Int. J. Indust. Entomol. Vol. 15, No. 1, 2007, pp. 23~33

International Journal of *Industrial Entomology*

Cryopreservation of Mulberry Germplasm Core Collection and Assessment of Genetic Stability through ISSR Markers

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(Received June 10 2007; Accepted August 3 2007)

A simple and reliable cryo technique using desiccation and slow freezing of winter dormant buds was employed for 238 core collection of mulberry germplasm collected from diverse geographical regions and maintained under tropical conditions in the *ex situ* field gene bank to develop long-term biodiversity conservation for ensuring sustainable utilization of these valuable resources. Desiccation and freezing tolerance of bud grafts and excised shoot apices in the axillary buds of different *Morus* species under *in vivo* and *in vitro* condition indicated species-specific variation and most of the wild *Morus* species were found sensitive. *In vitro* regeneration and cryopreservation (-196°C) protocols using differentiated bud meristem like axillary winter dormant buds were worked out for a wide range of *Morus* species, landraces, wild and cultivated varieties. Successful cryopreservation of mulberry winter dormant buds of different accessions belonging to *M. indica, M. alba, M. latifolia, M. cathayana, M. laevigata, M. nigra, M. australis, M. bombycis, M. sinensis, M multicaulis and M. rotundiloba* was achieved. Among wild species *Morus tiliaefolia*, and *M. serrata* showed moderate recovery after cryopreservation. Survival rates did not alter after three years of cryopreservation of different *Morus* species. ISSR markers were used to ascertain the genetic stability of cryopreserved mulberry, which showed no difference detected among the plantlets regenerated from frozen apices in comparison to the non-frozen material.

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Key words: Mulberry, Core collection, Cryopreservation, *in vitro*, Desiccation, Slow freezing.

Introduction

The resilience of *Morus* biodiversity is greatly threatened in recent times and significant impetus has been given to develop long-term biodiversity conservation programme for ensuring sustainable utilization of these valuable resources mostly after the adoption of Global Action Plan (GAP) by Food and Agriculture Organization (FAO, 1996). Fourth International Technical Conference on Plant Genetic Resources of FAO held in Leipzig, Germany in 1996 laid great stress on conservation of agri biodiversity and especially the endangered species. Presently there are about 1300 gene banks on agro-biodiversity, which are operating worldwide maintaining about 6 million Plant Genetic Resource (PGR) samples (FAO, 1998). Mulberry (*Morus* species) is out-breeding and highly heterozygous perennial tree and its high biomass production and protein rich foliage are used extensively for sericultural, agro-forestry and horticulture programmes. Among the sericultural countries, China is holding largest mulberry germplasm collections (2600 accessions) followed by Japan (1375), India (1053), Korea (615) and Bulgaria (140) in the *ex situ* field gene bank (Ananda Rao, 2003). Central Sericultural Germplasm Resources Centre (CSGRC) is a National Active Germplasm Site (NAGS) for mulberry in India, and presently conserves 1100 diverse mulberry germplasm accessions representing 13 *Morus* species collected from 26 countries in the *ex-situ* clonal field gene bank. Space and operational constraints impose limitations on the amount of mulberry germplasm material that can be conserved in the *ex-situ* field gene bank repository. Wide genetic variability exists in wild

and cultivated mulberry germplasm, which needs a well-planned efficient and cost effective long-term conservation strategy. Long-term conservation of recalcitrant seed, high desiccation and freezing sensitive and vegetatively propagated cold tender plant species is the priority areas of the conservation activities to safeguard clonal gene banks in the tropics (Engelmann, 1997). *In vitro* culture and cryogenic technology using liquid nitrogen (– 196°C) have come up in a great way in recent times and become a powerful biotechnological tool for conservation and utilization of plant genetic resources (Niino, *et al.*, 1995; Razdan and Cooking, 2000; Engelmann, 2000; Bisht and Dhillon, 2003 and Chaudhury & Malik, 2004). The National gene bank at NBPGR, New Delhi presently conserves a total of 1783 accessions (*in vitro* conservation) and 7265 accessions (cryopreservation) of diverse plant germplasm (Anonymous, 2006). Hence, the present study was undertaken to cryopreserve the diverse mulberry germplasm resources using dehydration and slow freezing cryo-technique and its fast regeneration under *in vitro* condition and assessing the genetic fidelity of the cryopreserved germplasm with ISSR markers.

Materials and Methods

Development of core collection of mulberry

The mulberry *ex situ* field gene bank at CSGRC, Hosur presently conserve 1100 mulberry accessions (Indigenous-836 and Exotic-264), which served as the source of explants for conservation through biotechnological approaches. These accessions are maintained as high bush with crown height of 1.5 m and spacing of 2.4 × 2.4 m between plants and rows with recommended cultural practices (latitude 12"45°N, longitude 77"51°E, altitude 942 MSL, temperature 13 - 32°C, humidity 36 - 86% and rainfall 1184 mm). For cryopreservation studies, 778 accessions, which are well characterized and evaluated (Ananda Rao, 2004), were analyzed and the core collection of 250 mulberry accessions representing maximum variability was selected (Ananda Rao *et al., 2005)*. The core collection was worked out by considering 15 yield attributes and 16 morpho-reproductive standard descriptors using principal components score strategy (Bisht *et al.,* 1998, 2003).

Dehydration and freezing tolerance and cryopreservation of different mulberry species

Desiccation tolerance was determined for different *Morus* species *in vivo and vitro* conditions. The bud grafts of different mulberry species were collected from the fresh vegetative stem cutting from the matured trees of field gene bank. The axillary buds along with the 5 mm mother tissue were dissected from the stem cuttings and placed in the desiccators (150 mm I.D) filled with activated 500 g activated silica gel. The desiccation hours ranged from 1 - 6 hours under the room temperature at 27°C. After each desiccation period the buds were grafted to the local stock material and planted in the pots kept in the glass house. The survival of the grafts under *in vivo* condition was recorded up to 45 days. Moisture content of the bud grafts was recorded after each desiccation period by low constant temperature oven method by drying at 103°C ± 2°C temperature in an oven for 17 h. The viability of the desiccated buds was also determined under *in vitro* condition.

In order to test the cold hardiness and storage potentiality of different mulberry germplasm resources, the one year old vegetative stem cuttings of different mulberry accessions colleted from ex situ field gene bank were maintained at – 1.5°C in the cold storage for three months. At monthly intervals the stored stem cuttings were retrieved from cold storage and the vegetative bud grafts (scions) along with small portion of the mother tissue were harvested and grafted to the fresh local stock and planted in the nursery. The survival of bud grafts (sprouting) was recorded after 45 days of plantation and the freezing tolerance was determined.

Cryopreservation of dormant winter buds by dehydration and slow freezing

Axillary winter dormant buds were collected from one year old lateral shoots of matured trees in the field gene bank at CSGRC, Hosur in the month of January-February and cryopreserved at super low temperatures in liquid nitrogen (– 196°C) using dehydration in silica gel and slow freezing (Niino *et al.*, 1995). The axillary buds were harvested and 5 - 7 scales were removed. The dehydration period of 4 - 6 hours in silica gel (500 g/150 mm I.D desiccators) was determined by considering initial moisture levels of the dormant buds using moisture analyzer (Mettler-LJ-16, Switzerland). The slow freezing was achieved by storing the buds sequentially lowering temperature – 5°C/ day up to the terminal temperature of – 30°C and placed in 2.5 ml polypropylene cryo-vials and stored in the cryocan (Air Liquide, France) containing liquid nitrogen (– 196°C).

In vitro regeneration of cryopreserved germplasm

For recovery, the cryopreserved axillary buds were thawed rapidly under 38°C in water bath for 5 minutes. Rehydration treatment of 24 hours was given by keeping the buds in sterile filter papers in the desiccators filled with sterile soil-rite mixture. The rehydrated buds were surface sterilized with liquid detergent Tween-20, disinfected with 70% ethanol for 25 seconds followed with 0.1

% mercuric chloride for 6 to 8 minutes, rinsed with repeated three sterile water washes, and placed in culture tubes (25 × 150 mm, Borosil make, fitted with polypropylene caps) with MS (Murashige and Skoog, 1962) medium with 3% (W/V) sucrose and gelled with 0.7% (W/V) agar (Hi-Media Laboratories Pvt. Ltd, Mumbai, India). The medium was supplemented with 1.0 mg l^{-1} BAP, 0.2 mg l^{-1} GA_3 and 0.2 mg l^{-1} NAA (Sigma, USA). The pH of the medium was adjusted to 5.8 prior to autoclaving. Initially the cultures were kept under dark for 4 days and then transferred to normal growing conditions at 25° ± 1°C with 55 to 60% relative humidity and light intensity of 3000 lux with 16 hr photoperiod *in vitro* culture room. After emergence of 3-5 young leaves they were sub cultured. The sprouting of axillary buds and percentage of shoot formation was recorded after 30 days for each treatment. For shoot elongation, the *in vitro* sprouted buds were sub cultured after 30 days of inoculation to the fresh MS medium supplemented with cytokinins and auxins used for bud sprouting. The accessions, which have high phenolic exudation, were repeatedly sub cultured in the medium supplemented with activated charcoal 400-500 mg l^{-1}. The shoot length was recorded after 40 days. The micro shoots were transplanted to half strength MS medium supplemented with IBA 0.5 mg l^{-1} and activated charcoal (AC) 100 mg l^{-1} and incubated at 25° ± 1°C with 55 to 60% relative humidity and light intensity of 3000 lux. The rooting parameters were recorded after 25 days of initiation of roots for each variety separately. The *in vitro* developed rooted plants were successfully hardened in the plastic pots using autoclaved soil-rite mixture covered with polyethylene bags and kept in the poly house with 75% relative humidity. The plants were watered once in two days with half strength MS liquid media. The polyethylene bags were punctured after 25 days and completely removed after 40 days.

Genetic stability studies of cryopreserved germplasm through ISSR markers

Total genomic DNA was isolated from fresh young leaves from the *ex situ* field gene bank and *in vitro* regenerated cryo samples after hardening of mulberry accessions using CTAB (Cetyl trimethyl ammonium bromide) protocol of Murray and Thompson, 1980: Vijayan, 2006). About 500 mg of the leaf lamina was grounded in liquid nitrogen using pre cooled mortar and pestle. The leaf powder thus obtained was transferred to 50 ml polypropylene tube and 5 ml of extraction buffer containing 1% CTAB, 1.5% PVP (poly vinyl pyrrolidone), 1.4 M NaCl, 50 mM EDTA, 50 mM Tris HCl, 0.1% b-mercaptoethanol. The mixture was incubated at 65°C for 30 min with 1.5 ml of 15% SDS, followed by phenol-chloroform extraction method. After ethanol precipitation, DNA was resuspended in TE buffer (10 mM Tris HCl (pH 8.0) and 1 mM EDTA (pH 8.0) and incubated for 30 min at 37°C with 100 mg DNAse free RNAse A. DNA was further precipitated following the steps of phenol-chloroform-isoamyl alcohol (24 : 24 : 1), chloroform and precipitation with ethanol in the presence of 3 M sodium acetate. Quantification of the DNA was done by electrophoresis on 0.8% agarose gel (1 × TBE) stained with ethidium bromide (0.5 μg/ml) and using λ DNA (10 ng/μl) as standard.

Ten ISSR primers viz. UBC-807, 808, 810, 811, 812, 825, 827, 841, 855 and 864 (University of British Colombia, Canada) which have given polymorphism were selected (Vijayan and Chaterjee, 2003; Srivastava, *et al.*, 2004; Vijayan, 2004 and Vijayan *et al.*, 2006). The PCR amplification of the total genomic DNA was carried out on a PTC 200 MJ Research Thermal Cycler (MJ Research Inc. Waltham, Mass) as described by Vijayan *et al.*, 2006. The PCR product was resolved on 2.0% agarose gel in TBE (89 mM Tris HCl, 89 mM Borate, 2 mM EDTA) buffer at a constant current of 200 mA for approximately 4 hrs, and visualization with ethidium bromide (0.5% μg/ml) under UV transilluminator. Photographs were taken with Kodak DC 4800 zoom digital camera using Kodak 400 ASA film. Scoring of bands was on the basis of presence (1) or absence (0) of a particular band and those bands, which were prominent and reproducible, were selected for the analysis. Statistical analysis Pair-wise genetic similarity coefficients (GS) for each pair of the accessions were calculated following the methods of Nei and Li (1979), Clustering analysis was carried out with dissimilarity matrix using un-weighted pair group method using arithmetic average (UPGMA) and a clustering program, PHYLIP 3.5c software program (Felsenstein, 1993).

Results and Discussion

The genetic diversity of core set of mulberry used *in vitro* conservation and cryopreservation is presented in Table 1. The core set represents about 32.13% of whole collection of 778 accessions. The data indicate significant differences for most of the quantitative variables. The high coefficient of variation for different quantitative characters indicated skewed distribution on both positive and negative direction in the core collection than whole collection with wide diversity. A good core set needs to define genetic diversity more precisely and it is envisaged that core collection receives priority for germplasm management system (Marshal, 1989).

The desiccation tolerance of different mulberry acces-

Table 1. Genetic variability of the growth attributing parameters in the core collection

Parameters	Lowest	Highest	Kurtosis	Skewness	Range	Mean	Std. Dev.	Std. Error	C.V
Indigenous accessions (147 acc) (cultivated species- *M. indica and M.alba*)									
NBR	2.17	80.67	2.2239*	1.1290*	78.50	22.71	12.95	1.07	57.01
TSL	190.67	10143.00	1.8691*	1.2103*	9952.33	2512.05	1781.15	146.91	70.90
MRC	20.38	85.83	−0.40	−0.6579*	65.45	59.18	14.42	1.19	24.36
LT	83.62	220.21	0.04	0.02	136.59	162.41	25.85	2.13	15.92
HLW	53.33	1059.76	0.33	0.9405*	1006.43	341.14	219.49	18.10	64.34
LLS	52.17	248.83	−0.31	−0.20	196.66	157.17	41.20	3.40	26.21
IND	2.68	8.02	−0.33	0.15	5.34	4.98	1.09	0.09	21.87
LSR	0.32	3.51	5.4341*	2.0114*	3.19	1.31	0.50	0.04	38.11
LYD	0.18	5.08	0.03	0.9211*	4.90	1.63	1.21	0.10	74.60
TBIO	0.26	10.43	0.27	0.9909*	10.17	3.12	2.43	0.20	77.93
Indigenous accessions (33 acc) (Wild species- *M.serrata and M.laevigata*)									
NBR	5.17	53.67	11.5004*	2.8250*	48.50	15.31	8.68	1.51	56.72
TSL	197.50	6984.00	5.7289*	2.1038*	6786.50	1719.17	1387.17	241.48	80.69
MRC	48.99	83.62	−0.52	−0.01	34.63	67.38	8.82	1.54	13.09
LT	138.98	340.89	2.0258*	0.9732*	201.91	206.20	42.50	7.40	20.61
HLW	246.12	1455.10	−0.69	0.17	1208.98	811.39	309.51	53.88	38.15
LLS	57.50	230.33	−0.37	−0.02	172.83	145.37	40.17	6.99	27.63
IND	3.97	10.64	−0.23	0.10	6.67	6.89	1.58	0.28	22.96
LSR	0.88	2.19	3.6193*	1.4736*	1.31	1.28	0.26	0.05	20.28
LYD	0.18	4.20	0.01	0.8429*	4.02	1.62	1.04	0.18	64.21
TBIO	0.39	7.33	-0.03	0.8795*	6.94	2.91	1.88	0.33	64.73
Exotic accessions (70 acc)									
NBR	2.00	66.67	1.3844*	1.4070*	64.67	17.46	15.74	1.88	90.16
TSL	42.33	8203.00	1.4992*	1.4532*	8160.67	1795.10	1884.53	225.24	104.98
MRC	26.80	88.56	4.3524*	−1.1801*	61.76	68.63	9.54	1.14	13.90
LT	99.71	283.52	0.46	0.48	183.81	175.27	35.12	4.20	20.04
HLW	144.89	1311.32	1.2028*	1.1469*	1166.43	501.23	260.55	31.14	51.98
LLS	23.67	242.67	−0.33	0.00	219.00	130.75	49.63	5.93	37.96
IND	2.51	7.45	−0.64	0.15	4.94	4.71	1.20	0.14	25.47
LSR	0.91	5.93	1.7554*	1.6168*	5.02	2.03	1.28	0.15	63.20
LYD	0.10	7.08	5.4419*	2.0738*	6.98	1.46	1.41	0.17	96.60
TBIO	0.15	12.70	5.2254*	2.0386*	12.55	2.57	2.59	0.31	100.85

NBR-number of branches, TSL-total shoot length (cm), MRC-moisture retention capacity (%), LT-leaf thickness (μm), HLW-hundred leaf wt (gm), Length of longest shoot (cm), Internodal distance(cm), LSR-leaf shoot ratio, LYD-leaf yield (kg), TBIO-(total biomass (kg)

sions was tested both under *in vitro* and *in vivo*. The genotypic response was observed for desiccation tolerance of different mulberry species. Viability of all the species declined with desiccation (Fig. 1). With increasing desiccation hours there was a steady fall in moisture content accompanied with fall in viability. Higher desiccation rates were obtained in case of *M. indica, M. alba, M. bombycis* and *M.latifolia* compared to the wild species of *M. laevigata* and *M. serrata*. The desiccation rates are highly correlated to the size of the dormant bud (Tyler and Stushnoff, 1998). At moisture content below 20%, survival of 30% and 53% was achieved in *M. indica* and *M. alba in vitro*. Bud grafts of *M. laevigata* showed a drastic decline in viability below 40% moisture content. *M. serrata* grafts did not survive *in vivo* after desiccation. The accessions belong to *M.latifolia* sustained higher desiccation where 73% survival was recorded after 6 hours of desiccation with moisture content of 10%. The wild species *M.nigra*,

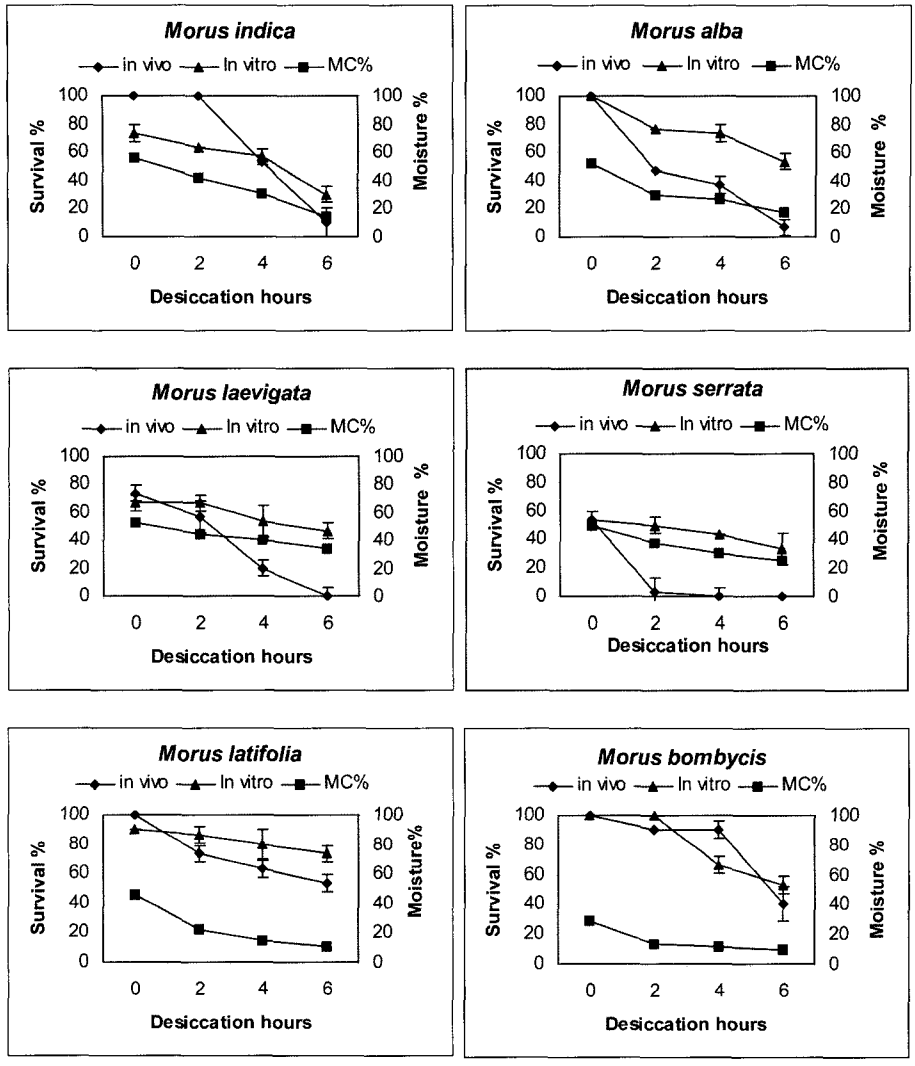

Fig. 1. Dehydration tolerance of different *Morus* species under *in vitro* and *in vivo* condition.

M. laevigata and *M. serrata, M. tiliaefolia are* cold sensitive and showed lesser freezing tolerance, which could not be stored beyond three months (Fig. 2). These accessions have showed moderate recovery rates after cryo exposure. The degree of freezing tolerance of different mulberry accessions supported the relationship of cold hardiness and cryopreservability in general with some exceptions as in the case of Kalimpong local and *M.nigra* which did not survive three month cold exposure (Fig. 2) but showed higher survival (93% and 63 % survival) after cryo exposure as in the case of apple germplasm where poor survival was recorded in some cold hardy germplasm (Forsline *et al.*, 1998). Increased carbohydrate levels are often correlated to cold hardiness and high survival rates after cryopreservation (Seufferheld *et al.*, 1999). The regeneration after cryopreservation was tested for different mulberry accessions (Table 2). Direct organogenesis was observed *in vitro* from dormant winter buds obviating intermediate callus phase ensuring production of genetically stable cultures, which is a prerequisite for *in vitro* conservation of mulberry germplasm accessions. All the

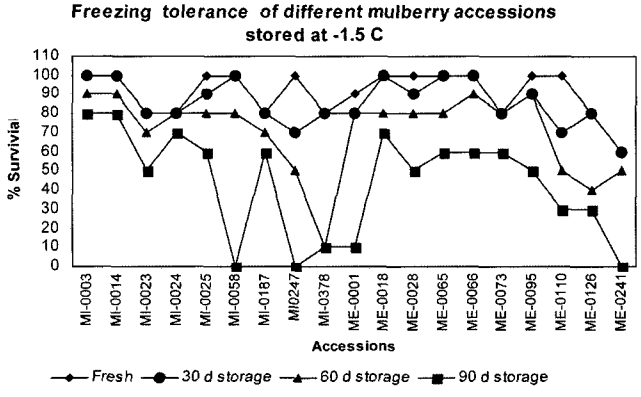

Fig. 2. Freezing tolerance of different mulberry accessions stored at −1.5°C.

Table 2. Recovery of shoot apices of mulberry winter dormant buds cryopreserved in liquid nitrogen after partial dehydration and slow freezing

Sl.no	Accession no.	Variety/species	Moisture % of the buds		Survival % under *in vitro*		
					Control (unfrozen)	Desiccation	LN_2 storage (−196°C)
			Fresh	Desiccated	Mean ± SD	Mean ± SD	Mean ± SD
1	MI-0003 IC-313965	Surat (*M. indica* L.)	28.99	14.50	73.33 ± 5.77	56.67 ± 15.28	43.33 ± 5.77
2	MI-0014 IC-313679	K2 (*M. indica* L.)	46.84	12.43	60.00 ± 10.00	50.00 ± 0.00	40.00 ± 10.00
3	MI-0023 IC-313684	Chatatul (*M. indica* L.)	47.62	16.44	76.67 ± 11.55	53.33 ± 15.28	50.00 ± 0.00
4	MI-0024 IC-313685	Assambola (*M. indica* L.)	36.31	13.63	80.00 ± 0.00	70.00 ± 10.00	46.67 ± 15.28
5	MI-0025 IC-313974	MR-2 *M. sinensis* Hort.	45.47	14.5	80.00 ± 0.00	70.00 ± 10.00	73.33 ± 5.77
6	MI-0058 IC-313703	Kailmpong (*M. indica* L.)	45.54	15.31	96.67 ± 5.77	80.00 ± 10.00	93.33 ± 5.77
7	MI-0187 IC-313992	TR-4 (*M .indica* L.)	56.9	17.29	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
8	MI-0247 IC-313791	Nao khurkul (*M. laevigata Wall.*)	52.99	15.17	43.33 ± 5.77	40.00 ± 0.00	20.00 ± 0.00
9	MI-0378 IC-314167	*M. serrata* Roxb.	45.38	15.38	50.00 ± 10.00	40.00 ± 10.00	30.00 ± 10.00
10	ME-0001 EC-493758	*M.australis* Poir.	28.07	14.83	70.00 ± 10.00	30.00 ± 10.00	26.67 ± 5.77
11	ME-0018 EC-493775	M. cathayana Hemsl.	57.48	13.79	46.67 ± 5.77	36.67 ± 5.77	30.00 ± 0.00
12	ME-0028 EC-493785	*M. bombycis* Koidz.	40.72	13.30	80.00 ± 0.00	70.00 ± 0.00	66.67 ± 5.77
13	ME-0065 EC-493822	S1 (*M. alba* L.)	49.98	14.63	73.33 ± 10.00	56.67 ± 5.77	53.33 ± 15.33
14	ME-0066 EC-493823	Kosen *M. latifolia* Poir.	51.64	14.87	70.00 ± 10.00	46.67 ± 5.77	40.00 ± 0.00
15	ME-0073 EC- 493830	Kasuga (*M. latifolia Poir.*)	32.91	14.61	86.67 ± 15.28	63.33 ± 5.77	60.00 ± 10.00
16	ME-0095 EC-493973	*M. rotundiloba* Koidz.	48.78	15.63	46.67 ± 5.77	30.00 ± 10.00	10.00 ± 10.00
17	ME-0110 EC-493988	*M.rubra* L.	37.55	15.50	50.00 ± 10.00	30.00 ± 10.00	26.67 ± 5.77
18	ME-0126 EC-493853	*M. teliaefolia* Makino.	52.44	12.82	56.67 ± 5.77	36.67 ± 5.77	23.33 ± 5.77
19	ME-0241 EC-493968	*M. nigra* L.	49.26	12.56	86.67 ± 5.77	76.67 ± 5.77	63.33 ± 5.77

cryopreserved accessions retained morphogenetic potential after 3 years of cryopreservation. After retrieval the buds started sprouting *in vitro* after 20 - 30 day of *in vitro* culture (Fig. 3). Maximum shoot formation (93.3%) was recorded *in vitro* for *Morus indica* (Var Kalimpong local) when retrieved after cryopreservation. Low survival and shoot formation with incase of *M.laevigata* Wall., *M. serrata* Roxb., *M. tiliaefolia* Makino., *M. rubra* L, *M. rotundiloba* Koidz. and *M. cathayana* Hemsl. indicated requirement of higher concentration of BAP and GA_3 and modification of $NO_3 : NH_4$ ratio in the MS basal media (Pattnaik and Chand, 1997 and Wen and Deng, 2005).

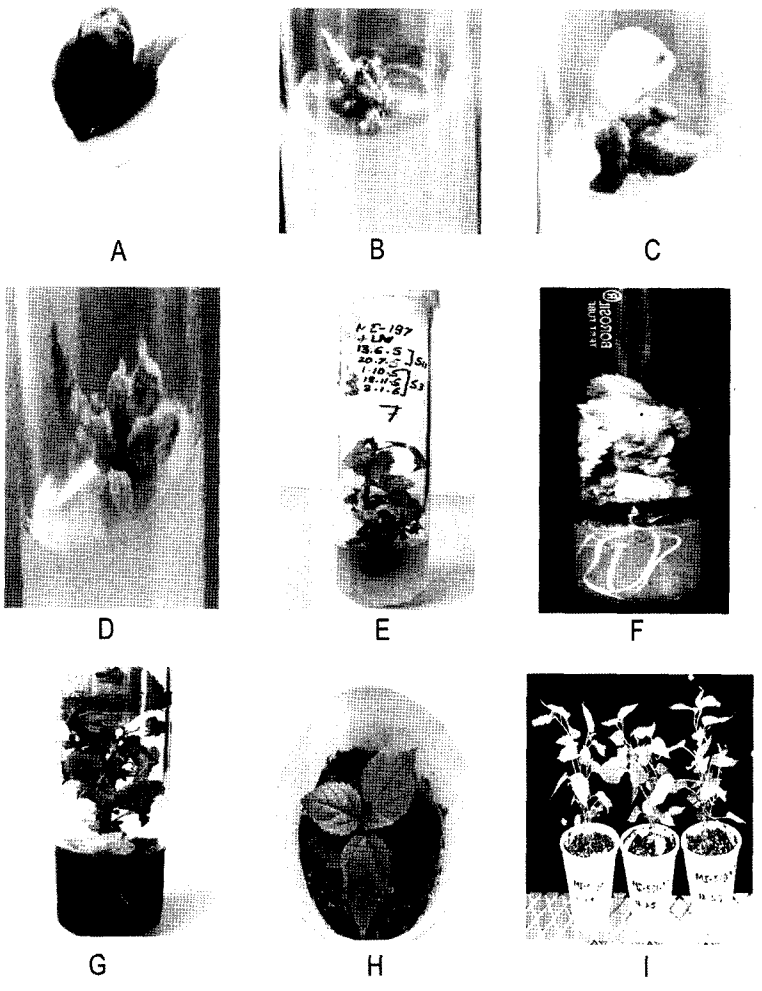

Fig. 3. Captions for legends.
A. *In vitro* sprouting of cryopreserved dormant bud of *M.nigra*
B. *In vitro* sprouting of cryopreserved dormant bud *M.alba*
C. *In vitro* sprouting of cryopreserved dormant bud of *M.laevigata*
D. *In vitro* sprouting of cryopreserved dormant bud of *M. tiliaefolia*
E. Shoot formation *in vitro* TR-14 (MI-0197) after preservation in LN
F. Rooting in *M.nigra* (MI-0241) in MS media supplemented with IBA
G. Rooting in *M.indica* (MI-0003) in MS media supplemented with IBA and AC
H. Hardening of *in vitro* generated cryopreserved mulberry germplasm (S1) in soil-rite
I. Hardening of *in vitro* generated cryopreserved mulberry germplasm (MI-0058)) in soil-rite

Inclusion of low concentration of auxins (0.2 to 0.5 mg l^{-1} NAA or GA_3) along with cytokinin BAP (1 mg l^{-1}) in the MS media enhanced *in vitro* shoot formation after cryopreservation in many of the accessions (Abdelnour *et al.*, 1992 and Khurana *et al.*, 2003). Among the wild species *M.nigra* survived better with 63.33% survival *in vitro*. Majority of the accessions rooted *in vitro* within 20-25 days of culture in the auxin rich rooting media (0.5 mg l^{-1} IBA and 0.01% AC) except some wild species. *Morus latifolia* Poir. (Var. Kosen) and *M. laevigata* Wall. accessions took 45 to 60 days for root development. Using low concentration of cytokinins after initial establishment and polypropylene caps for the culture tubes enhanced conservation period for more than one year. The cold preserved buds did not survive cryoexposure with out dehydration. Prior desiccation of buds with moisture content between 12-16 percent before slow freezing significantly enhanced the survival rates ranging between 40-100% in majority of the accessions studied. Survival rates after cryopreservation above 40 to 100% was achieved in 47% accessions studied (Fig. 4). The rehydration of cryopreserved buds after thawing and incubation in dark of *in*

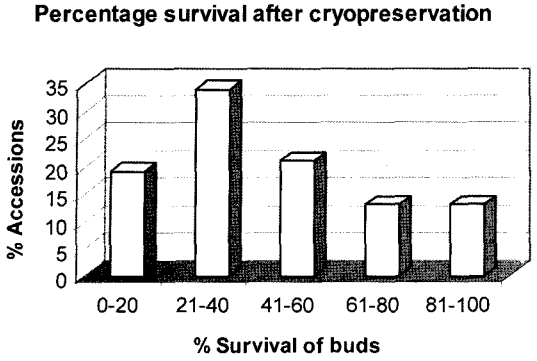

Fig. 4. Percentage survival of mulberry accessions after cryopreservation.

Table 3. Cryopreservation of different *Morus* species in the Nationa cryogene bank

Morus species	No of accessions	*Morus* species	No of accessions
M.rubra	1	M.indica	108
M.nigra	1	M.alba	36
M.serrata	3	M.australis	2
M.laevigata	25	M.multicaulis	2
M.sinensis	2	M.bombycis	6
M.cathayana	1	M.latifolia	11
M.tiliaefolia	1	Morus spp.	37
M.rotundiloba	1	Total	238
M.macroura	1		

vitro cultures further increased the survival rates. The species wise distribution of cryo storage of different mulberry accessions was given in table 3. The results are in agreement with other reports. An average recovery of about 40% survival was achieved after cryopreservation with 219 varieties tested in potato (Schafer, 1996) and 50% survival in mulberry with 376 varieties without any significant changes after 8 years of cryopreservation (Niino, *et al.*, 1995). It is essential to critically dehydrate the tissues to avoid lethal intracellular freezing prior to rapid cooling to produce high level of survival of organized regrowth of the tissues after cryopreservation (Sakai, 1995). The survival rates were widely varied between populations of different *Morus* species. Low survival was recorded for most of the wild species viz. *M. tiliaefolia* Makino, *M. rubra* L, *M. rotundiloba* Koidz., *M. serrata* Roxb. *and M. laevigata Wall.* which are not easily amenable for higher desiccation and freezing and needs employment of protocols like encapsulation and vitrification and optimization of *in vitro* culturing conditions (Scocchi *et al.*, 2004). During freezing, water migrates from the super cooled shoot primordial tissue to the bud scales and the dehydrated shoot tips are attained high degree of freezing tolerance preventing intra cellular freezing and lethal ice nucleation under cryopreservation (Niino *et al.*, 1995).

Considerable genetic diversity was observed among the genotypes selected for the genetic stability studies based on DNA markers generated with ISSR primers (Table 4). The PCR amplification products generated by the ISSR primers namely UBC-807 and UBC-825 have revealed higher polymorphism. The ten ISSR primers generated a total of 110 bands, of which 83 were polymorphic, thus generating 75.45% polymorphism (Vijayan, 2003; Vijayan and Chatteerjee, 2003; Naik and Dandin, 2005 and Vijayan *et al.*, 2006). The highest genetic distance 0.481 was observed between Kanva-2 and TR-12 and genetic similarity 0.808 between Surat and Kalimpong local. The genetic similarity co-efficient among the genotypes estimated indicated no difference between the fresh and *in vitro* regenerated cryo samples (Table 5, Fig. 6). From UPGMA dendogram based on ISSR profile it is possible to observe seven major groups and both the control and cryopreserved plants of the accessions grouped jointly in the same cluster (Fig. 5). The variety S1 (*M.alba* L.) and

Table 4. ISSR Primer with their nucleotide sequence and number of total and polymorphic bands generated by 14 genotypes (control and cryo samples)

Sl. No.	Primers	Sequence5' - 3'	Total bands	Polymorphic bands
1	UBC807	AGAGAGAGAGAGAGAGT	15	13 (86.67 %)
2	UBC808	AGAGAGAGAGAG AGAGC	12	10 (83.33 %)
3	UBC810	GAGAGAGAGAGAGAGAT	11	7 (63.63 %)
4	UBC811	GAGAGAGAGAGAGAGAC	8	5 (62.5%)
5	UBC812	GAGAGAGAGAGAGAGAA	15	11 (73.33 %)
6	UBC825	ACACACACACACACACT	10	9 (90.00%)
7	UBC827	ACACACACACACACACG	13	10 (76.92)
8	UBC841	GAGAGAGAGAGAGAGAC	12	9 (75.00)
9	UBC855	ACACACACACACACACYT	8	6 (75 %)
10	UBC864	ATGATGATGATGATGATG	6	3 (50.00 %)

Table 5. Genetic similarity coefficients (above diagonal) and genetic distance (below diagonal) among 7 genotypes (7 controls and respective 7 respective cryo samples) of mulberry realized from ISSR markers

Geno types	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	***	1.00	0.700	0.700	0.755	0.755	0.664	0.664	0.636	0.636	0.627	0.627	0.664	0.664
2	0.000	***	0.700	0.700	0.755	0.755	0.664	0.664	0.636	0.636	0.627	0.627	0.664	0.664
3	0.357	0.357	***	1.000	0.727	0.727	0.709	0.709	0.718	0.718	0.709	0.709	0.727	0.727
4	0.357	0.357	0.000	***	0.727	0.727	0.709	0.709	0.718	0.718	0.709	0.709	0.727	0.727
5	0.282	0.282	0.319	0.319	***	1.000	0.746	0.746	0.809	0.809	0.709	0.709	0.673	0.673
6	0.282	0.282	0.319	0.319	0.000	***	0.746	0.746	0.809	0.809	0.709	0.709	0.673	0.673
7	0.410	0.410	0.344	0.344	0.294	0.294	***	1.000	0.646	0.646	0.691	0.691	0.618	0.618
8	0.410	0.410	0.344	0.344	0.294	0.294	0.000	***	0.646	0.646	0.691	0.691	0.618	0.618
9	0.452	0.452	0.331	0.331	0.212	0.212	0.438	0.438	***	1.000	0.682	0.682	0.664	0.664
10	0.452	0.452	0.331	0.331	0.212	0.212	0.438	0.438	0.000	***	0.682	0.682	0.664	0.664
11	0.466	0.466	0.344	0.344	0.344	0.344	0.370	0.370	0.383	0.383	***	1.000	0.673	0.673
12	0.466	0.466	0.344	0.344	0.344	0.344	0.370	0.370	0.383	0.383	0.000	***	0.673	0.673
13	0.410	0.410	0.319	0.319	0.396	0.396	0.481	0.481	0.410	0.410	0.396	0.396	***	1.00
14	0.410	0.410	0.319	0.319	0.396	0.396	0.481	0.481	0.410	0.410	0.396	0.396	0.000	***

1, 2 - *M. australis*, 3, 4 - S1 (*M.alba*), 5, 6- Surat (*M.indica*), 7, 8- Kanava-2 (*M.indica*), 9, 10- Kalimpong local (*M.indica*), 11, 12- Matigara white (*M.alba*), 13, 14 -TR 14 (triploid of *M.indica and M.alba*)

TR-14 (hybrid of S1) grouped together indicated their similarity due to same genetic origin. Maintenance of genetic stability of cryopreserved germplasm has been reported in Melia (Scocchi, *et al.*, 2004); Dioscorea (Dixit, *et al.*, 2003) grape and Kiwi (Zhai, *et al.*, 2003). Any accumulative DNA polymorphism may not be induced by cryopreservation *per se* but are the result of whole culture-cryoprotection-regeneration process (Harding, 2004). Using organized and well differentiated explants like apical shoots and dormant buds avoiding callus phase minimizes the genetic variations in regenerating plants (Amato, 1985).

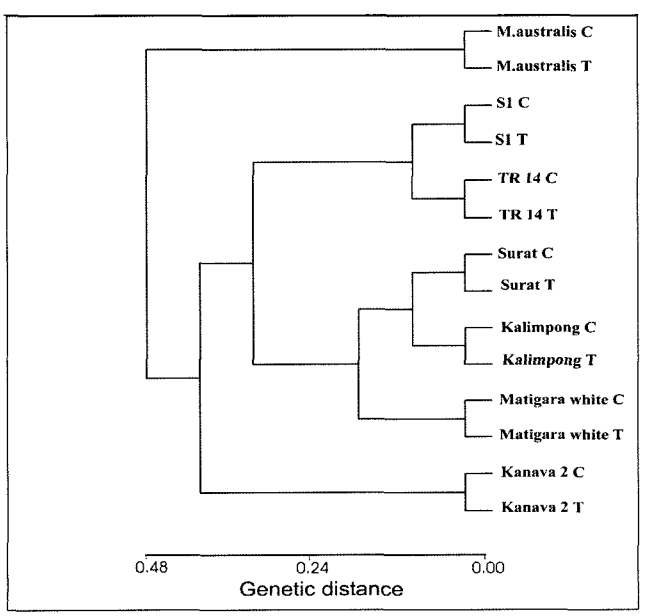

Fig. 5. Dendrogram of mulberry accessions based on 110 ISSR markers (C-Control, T- *in vitro* regenerated cryopreserved germplasm).

Conclusion

It is well recognized that for efficient and cost effective conservation of any gene pool different approaches are necessary. Dormant buds collected in the month of January-February were found suitable for the cryopreserva-

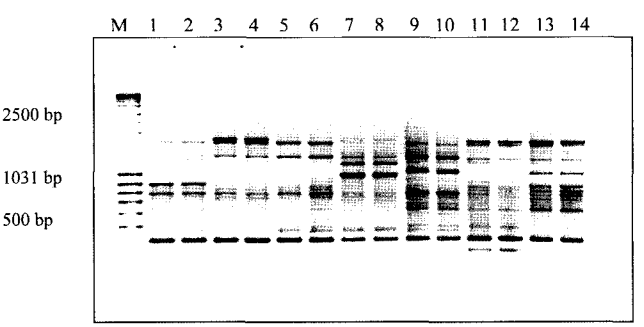

Fig. 6. ISSR band profiles of genotypes of frozen and non frozen samples of *Morus* species as revealed by the PCR amplification products generated by a) UBC-807 primer resolved on 2.0%gel. Serial numbers -1, 2 - *M.australis*; 3, 4 - S1; 5, 6 - Surat; 7, 8 -Kanava-2; 9, 10 - Kailmpong local; 11, 12 - Matigara white; 13, 14 -TR 14

tion in LN. Partial desiccation of winter buds with moisture content between 12 - 19% before slow freezing enhanced the survival rates up to 60% after cryopreservation. The present study on dehydration and slow freezing of winter dormant buds without using any growth retardants and toxic cryoprotectants maintaining high degree of genetic stability clearly demonstrates the success of this technology for long-term cryopreservation of tropical mulberry germplasm representing wide range of different *Morus* species for future use.

Acknowledgements

The authors are grateful to Department of Biotechnology (DBT), Govt. of India, New Delhi for providing financial support.

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