group Ltd., New York.

Smith J W, Walters C, Pammenter N W & Berjak P 2001 Interactions of water content,rapid (non-equilibrium) cooling to -196 °C and survival of embryonic axes of *Aesculus hippocastanum* seeds.Cryobiology. 42 : 196 –206.

Sonali-Dixit S, Ahuja-Ghosh S, Mandal B B & Srivastava P S 2005 Metabolic stability of plants regenerated from cryopreserved shoot tips of *Dioscorea deltoidea* - an endangered medicinal plant. Scientia Horticulturae. 105 : 513-517.

Sreedhar R V, Venkatachalam L & Bhagyalakshmi N 2007 Genetic fidelity of long-term micropropagated shoot cultures of vanilla (*Vanilla planifolia* Andrews)as assessed by molecular markers Biotechnol.J. 2.

Steponkus P L & Lanphear F O 1967 Refinement of the triphenyl tetrazolium chloride method of determining cold injury. Plant . Physiol. 42:1432-1436.

Steponkus P L, Langis R & Fujikawa S 1992 Cryopreservation of plant tissues by vitri W cation,in:P.L.Steponkus (Ed.),Advances in Low-Temperature Biology,JAI Press,Greenwich,CT. pp : 1 –61.

Steponkus P L, Uemura M & Webb M S 1995 Freeze-induced destabilization of cellular membranes and lipid bilayers,in: E.A.Disalvo,S.A.Simon (Eds.),Permeability and Stability of Lipid Bilayers,CRC Press,Boca Raton. pp : 77 –104.

Subramanyam K & Henry A N 1970 Rare or little known plants from South India. Bull. Bot. Surv. India. 12(1-4):1-5.

Sudharshan M R, Kuruvilla K M & Madhusoodanan K J 1991 A key to the identification of types in cardamom. J. Plantation Crops. 18 : 52-55.

Suzuki T, Oishi M, Marshak D R, Czernik A J, Nairn A C & Greengard P 1994 Cell cycle-dependent regulation of the phosphorylation and metabolism of the Alzheimer amyloid precursor protein. EMBO J. 1;13(5):1114-22

Swan T W, Deakin E A, Hunjan G, Souch G R, Spencer M E, Stafford A M & Lynch P T 1998 Cryopreservation of cell suspensions of polygonum aviculare using traditional controlled rate freezing and encapsulation/dehydration protocols, a comp arison of post-thaw cell recovery. CryoLetters. 19 : 237-248.

Swartz H J 1999 in *Micropropagation. Technology and Application,* (eds) PC Dehergh & RE Zimmennann, Kluwer Academic Publishers, Dordrecht. pp : 95-121.

Takagi 2000 Recent developments in cryopreservation of shoot apices of tropical species. in Cryopreservation of Tropical Plant Germplasm, Current Progress and Application, F Engelmann & H Takagi (eds), IPGRI, Rome, pp 178-192.

Takagi H, Thinh N T & Kyesmu P M 1998 Cryopreservation of vegetatively propagated tropical crops by vitrification. Acta Horticulturae. 461 : 485-494.

Tanaka D, Niino T, Isuzugawa K, Hikage T & Uemura M 2004 Cryopreservation of shoot apices of *in-vitro* grown gentian plants: comparison of vitrification and encapsulation-vitrification protocols. CryoLetters. 25 : 167-176.

Tannoury M 1993 *Cryoconservation d'Apex d'Oeillet* (Dianthus caryophyllus *L.) et d'Embryons Somatiques de Carotte* (Daucus carota *L.) par les Procédés d'Enrobage-Déshydratation et d'Enrobage-Vitrification. Thèse d'Universit*é, Université Paris. 6.

Tannoury M, Ralambosoa J, Kaminski M & Dereuddre J 1991 *Comptes Rendus de l'Académie des Sciences Paris,* 313, *Série III. :* 633-638.

Tashiro H, Niino T, Magoshi J & Akihama T 1995 in *Japanese Society of Plant Tissue Culture 14 th Annual Meeting Repor*t. p : 120.

Tautz D 1989 Hypervariability of simple sequences as a general source of polymorphic DNA markers. - Nucl. Acids Res. 17 : 6463-6467.

Thammasiri K 1999 Cryopreservation of embryo axes of Jackfruit. CryoLetters. 20 : 21-28.

Thammasiri K 2000 Cryopreservation of seeds of a thai orchid (*doritis pulcherrima* lindl.) by vitrification. CryoLetters. 21 : 237-244.

Thierry C, Florin B & Petiard V 1999 Changes in protein metabolism during the acquisition of tolerance to cryopreservation of carrot somatic embryos. Plant Physiology and Biochemistry. 37: 145–154.

Thinh N T & Takagi H 2000 Cryopreservation of *in vitro* grown apical meristems of terrestrial orchids (*Cymbidium spp*.) by vitrification in *Cryopreservation of Tropical Plant Germplasm - Current Research Progress and Application*s, (eds) F Engelmann & H Takagi, JIRCAS, Tsukuba and IPGRI, Rome. pp : 441-443.

Thinh N T 1997 *Cryopreservation of Germplasm of Vegetatively Propagated Tropical Monocots by Vitrificatio*n. Doctoral Paper, Faculty of Agriculture, Kobe University, Japan.

Thinh N T, Takagi H & Yashima S 1999 Cryopreservation of in vitro-grown shoot tips of banana (*Musa spp*.) by vitrification method. Cryoletters. 20 : 163–174.

Thinh Thinh N T, Takagi H & Sakai A 2000 Cryopreservation of *in vitro* grown apical meristems of some vegetatively propagated tropical monocots by vitrification. In *Cryopreservation of Tropical Plant Germplasm - Current Research Progress and Application*s, (eds) F Engelmann & H Takagi, JIRCAS, Tsukuba and IPGRI, Rome. pp : 441-443.

Tindall H D 1968 Commercially grown vegetables. In: *Commercial Vegetable Growing,* Oxford Univ. Press, London.

Tonon G, Lambardi M, De Carlo A & Rossi C 2001 in Proceedings V1^{0} Convegno Nazionale Biodiversita (ed) G Russo, Bari, Italy, pp 619-625.

Tonon G, Lambardi M, De Carlo A & Rossi C 2005 Crioconservazione di linee embriogeniche di *Fraxinus angustifolia* Whal. Proc. "VI Convegno Nazionale Biodiversità". Bari, Italy. 6-7/9/2001.

Touchell D H & Dixon K W 1996 in *International Workshop on the* In Vitro *Conservation of Plant Genetic Resources* (eds) MN Normah, MK Narimah & MM Clyde, Plant Biotechnology Laboratory, University Kebangsaan Malaysia, Kuala Lumpur. Pp : 169-180.

Touchell D H, Chiang V L & Tsai C J 2002 Cryopreservation of embryogenic cultures of *Picea mariana* (black spruce) using vitrification. Plant Cell Reports 21: 118-124.

Touchell D H**,** Dixon K W & Tan B 1992 Cryopreservation of shoot-tips of *Grevillea scapigera* (Proteaceae): a rare and endangered plant from Western Australia. Australian Journal of Botany. 40: 305 - 310.

Touchell D H, Turner S R, Senaratna T, Bunn E & Dixon K W 2002 Cryopreservation of Australian species – the role of plant growth

regulators. In: Towill L E & Bajaj Y P S (eds) Biotechnology in agriculture and forestry, vlo. 50. Cryopreservation of plant germplasm 11. Berlin: Springer Verlag; 373-390.

Towill L E & Bonnart R 2003 Cracking in a vitrification solution during cooling or warming does not affect growth of cryopreserved mint shoot tips. CryoLetters. 24 : 341-346.

Towill L E & Jarret R L 1992 Cryopreservation of sweet potato (*Ipomoea batatas* [L.] Lam.) shoot tips by vitrification. Plant Cell Reports. 11 : 175-178.

Towill L E 1984 CryoLetters. 5 : 319-326.

Towill L E 1990 Cryopreservation of isolated mint shoot tips by vitrification. Plant Cell Reports. 9 : 178-180.

Towill L E 1995 Cryopreservation by vitrification. p. 99-111 In B.W.W. Grout (ed.) Genetic preservation of plant cells *in vitro*. Springer-Verlag, New York.

Towill L E, Bonnart R & Volk G M 2006 Cryopreservation of *Arabidopsis thaliana* shoot tips. CryoLetters 27: 353-360.

Tsukazaki H, Mii M, Tokuhara M & Ishikawa K 2000 Cryopreservation of Doritaenopsis suspension culture by vitrification. Plant Cell Reports. 19: 1160-1164.

Tsuruta T, Ishimoto Y & Masuoka T 1998 Effects of glycerol on intracellular ice formation and dehydration of onion epidermis. Ann. NY Acad. Sci. 858: 217-226

Turner S R, Krauss S L, Bunn E, Senaratna T, Dixon K, Tan, B & Touchell D H 2001 Genetic fidelity and viability of *Anigozanthos viridis* following tissue culture, cold storage and cryopreservation. *Plant Science*. 161 : 1099-1106.

Turner S R, Senaratna T, Bunn E, Tan B, Dixon K W & Touchell D H 2001 Cryopreservation of shoot tips from six endangered Australian species using a modified vitrification protocol. *Annals of Botany.* 87 : 371-378.

Turner S R, Tan B, Senaratna T, Bunn E, Dixon K W & Touchell D H

2000 Cryopreservation of the Australian species *Macropidia fuliginosa* (Haemodoraceae) by vitrification. CryoLetters. 21 : 379-388.

Turner S, Krauss S L, Bunn E, Senaratna T, Dixon K, Tan B & Touchell D 2001 Genetic fidelity and viability of *Anigozanthos viridis* following tissue culture, cold storage and cryopreservation, Plant Sci. 161 : 1099–1106.

Uragami A, Lucas M O, Ralambosoa J, Renard M & Dereuddre J 1993 Cryopreservation of microspore embryos of oilseed rape (*Brassica napus L*.) by dehydration in air with or with out alginate encapsulation. CryoLetters. 14:83-90.

Uragami A, Sakai A, Nagai M & Takahashi T 1989 Survival of cultured cells o*fAsparagus officinalis* L cryopreserved by vitrification. Plant Cell Reports. 8 : 418-421.

Valladares S, Toribio M, Celestino C & Vietiez A 2004 Cryopreservation of embryogenic cultures from mature *Quercus suber* trees using vitrification. CryoLetters. 25 : 177-186.

Van Waes M, Debergh P C 1986 Adaptation of the tetrazolium method for testing the seed viability, and scanning electron microscopy study of some Western European orchids. Physiologia Plantarum 66 (3), 435–442.

Vandenbussche B & De Proft M P 1996 cryopreservation of in vitro sugar beet shoot-tips using the encapsulation-dehydration technique : development of a basic protocol. CryoLetters. 17 : 137-140.

Vandenbussche B, Demeulemeester M A C & De Proft M P 1993 Cryopreservation of alginate-coated in-vitro grown shoot-tips of chicory (*Cichorium-intybus*). CryoLetters. 14 : 259-266.

Vandenbussche B, Leuridan S, Verdoodt V, Gysemberg M, De Proft M 1999 Changes in sugar content and fatty acid composition of in vitro sugar beet shoots after cold acclimation: influence on survival after cryopreservation. Plant Growth Regulation. 28 : 157–163.

Vandenbussche B, Weyens G & De Proft M 2000 Cryopreservation of in

vitro sugar beet (Beta vulgaris L.) shoot tips by a vitrification technique. Plant Cell Reports. 19 : 1064-1068.

Venkatachalam L, Sreedhar R V & Bhagyalakshmi N, 2007 Molecular analysis of genetic stability in long-term micropropagated shoots of Banana using RAPD and ISSR markers. Electronic J. Biotechnol. *1*0 : 1–8.

Venugopal M N 2001 Viral diseases of cardamom. In. Ravindran, P. N. and Madhusoodanan, K. J. (eds.) Cardamom – The Queen of Spices. Harwood Academic Publishers (In Press).

Verleysen H, Fernandes P, Sanchez Pinto I, Van Bockstaele E & Debergh P 2005 Cryopreservation of *Robinia pseudoacacia*. Plant Cell Tiss. Org. Cult.81: 193-202

Vertucci C W & Stushno C V 1992 The state of water in acclimating vegetative buds of apple and *Amelanchier* and its relation- ship to winter hardiness.Physiol. Plant. 86: 503 –511.

Vidal N, Sanchez C, Jorquera L, Ballester A & Vieitez A M 2005 Cryopreservation of chestnut by vitrification of in vitro-grown shoot tips. In Vitro Cellular and Developmental Biology-Plant. 41 : 63-68.

Virk P S, Zhu J, Newbury H J & Bryan G J *et a*l 2000 Effectiveness of different classes of molecular markers for classifying and revealing variation in rice *(Oryza sativa)* germplasm. Euphytica. 112 : 275–284.

Volk G .M., Harris J L & Rotindo K .E 2006 Survival of mint shoot tips after exposure to cryoprotectant solutions components. Cryobiology. 52:305-308.

Volk G M & Richards K 2006 Preservation methods for Jerusalem artichoke cultivars. HortScience. 41: 80-83.

Volk G M & Walters C 2006 Plant vitrification solution 2 lowers water content and alters freezing behavior in shoot tips during cryoprotection Cryobiology. 52 : 48 –61.

Volk G M, Maness N & Rotindo K 2004 Cryopreservation of garlic (*Allium sativum* L.) using plant vitrification solution 2.

CryoLetters. 25 : 219-226.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Homes M, Frijters A, Pot J, Peleman J, Kuiper M & Zabeau M 1995 AFLP: a new technique for DNA fingerprinting. Nucleic Acid; Research. 23 : 4407-4414.

W u Y, Zhao Y, Engelmann F & Zhou M 2001 Cryopreservation of Kiwi shoot tips. CryoLetters.22:375-380.

Wang J H, Bian H W, Zhang Y X & Cheng H P 2001 The dual effect of antifreeze protein on cryopreservation of rice (*Oryza sativa* L.) embryogenic suspension cells. CryoLetters. 22 : 175–182.

Wang J F, Liu Y X & Lin S Q 2006 J Plant Resourc Environ. 15 : 75-76.

Wang J H, Bian H W, Zhang Y X & Cheng H P 2001 The dual effect of antifreeze protein on cryopreservation of rice (*oryza sativa* l.) embryogenic suspension cells. CryoLetters. 22 : 175-182.

Wang J H, Liu F, Huang C N, Yan Q S & Zhang X Q 1998 *Chinese Rice Research Newsletter.* 6 : 5-6.

Wang J H, Yan Q F & Huang C N 1996 Plant regeneration from barly (Hordeum vulgare) immature inflorescences cryopreserved by vitrification. Acta Botanica Sinica. 38 : 730-734.

Wang Q C, Batuman Ö, Li P, Bar-Joseph M & Gafny R 2002 A simple and efficient cryopreservation of in vitro-grown shoot tips of 'Troyer' citrange [Poncirus trifoliata(L)Raf × Citrus sinensis(L)Osbeck] by encapsulation-vitrification. Euphytica. 128 : 135-142.

Wang Q C, Laamanen J, Uosukainen M & Valkonen J P T 2005 Cryopreservation of in vitro-grown shoot tips of raspberry (Rubus idaeus L.) by encapsulation. Plant Cell Reports. 24 : 280-288.

Wang Q C, Li P, Batuman O, Gafny R & Mawassi M 2003 effect of benzyladenine on recovery of cryopreserved shoot tips of grapevine and citrus cultured *in vitro.* CryoLetters. 24 : 293-302.

Wang Q C, Mawassi M, Sahar N, Li P, Violeta C T, Gafny R, Sela I, Tanne

E & Perl A 2004 Cryopreservation of Grapevine (*Vitis* spp.) Embryogenic Cell Suspensions by Encapsulation–Vitrification. Plant Cell Tissue and Organ Culture. 77 : 267-275.

Wang Q, Tanne E, Arav A & Gafny R 2000 Cryopreservation of in vitro grown shoot tips of grapevine by encapsulation dehydration. Plant Cell Tiss. Organ Cult. 63: 41-46.

Wang Z C & Deng X X 2004 Cryopreservation of shoot-tips of citrus using vitrification: effect of reduced form of glutathione. CryoLetters. 25 : 43-50.

Ward A C W, Benson E E, Blackhall N W, Cooper–Bland S, Powell W, Power J B & Davey M R. 1993 Flow cytometric assessments of ploidy stability in cryopreserved dihaploid *Solanum tuberosum* and wild *Solanum* species. CryoLetters . 14: 145–152.

Watanabe K & Steponkus P L 1995 Vitrification of *Oryza sativa* L. cell suspensions CryoLetters. 16 : 255-262.

Watanabe K, Kuriyama A, Kawai F & Kanamori M 1999 Effect of cryoprotectant treatment and post–thaw washing on the survival of cultured rice (*Oryza sativa* L.) cells after cryopreservation. CryoLetters. 20 : 377–382.

Welsh N, Oberg C & Welsh M 1991 GTP-binding proteins may stimulate insulin biosynthesis in rat pancreatic islets by enhancing the signal-recognition-particle-dependent translocation of the insulin mRNA poly-/mono-some complex to the endoplasmic reticulum. Biochem. J. 275:23–28

Westman A L & Kresovich S 1997 in *Biotechnology and Plant Genetic Resources: Conservation and Use,* (eds) BV Ford-Lloyd, ill Newburry & JA Callow, CAB, UK. pp : 9-48.

Wilkes G 1983 CRC Crit. Rev. 1:131.

Wilkinson T, Wetten A & Fay M F 1998 Cryopreservation of cosmos atrosanguineus shoot tips by a modified encapsulation/dehydration method. CryoLetters. 19 : 293-302.

Wilkinson T, Wetten A, Prychid *C* & *Fay* M F 2003 Suitability of Cryopreservation for the Long-term Storage of Rare and Endangered Plant Species: a Case History for Cosmos

atrosanguineus..Annals of Botany. 91(1) : 65-74.

Williams J G K, Kubelik A R, Livak K J, Rafalski J A & Tingey S V 1990 DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18:6531-6535.

Williams MNV, Pande N, Nair M, Mohan M, Bennet J 1991 Restriction fragment length polymorphism analysis of polymerase chain reaction products amplified from mapped loci of rice (Oryza sativa L.) genomic DNA. Theor Appl Genet. 82: 489-498.

Withers L A 1980 Preservation of germplasm. Int. Rev. Cytol., S u ppl . II B: 101-136.

Withers L A 1985 Cryopreservation and storage of germplasm. In: Dixon, R.A. (Ed.), *Plant Cell Culture-a Practical Approach*, IRL Press, Oxford, U.K. pp : 169-19l.

Withers L A 1985a Cryopreservation of cultured cells and meristems., In *Cell culture and somatic cell genetics of plants.* Vol.2: Cell growth , nutrition, cytodifferentiation and cryopreservation. (I. K. Vasil Ed.). Academic Press, Orlando, Florida. pp : 253– 316.

Withers L A 1985b Cryopreservation and storage of germplasm.. *In*. Dixon, R.A. (ed.). *Plant Cell Culture – a Practical Approach*, IRL Press, Oxford. Pp : 169–191.

Withers L A & Engelmann F 1997 *In vitro* conserva-tion of plant genetic resources. In: Biotechnology in Agri-culture (Altman A., ed.). Marcel Dekker, NY. Pp : 57-88.

Withers L A 1985c Cryopreservation of cultured plant cells and protoplasts. In. *Cryopreservation of Plant Cells and Organs*. Kartha, K. K. (ed.) CRC Press, Boca Raton, Florida. pp : 243–267.

Withers L A. 1980 Tissue culture storage for genetic conservation. IBPGR Technical Report. International Board for Plant Genetic Resources, Rome. p. 91.

Withers LA 1979 Freeze preservation of somatic,embryos and clonal plantlets of carrot (*Daucus carota*). Plant Physiol. 63 : 460-467.

Wolf J & Bryant G 2001 Cellular cryobiology: thermodynamic and mechanical effects. Int. J. Refrig. 24: 438-450.

Wolfe A D & Liston A 1998 Contributions of the polymerase chain reaction to plant systematics and evolutionary biology. In: Soltis, D. E.., P. S.. Soltis and J. J. Doyle (eds.): Molecular Systematics of Plants II, Kluwer pp. 43-86.

Wood C B, Pritchard H W & Miller A P 2000 Simultaneous preservation of orchid seed and its fungal symbiont by encapsulation-dehydration is dependent on storage temperature and moisture content. CryoLetters 21: 125-136.

Wood P, Stedman-Edwards & Mang J 2000 editors *The root causes of biodiversity loss.* World Wildlife Fund and Earthscan Publications Ltd., London, UK. Conservation Ecology **5**(1): 12. [online] URL: http://www.consecol.org/vol5/iss1/art12/

Wu Y J, Engelmann F, Zhao Y, Zhou M & Chen S 1999 Cryopreservation of apple shoot tips: importance of cryopreservation technique and of conditioning of donor plants. CryoLetters. 20 : 121-130.

Wu Y J, Huang X L, Xiao J N, Li X J, Zhou M D & Engelmann F 2003 Cryopreservation of mango (*Mangifera indica* L.) embryogenic. cultures. CryoLetters. 24 : 303-314.

Wu Y J, Zhao Y, Engelmann F, Zhou M, Zang D & Chen S 2001 Cryopreservation of apple dormant buds and shoot tips. CryoLetters. 22 : 375-380.

Wu Y, Zhao Y, Engelmann F & Zhou M 2001 Cryopreservation of Kiwi shoot tips CryoLetters. 22 : 277-284.

Xu X B, Cai Z G, Gu Q Q & Zhang Q M 2006 Cell Ultrastructure of Kiwifruit (*Actinidia chinensis*) Shoot Tips During Cryopreservation. Agricultural Sciences in China. 5 : 587-590.

Xu X B, Gu Q Q, Cai Z G, Deng X M & Zhang Q M 2006 Cryopreservation of in Vitro Cultured Kiwifruit Shoot-tips by Vitrification and their Regeneration. Acta Horticluturae Sinica. 33 : 842-844.

Xue J P, Zhang A M, Liu J & Shi L 2003 Cryopreservation of *Rehmannia glutinosa* shoot-tips *I nvitro* grown by vitrification. *J Agricult Biotechnol.* 11 : 430-431.

Yamada T, Sakai A, Matsumura T & Higuchi S 1991 Cryopreservation of apical meristems of white clover(Trifolium repens L.) by vitrification. Plant Science. 78 : 81-87.

Yamada T, Sakai A, Matsumura T & Higuchi S 1993 Plant regeneration of meristematic callus of white clover (Trifolium repens L.) cooled to −96°C by vitrification. Euphytica. 70 : 197-203.

Yoshimalsu K, Touno K & Shimomura K 2000 in *Cryopreservation of Tropical Plant Germplasm: current research progress and application.* (eds) F Engelmann & H Takagi. ISBN 92-9043-428-7. JIRCAS Int Agric Ser No. *8.* Japan. pp : 77-88.

Yoshimatsu K, Yamaguchi H & Shimomura K 1996 Traits of Panax ginseng hairy roots after cold storage and cryopreservation. Plant Cell Reports. 15 : 555-560.

Zachariah T J & Luckose R. 1992 Aroma profile of selected germplasm accessions in cardamom (*Elettaria cardamomum* Maton). *J. Plantation Crops*, 20 (Suppl.). : 310–312.

Zachariah T J, Mulge R & Venugopal M N 1998 Quality of cardamom from different accessions. In *Developments in Plantation Crop Research*, Mathew, N. M. and Jacob, C. K. (eds.). Allied Publishers, India. pp : 337–340.

Zeng J W, Yi J, Zhang Q M, Zhou B R & Wu Y L 2005 Cell ultrastructure of. papaya shoot-tip during cryopreservation. Acta Horticulturae Sinica. 32 : 15-19.

Zhang Y J, Zhang X X & Pang J 2001 *Acta Agronomica Sinica.* 27 : 97-102.

Zhang Y Q, Li XX & Ma Q 2004 *China Vegetables.* 4 : 11-13.

Zhang Y Z & Luo Z R 2004 Cryopreservation of dormant shoot tips of non-astringent types of Chinese persimmon by encapsulation-vitrification and plantlets regeneration. Scientia Agricultura Sinica. 37 : 2019-2022.

Zhao M Z, Wang Z W, Su J L & Qian Y M 2006 *Acta Horticulturae.* 708 : 265-270.

Zhao Y, Wu Y, Engelmann F & Zhou M 2001 cryopreservation of axillary buds of grape (*Vitis vinifera*) *in vitro* plantlets CryoLetters. 22 :

321-328.

Zhao Y, Wu Y, Engelmann F, Zhou M, Zhang D & Chen S 1999 Cryopreservation of apple shoot tips by encapsulation-dehydration : Effect of preculture, dehydration and freezing procedure on shoot regeneration. CryoLetters. 20 : 103-108.

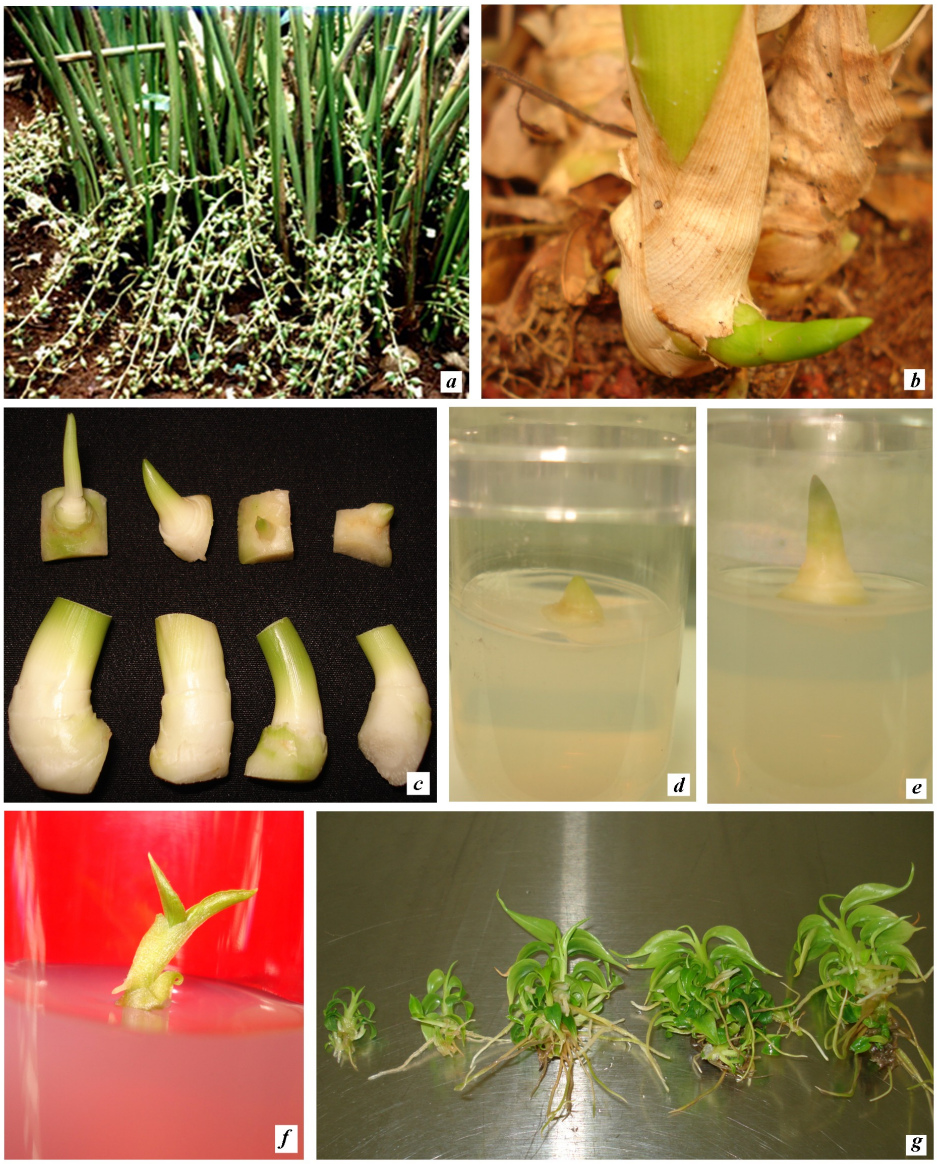

Fig 1. *In vitro* culture initiation in cardamom.
(a) Cardamom plant, (b) & (c) Buds used for culture initiation, (d) & (e) Culture initiation in MS + 0.5 mg/l Kin, (f) Growing plantlet, (g) Development of multiple shoots and roots

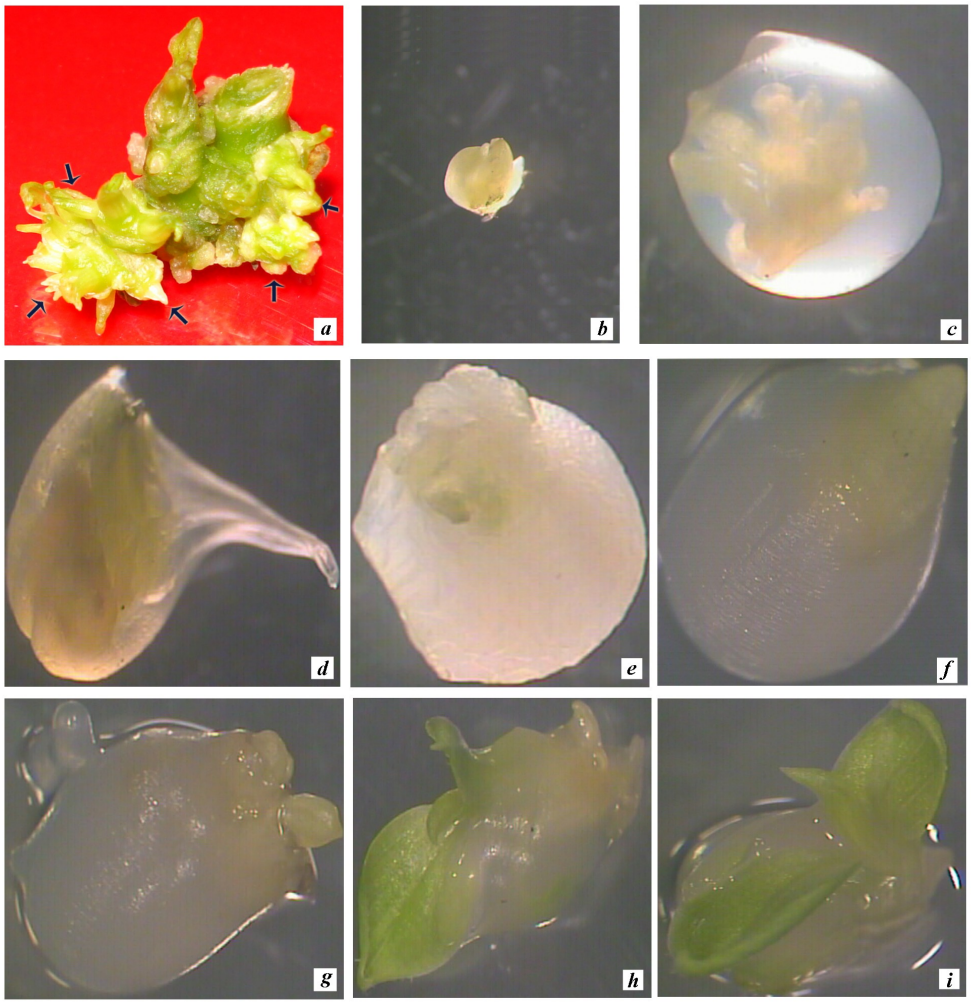

Fig 2. Plant regeneration from cryopreserved miniature shoots of cardamom by encapsulation dehydration.
(a) Group of multiplying cardamom cultures with miniature shoot buds (b) A typically excised cardamom miniature shoot used for cryopreservation, (c) Encapsulated cardamom shoot in 4% Na alginate, (d) Encapsulated and dehydrated shoot bud, (e), (f), (g), (h) & (i) Shoot development from dehydrated and cryopreserved miniature shoot buds of cardamom cooled in liquid nitrogen 7, 10, 14, 20 and 25 days after plating.

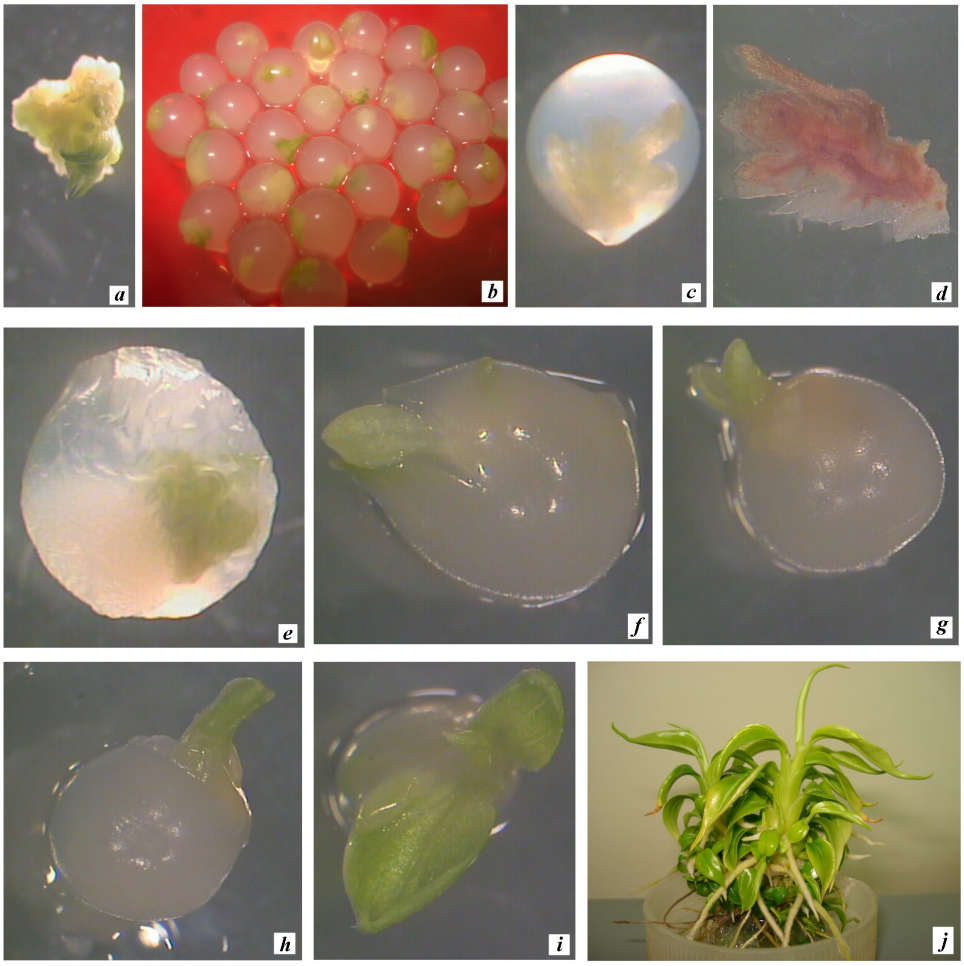

Fig 3. Plant regeneration from cryopreserved miniature shoots of cardamom by encapsulation vitrification.
(a) Cardamom miniature shoots used for cryopreservation, (b) & (c) Miniature shoots encapsulated in 4% Na alginate, (d) Viable tissues stained in TTC after liquid nitrogen storage (TTC staining), (e), (f), (g), (h) & (i) Shoot development from encapsulated and vitrified miniature shoots of cardamom cooled to -196°C, 2, 10, 12, 15 and 20 days after plating, (j) Development of multiple shoots and roots 90 days after plating.

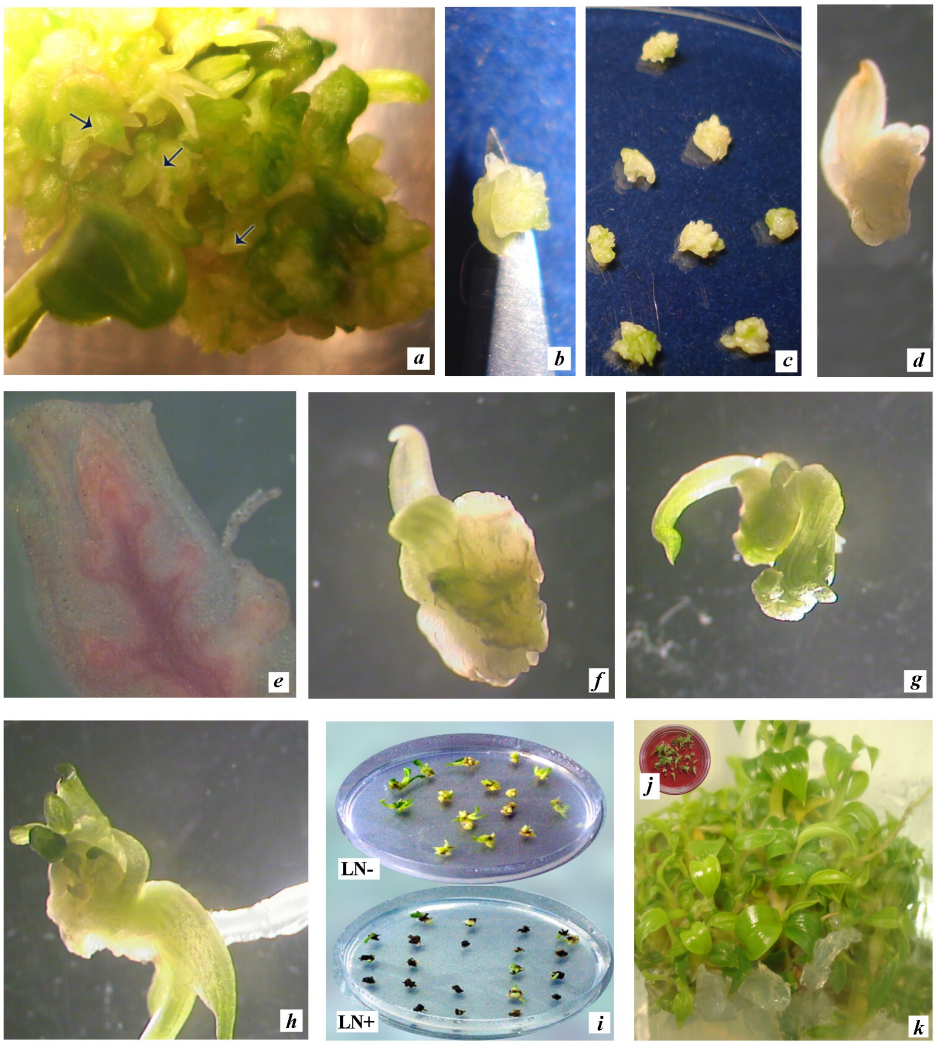

Fig 4. Plant regeneration from cryopreserved miniature shoots of cardamom by vitrification.
(a) Cardamom culture with miniature shoots, (b) & (c) Excised meristematic clumps used for cryopreservation, (d) Explant turned brown after cryopreservation, (e) Viable tissues stained in TTC after cryopreservation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

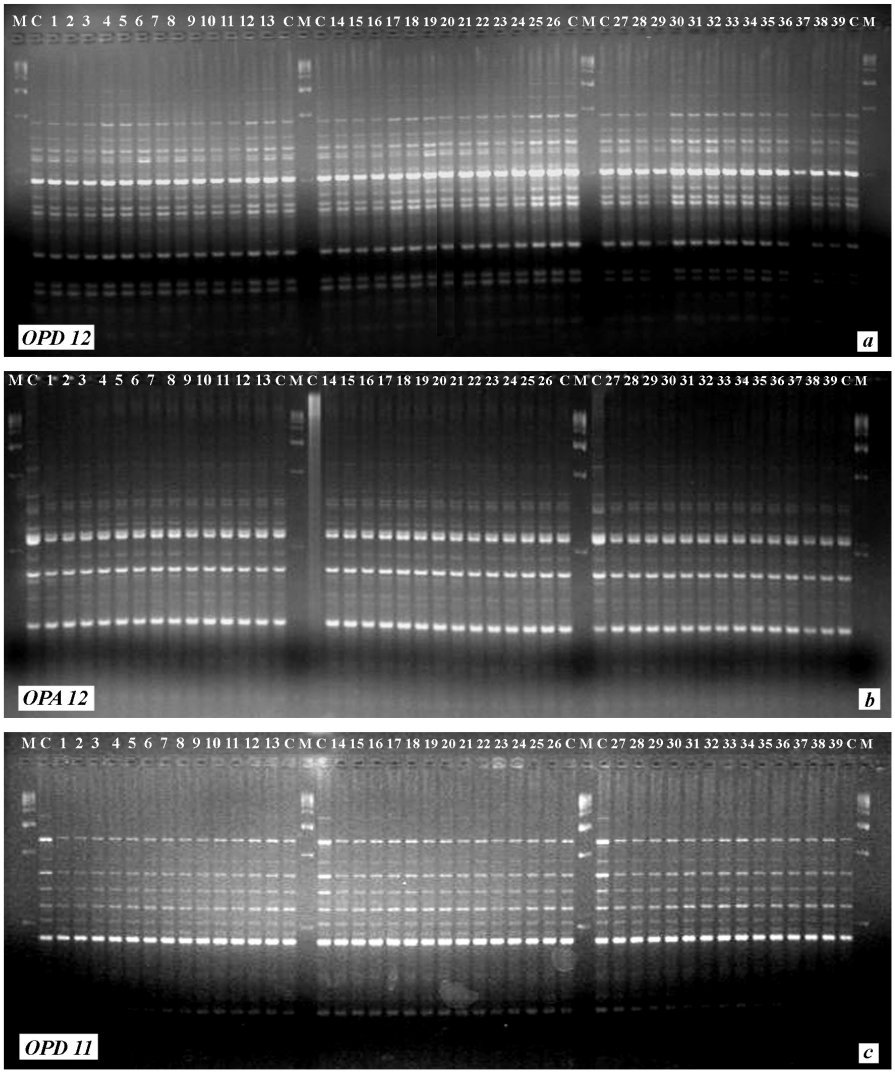

Fig 5. RAPD profiles generated for studying the genetic fidelity of cryopreserved shoots of cardamom.
RAPD profiles of randomly selected cardamom plants cryopreserved by (a) encapsulation dehydration , encapsulation vitrification and vitrification as amplified by OPERON primers OPD 12, OPA 12 and OPD 11. (M – 1 Kb ladder, C – Control plant, 1 – 13 ; 14-26 and 27-39 ; Cryopreserved lines)

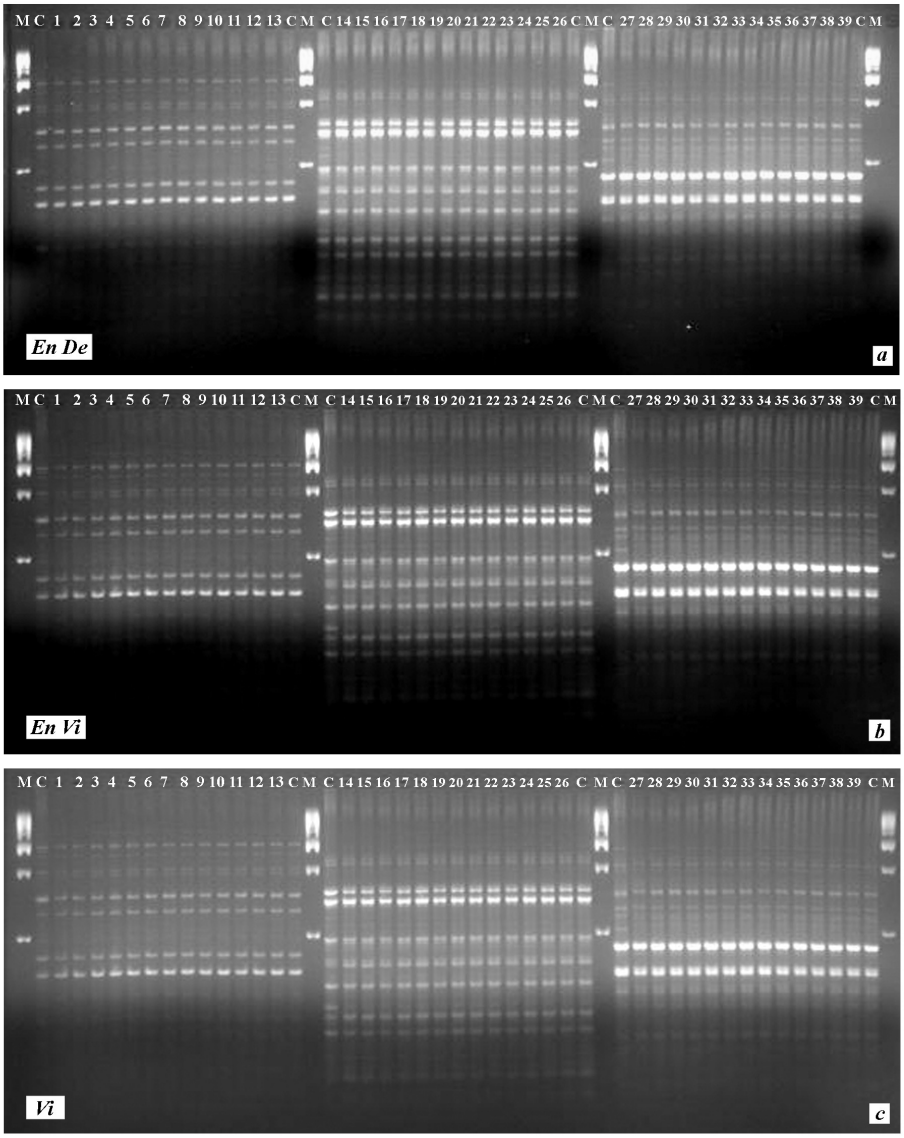

Fig 6. RAPD profiles generated for studying the genetic fidelity of cryopreserved shoots of cardamom. RAPD profiles of randomly selected cardamom plants cryopreserved by (a) encapsulation dehydration as amplified by OPERON primers OPD 13, OPE 18 and OPB 13, (b) encapsulation vitrification as amplified by OPERON primers OPD 13, OPE 18 and OPB 13, (c) vitrification as amplified by OPERON primers OPD 13, OPE 18 and OPB 13. (M – 1 Kb ladder, C – Control plant, 1 – 13 ; Cryopreserved lines)

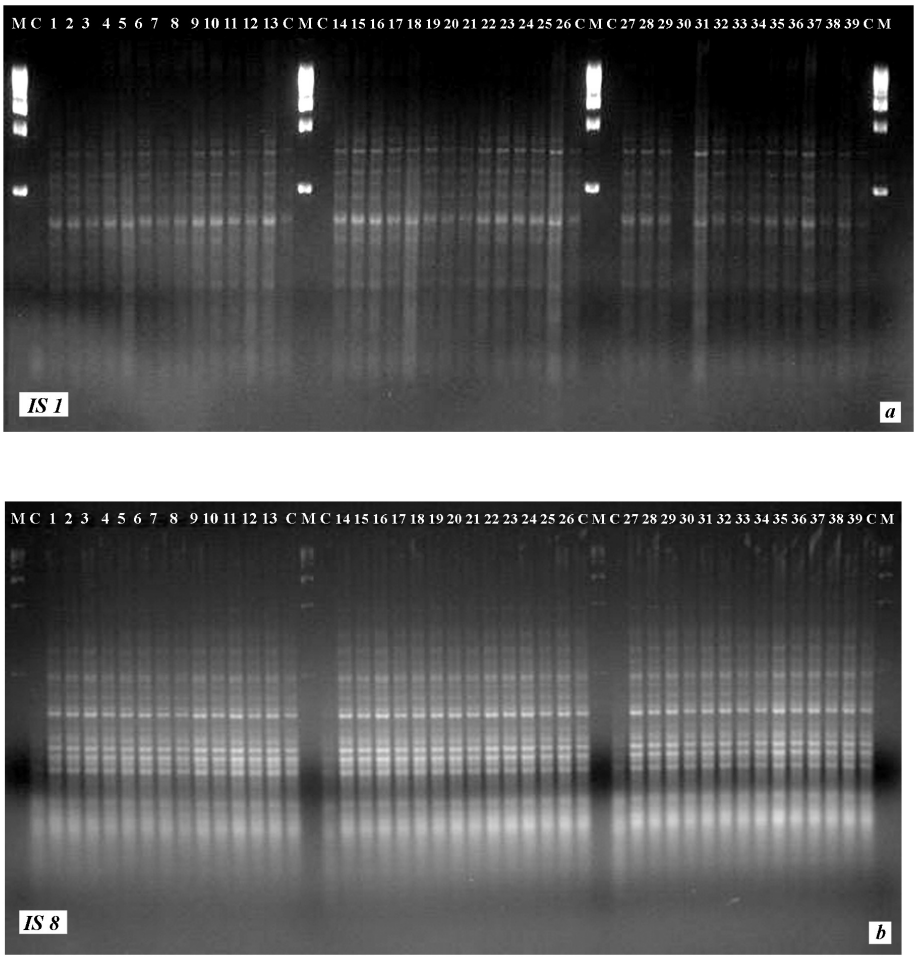

Fig 7. ISSR profiles generated for studying the genetic fidelity of cryopreserved shoots of cardamom.
ISSR profiles of randomly selected cardamom plants cryopreserved by encapsulation dehydration ,encapsulation vitrification and vitrification as amplified by ISSR primers IS 1 and IS 8.
(M – 1 Kb ladder, C – Control plant, 1 – 13, 14-26 and 27-39 ; Cryopreserved lines)

Fig 8. *In vitro* culture initiation in ginger (*Zingiber officinale* Rosc.)
(a) Ginger plant, (b) & (c) Vegetative bud as explant, (d) Sterilized buds in a petri plate,
(e) & (f) Culture initiation in MS + 0.5 mg/l Kin, (g) Development of multiple shoots and roots

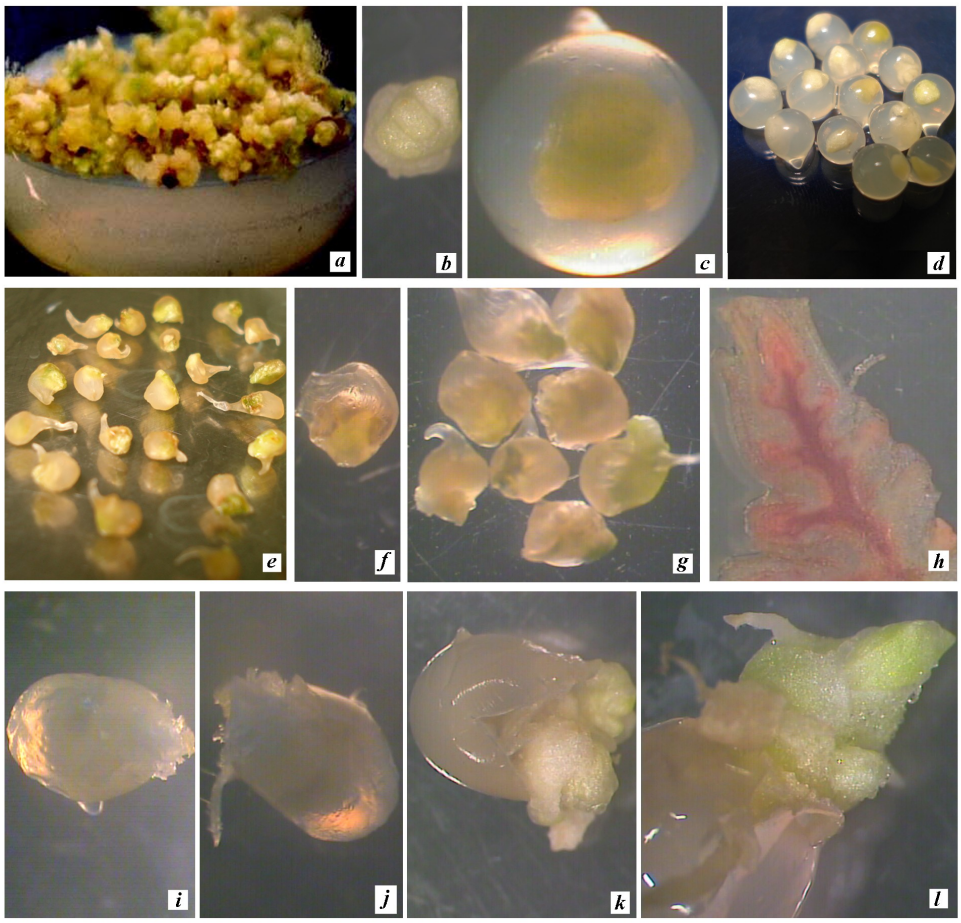

Fig 9. Plant regeneration from cryopreserved shoot buds of ginger by encapsulation dehydration.
(a) *In vitro* culture of ginger, (b) Shoot bud used for cryopreservation, (c) & (d) Shoot buds encapsulated in 4% Na alginate, (e), (f) & (g) Encapsulated shoot buds dehydrated in the laminar air flow, (h) Viable tissues stained in TTC after liquid nitrogen storage, (I), (j), (k) & (l) Shoot development from dehydrated and cryopreserved shoot buds of ginger 7, 10, 20 and 30 days after plating.

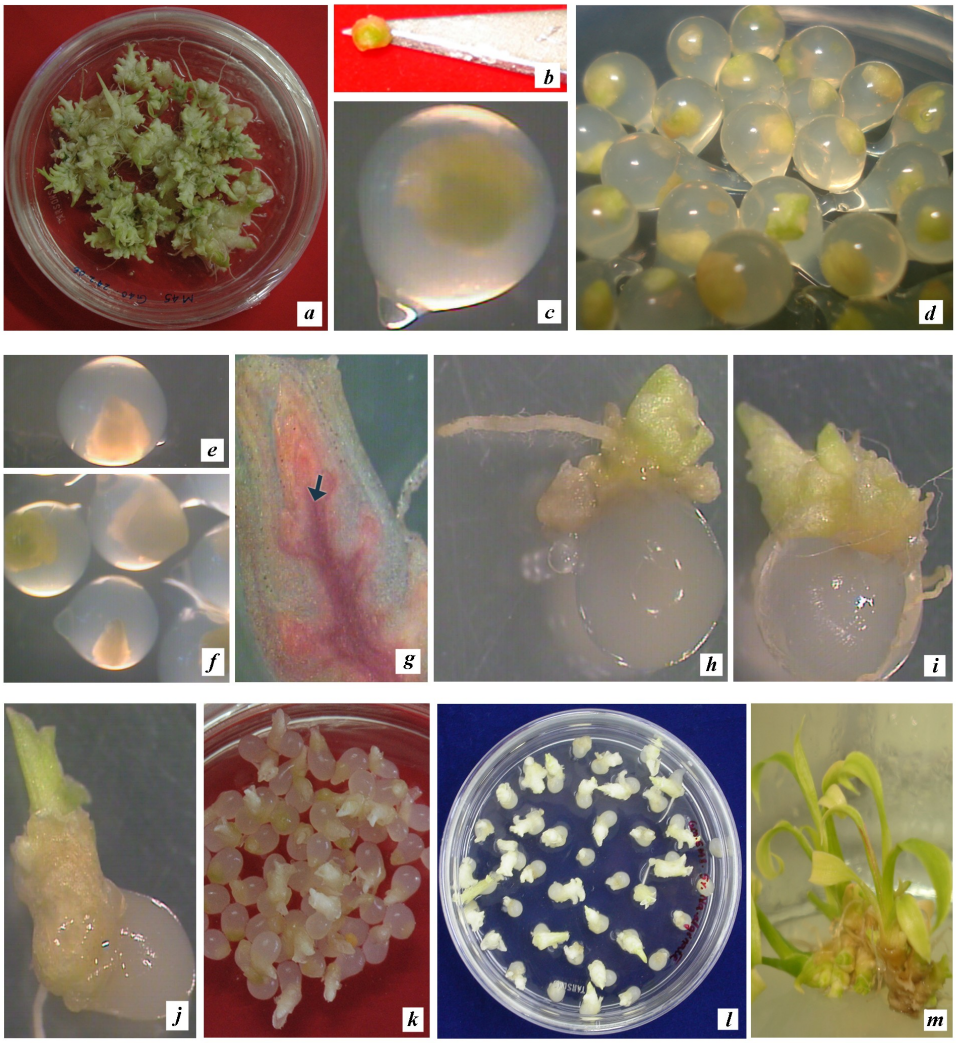

Fig 10. Plant regeneration from cryopreserved shoot buds of ginger by encapsulation vitrification.
(a) *In vitro* culture, (b) A typically excised shoot bud used for cryopreservation, (c) & (d) shoot buds encapsulated in Na -alginate, (e) & (f) shoot buds turned brown after thawing, (g) Viable apical 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 regenerated from cryopreserved shoot bud.

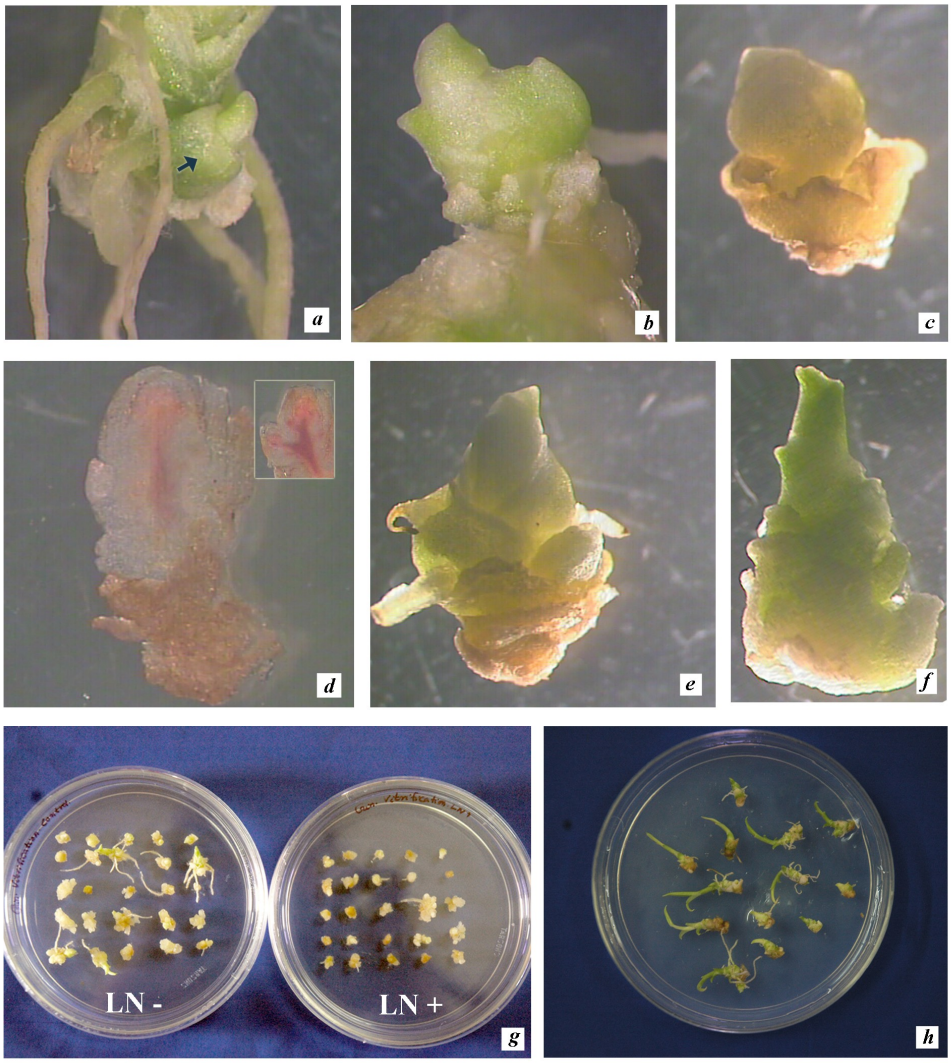

Fig 11. Plant regeneration from cryopreserved shoot buds of ginger genotype Maran by vitrification
(a) & (b) Shoot buds used for cryopreservation, (c) Shoot bud turned brown after thawing (d) Viability of shoot bud after cryopreservation (TTC staining) (e) regenerating bud 14 days after post culturing, (f, g) One month after post culturing (h) 2 months after post culturing.

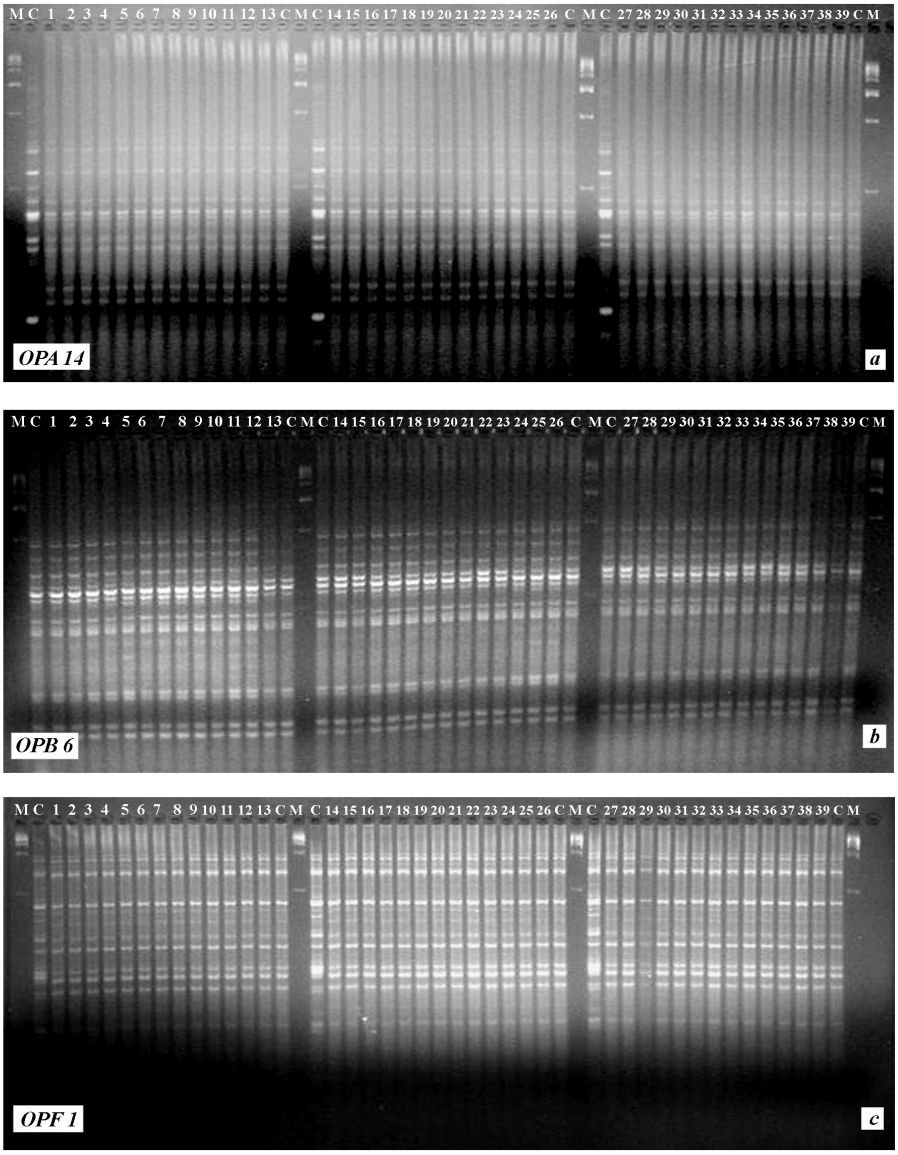

Fig 12. RAPD profiles generated for studying the genetic fidelity of cryopreserved shoot buds of ginger
RAPD profiles of randomly selected ginger plants cryopreserved by (a) encapsulation dehydration , encapsulation vitrification and vitrification as amplified by OPERON primers OPA 14, OPB 06 and OPF 01. (M – 1 Kb ladder, C – Control plant, 1 – 13 ; 14-26 and 27-39 ; Cryopreserved lines)

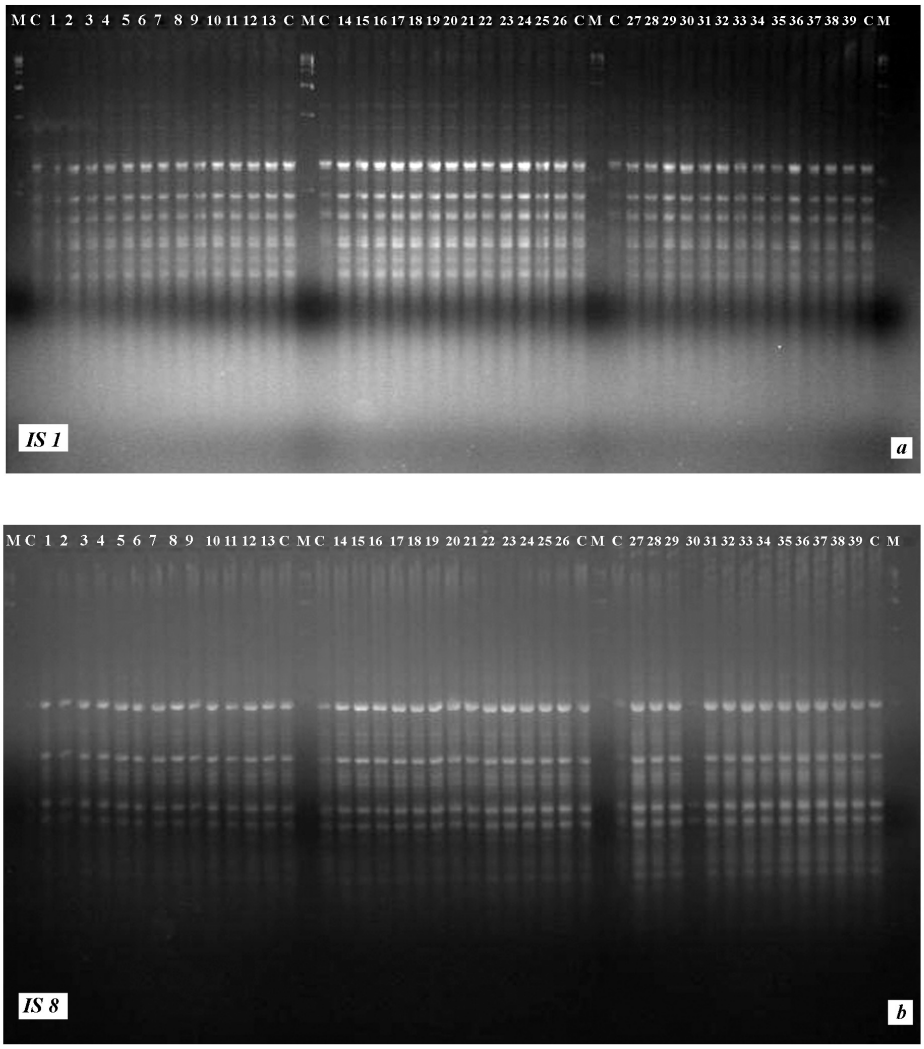

Fig 14. ISSR profiles generated for studying the genetic fidelity of cryopreserved shoot buds of ginger.
ISSR profiles of randomly selected ginger plants cryopreserved by encapsulation dehydration , encapsulation vitrification and vitrification as amplified by ISSR IS 1 and IS 8.
(M – 1 Kb ladder, C – Control plant, 1 – 13, 14-26 and 27-39 ; Cryopreserved lines)

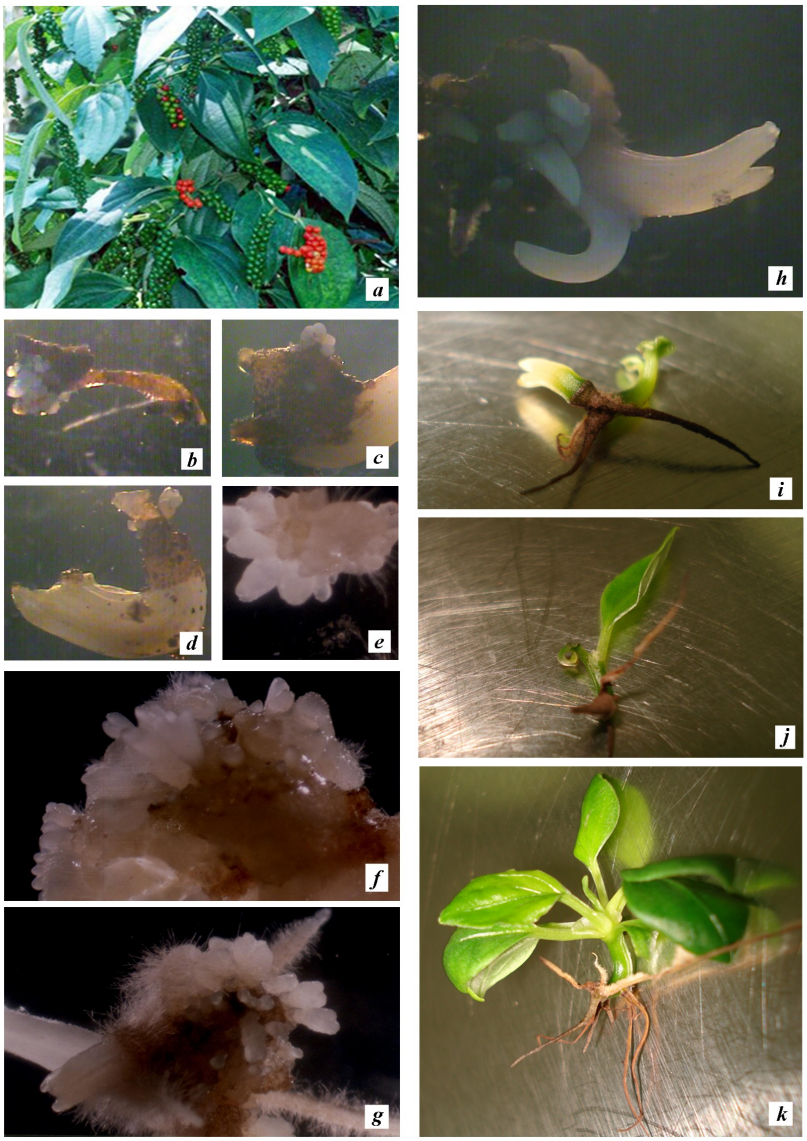

Fig 15. Somatic embryogenesis in black pepper.
(a) Black pepper plant, (b) Somatic embryos of black pepper directly formed on the surface of stressed zygotic embryos, (c) & (d) Secondary somatic embryos originating from the basal part of a primary embryo, (e), (f) & (g) Embryos appearing in clusters, (h) Somatic embryos germinated and developed into multiple buds and shoots, (i) Two somatic embryos have developed and greened soon after transferring to the light, (j) & (k) Fully developed plantlet from a somatic embryo

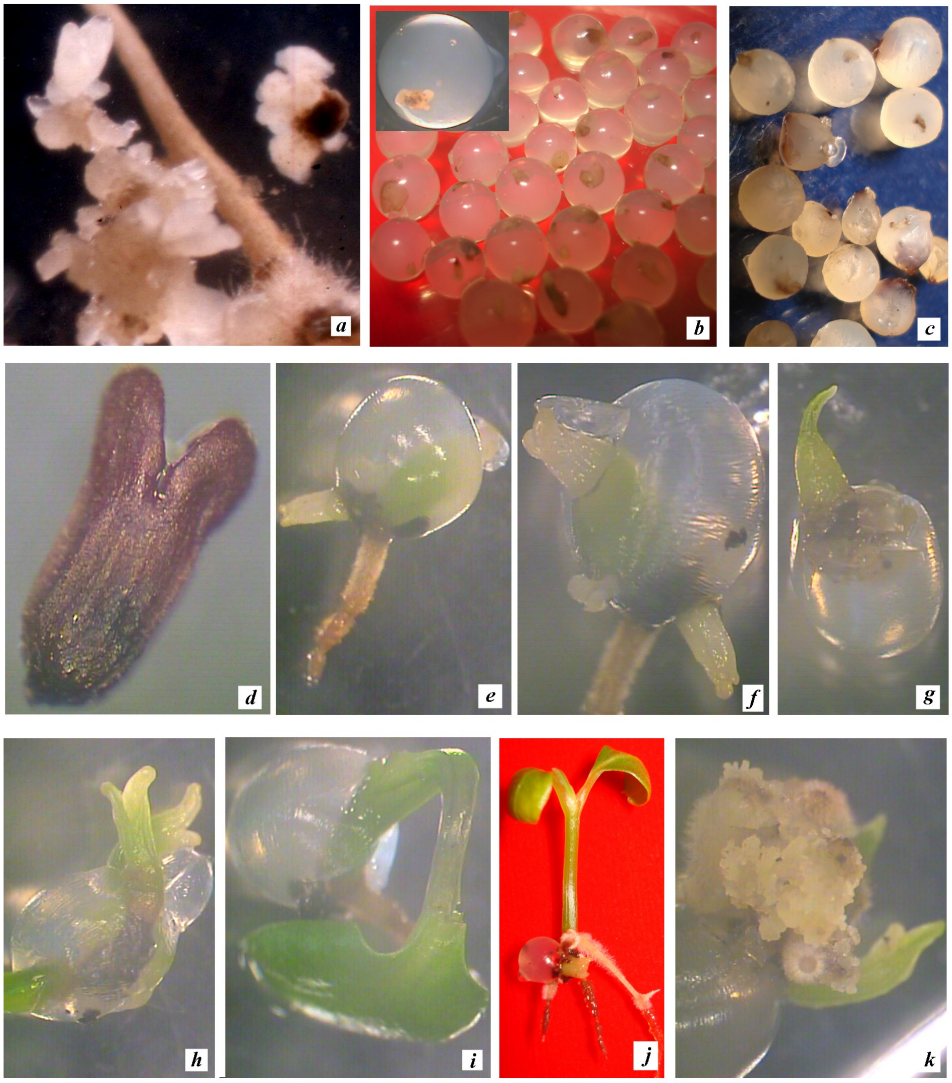

Fig 16. Cryopreservation of black pepper somatic embryos by encapsulation dehydration. (a) Somatic embryos used for cryopreservation, (b) Somatic embryos encapsulated in Na alginate, (c) Encapsulated and dehydrated somatic embryos, (d) Viable somatic embryo stained in red colour after cryopreservation, (e), (f), (g), (h) & (i) Various stages of development of somatic embyos to plantlet after cryopreservation, (j) Fully developed plantlet from a somatic embyo cryopreserved by encapsulation dehydration, (k) A cluster of somatic embryos at different stages of development, originated from an embryogenic line after cryopreservation.