


Genetic and biochemical stability assessment of plants regenerated from cryopreserved shoot tips of a commercially valuable medicinal herb *Bacopa monnieri* (L.) Wettst

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Abstract Shoot tips from four accessions (IC249250, IC 426442, IC 375976, and IC468878) of *Bacopa monnieri* (L.) Wettst., a commercially valuable memory revitalizing medicinal plant, were cryopreserved using a vitrification technique. Depending on the genotype, 0 to 20% plant regeneration without intermediary callus was achieved from cryopreserved shoot tips. Genetic stability of plants derived from cryopreserved shoot tips was assessed using biochemical and molecular markers. The regenerated plants from non-frozen controls and cryopreserved shoot tips exhibited morphological similarity to respective parental material when transferred to soil. On the basis of ten random amplified polymorphic DNA (RAPD) and bacoside A content using HPLC analysis, no significant reproducible variation was observed between the controls and *in vitro*-cryopreserved plants. Thus, after cryopreservation treatment, the regenerated plants exhibited molecular and biochemical genetic stability.

Keywords *Bacopa monnieri* · Bacoside A · Cryopreservation · RAPD

Introduction

The Indian gene centre is immensely rich with a diversity of medicinal plants including 750 species with established folklore records and proven healthcare benefits in both traditional and modern pharmacopoeias. More than 80% of these bio-resources for industrial use still come through collection from wild stands (Chandel *et al.* 1996; Sharma and Pandey 2013). The threat of losing valuable gene pools of medicinal plant biodiversity is alarming and requires multiple approaches to prevent the loss. First, the available biodiverse and priority medicinal plants must be catalogued and characterized to promote their domestication and organized cultivation to meet industrial demands. Second, efficient methods must be developed for *in-situ* and *ex-situ* conservation of threatened gene pools. The last three decades have witnessed an increased use of *in vitro* cell and tissue culture techniques for biodiversity conservation of medicinal plants (Sarasan *et al.* 2006; Sharma and Pandey 2013).

Bacopa monnieri (L.) Wettst. is a popular ancient Indian Ayurvedic herb ('Brahmi') used as a nootropic, nervine tonic for neutralizing mental stress, epileptic disorders, and improving memory functions in humans (Anonymous 1988). It is also known to possess adaptogenic, anti-cancer, anti-oxidant, and immunomodulatory properties (Anonymous 1988; Sharma *et al.* 2007). The clinical efficacy of *B. monnieri* has been attributed to the presence of several triterpene saponins (bacosides) in its aerial tissues (Deepak *et al.* 2005). 'Brahmighritam', 'Brahmirasayanam', Memory Plus, and Mentat are some of the popular brand names of Bacopa-based drug preparations in the Indian herbal trade. As a predominantly vegetatively propagated species with a restricted habitat, *B. monnieri* has a narrow genetic base for herb and metabolite-contributing traits. Despite having abundant growth, large quantities of material are required to meet

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pharmaceutical demands due to a low drug content. Consequently, the intervention of biotechnological tools will be beneficial for conservation and genetic improvement. This herb is amenable to *in vitro* micropropagation and conservation techniques (Sharma *et al.* 2007, 2012, 2016). A single-step medium-term conservation protocol was successfully used to conserve over 20 accessions of *B. monnieri* from various ecogeographic regions of India (Sharma *et al.* 2007, 2016). For long-term conservation, cryogenic storage is the only method available. A basic protocol for cryopreservation of shoot tips using a vitrification technique has also been previously reported (Sharma *et al.* 2011). This protocol was developed using one accession. From the point of view of a conservationist, maintenance of genetic stability of plants regenerated after conservation is important to ensure sustainable use of conserved resources in the future.

The present study was, therefore, undertaken with the two objectives: (i) to assess the feasibility of cryopreserving shoot tips in three new accessions using a vitrification procedure and (ii) to monitor the genetic stability of plants regenerated from cryopreserved shoot tips using biochemical and molecular markers. This is the first report of genetic stability assessment of cryopreserved shoot tips in this important medicinal herb.

Material and Methods

Plant material A total of four accessions of *B. monnieri* (IC249250, IC426442, IC375976, and IC468878; hereafter referred to as BC1, BCM, BCJ, and BC7, respectively) conserved in the *In Vitro* Genebank at ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR), New Delhi, India, were used in the present study. The experimental material consisted of shoot tips (~1.0 mm in length) obtained from 24-wk-old proliferating shoot cultures. Stock cultures were multiplied on MS medium (Murashige and Skoog 1962) with 0.2 mg L⁻¹ 6-benzyladenine (BA) (Sigma-Aldrich®, St. Louis, MO) and 3% (*w/v*, 0.08 M) sucrose (Hi-Media®, Mumbai, India) at pH 5.8, henceforth, referred to as shoot multiplication (SM) medium. The medium was gelled using 0.8% (*w/v*) bacteriological agar (Hi-Media®) and autoclaved at 121°C and 1.06 kg cm⁻² for 15 min. For multiplication of the cultures, single-node cuttings were subcultured onto SM medium every 4 to 8 wk under standard culture room conditions of 25 ± 2°C and a 16-h photoperiod (30 μmol m⁻² s⁻¹) provided by 30-W cool-white fluorescent tubes (Philips, Mumbai, India) (Sharma *et al.* 2007).

Cryopreservation Cryopreservation of shoot tips was done following a slightly modified method described by Sharma *et al.* (2011) using BC1. Shoot tips (*ca.* 1.0 mm) dissected from 24-wk-old shoot cultures were precultured for 2 d on MS medium supplemented with 0.3 M sucrose, before dehydrating with

plant vitrification solution 2 (PVS2) at 0°C. The PVS2 solution consisted of 30% (*v/v*, 3.26 M) glycerol (Sigma-Aldrich®), 15% (*w/v*, 2.42 M) ethylene glycol (EG) (Sigma-Aldrich®), 15% (*v/v*, 1.9 M) dimethylsulphoxide (DMSO) (Sigma-Aldrich®), and 0.4 M sucrose dissolved in pH 5.8 liquid MS medium (Sakai *et al.* 1990). In each experimental treatment, ten shoot tips were precultured on 10 ml of medium in a Petri dish (Hi-Media®) (60-mm diameter) at 25°C for 2 d. The sucrose-precultured shoot tips were placed in sterile 1.0ml Nunc™ cryovials (Thermo Fisher Scientific® Roskilde, Denmark) containing 1.0 ml PVS2 at 0°C for 30 min. After the 30 min in PVS2, cryovials containing shoot tips were quickly plunged into liquid nitrogen (LN) for at least 1 h. Thawing was done by stirring the cryovials in a water bath at 45°C for 2 min. After thawing, the PVS2 solution was removed using a Pasteur pipette and shoot tips were rinsed five times over a 20-min period with a deloading solution of 1.2 M sucrose dissolved in pH 5.8 liquid MS medium. Shoot tips were then transferred onto sterile filter paper strips. For regrowth, shoot tips were placed on 10 ml of SM medium in Petri dishes. The control shoot tips (precultured and PVS2-treated but not frozen) were recultured in a similar manner. After 1-wk incubation in the dark, these were transferred to the light.

All the cultures were incubated at 25 ± 2°C at a 16-h photoperiod and a light intensity of 30 μmol m⁻² s⁻¹, provided by 30-W cool white fluorescent tubes (Philips). After 8 wk, surviving shoot tips were transferred onto the same medium in culture tubes (Qualigens Fine Chemicals Pvt., Ltd., Mumbai, India) (150 mm × 25 mm) for multiplication and root formation. Ten shoot tips were tested for each of two replications in every experiment. The experiment was repeated at least three times. Survival of the cultures was assessed by visual examination and by the ability of the explants to regrow on fresh SM. Regeneration was recorded as the percentage of the total number of shoot tips that formed normal shoots 8 wk after plating.

For *ex vitro* transfer, 12-wk-old plantlets were transferred to pots for hardening as described by Sharma *et al.* (2007). The plants were hardened in plastic pots (7.5-cm diameter) containing autoclaved (20 min at 121°C and 1.06 kg cm⁻² for 15 min) vermiculite (Glassil, New Delhi, India) moistened with 10–15-ml 1/2 MS major salt solution (Hi-Media®) and allowed to grow at 25 ± 2°C. The plantlets were covered with transparent perforated polythene bags (Ambay Biotech, Delhi, India) for 2–3 wk to maintain high humidity (75 to 85%) and subsequently exposed to *ex vitro* conditions by removing the polythene bags after 3 wk. The 3-wk-old hardened plantlets were transplanted to garden soil [10:1 sandy soil:FYM (locally produced farm yard manure)] in earthen pots (30-cm diameter) and allowed to grow in a net house (50 mesh in⁻², ~50% shade) under natural light.

Genetic stability assessment For assessment of genetic stability, plants from four different treatments were used. These

included (i) plantlets obtained after post-thaw regrowth, (ii) plantlets obtained from *in vitro* multiplied material (controls) and (iii) and (iv) 6-mo-old plants grown in pots after transfer of hardened plantlets from treatment as described above (i) and (ii), respectively. The age of plantlets regenerated from control and cryopreserved shoot tips was the same at the time of the *ex vitro* transplanting.

The genetic stability of *in vitro*-cryopreserved plants was studied using molecular (random amplified polymorphic DNA (RAPD) markers) and biochemical methods (bacoside A estimation) (Sharma *et al.* 2012).

RAPD analyses Genomic DNA extraction Total genomic DNA was extracted from 1 g leaves each of the *in vitro* cultures and *ex vitro* grown regenerants using the CTAB (cetyltrimethyl ammonium bromide) (Amresco®, Solon, OH) method described by Saghai-Marooof *et al.* (1984). After RNase (Thermo Fisher Scientific®, Waltham, MA) treatment, the DNA concentrations were estimated using NanoDrop® 1000 (Thermo Fisher Scientific®). The DNA samples were stored at -20°C until used. Working solutions of genomic DNA ($5\text{ ng }\mu\text{l}^{-1}$) were prepared after dilution with Tris-EDTA (Amresco®) buffer (0.1 mM Tris-HCl, 0.01 mM EDTA at pH 8.0) and stored at -20°C for subsequent use in RAPD analyses.

PCR reactions were carried out in a DNA thermal cycler (G-Storm™ Thermal Cycler Systems, Somerset, UK). Each 25 μl reaction mix contained 1 \times PCR reaction buffer (10 mM Tris-HCl, 50 mM KCl, at pH 8.8); 1.5 mM MgCl_2 ; 1 U of Taq DNA polymerase; 200 μM each of dATP, dTTP, dCTP, and dGTP (Thermo Fisher Scientific®); 0.4 μM of primer (Qiagen® Operon, Inc., Alameda, CA), and approximately 25 ng of template DNA.

RAPD analyses A total of 30 primers (Qiagen® Operon, Inc.) were screened for RAPD analysis. Of these, ten primers showing good amplification were used for further analyses (Table 1). The PCR conditions were as follows: initial extended step of denaturation at 94°C for 4 min followed by 39 cycles of denaturation at 94°C for 1 min, primer annealing at 37°C for 1 min, primer elongation at 72°C for 2 min, followed by an extended elongation step at 72°C for 7 min. PCR products were stored at 4°C until electrophoresis.

Reaction products were mixed with 5 μl of 6 \times loading dye (0.03% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol FF, and 60% (w/v) glycerol) (Sigma-Aldrich®) using a micropipette. The mixture was electrophoresed on 1.4% (w/v) agarose (Lonza, Rockland, ME) gels (prepared in 1 \times TBE buffer) at 80 V (BIO RAD® subcell GT, Hercules, CA) for 3 h. A 100-bp DNA ladder plus (Thermo Fisher Scientific®) was run next to the amplified products to determine their approximate size. The amplified fragments were visualized using a gel-

Table 1. List of primers used in the RAPD analysis of *in vitro* cryopreserved plants of *Bacopa monnieri*

Serial no.	Primer name	Primer sequence (5'-----3')
1	OPA03	AGT CAG CCA C
2	OPA07	GAA ACG GGT G
3	OPA08	GGT ACG TAG G
4	OPA16	ACG CAG CGA A
5	OPB01	GTT TCG CTC C
6	OPB02	TGA TCC CTG G
7	OPB03	CAT CCC CCT G
8	OPB13	TTC CCC CGC T
9	OPC15	GAC GGA TCA G
10	OPC17	TTC CCC CCA G

documentation system (Gel Doc Mega, Biosystematica, Llandysul, Wales, UK).

For genetic stability assessment, in each treatment, 48 *in vitro* plantlets and 12 *ex vitro*-transferred plantlets were analysed. For each marker system, the strong amplified fragments were scored for their presence (1), absence (0), and missing data (9). Genetic similarity values were computed based on Jaccard's coefficient (Jaccard 1908) using the NTSYS-PC version 2.11a software (Rohlf 2002). Cluster analyses were performed based on similarity matrices for combined scores of RAPD using unweighted pair group method with arithmetical averages (UPGMA) (Sneath and Sokal 1973).

Biochemical analysis For the estimation of bacoside A, shoots of *in vitro* grown plantlets and *ex vitro* grown plants (same as those used for molecular analysis) were harvested and lyophilized. The secondary metabolite content was assessed using HPLC following the modified procedure of Deepak *et al.* (2005). For plotting of the standard curve, bacosides standard was obtained from ChromaDex® (Irvine, CA).

Approximately 1 g of lyophilized sample was pulverized in a grinder (FOSS, Hamburg, Germany). The powdered sample (100 mg) was extracted with methanol (E Merck, Darmstadt, Germany) by reflux ($3 \times 2\text{ ml}$, 10 min each) at 70°C . The extracts obtained after each extraction were pooled. The solvent was dried on a rotary evaporator and then the volume was made up to 2 ml using HPLC grade methanol (E Merck). The sample was injected into a HPLC column (Phenomenex, Torrance, CA) after filtering through a 0.22 μm Millipore filter (E. Merck). Ten microliter of the standard per sample was injected into a Waters HPLC system (Waters® Corporation, Milford, MA) consisting of a M-600 E quaternary gradient pump, 717 autosampler, Nova-Pak® Reverse phase C-18

(3.9 × 150 mm) column, and photo diode array (PDA) detector. The separation of the bacosides was obtained on a C-18 column using an isocratic run of water (A) and acetonitrile (E Merck) (B) in the ratio of 67.5:32.5 at 1.6 ml min⁻¹ as mobile phase. The peaks were monitored at 200 nm. The peak identity was confirmed by comparing the retention time of the peaks and also by PDA spectrum.

Results and Discussion

Cryopreservation is the most appropriate strategy for long-term conservation of vegetatively propagated plants and those with recalcitrant seeds. It is evident from the literature (Chandel *et al.* 1996; Sarasan *et al.* 2006; Sharma and Pandey 2013) that most of the work focused on *in vitro* propagation has resulted in varying degrees of success in a large number of medicinal plants. However, in medicinal plants, there are limited publications related to *in vitro* conservation and few reports on *in vitro* cryopreservation (Sharma and Pandey 2013). Over the last two decades, although some success has been achieved with cryopreservation and plantlet regeneration from *in vitro* shoot tips of *B. monnieri*, *Dioscorea* spp., *Holostemma ada-kodien*, *Picrorhiza kurroa*, and *Rauvolfia serpentina* (Mandal and Dixit 2000; Sharma and Sharma 2003; Ray and Bhattacharya 2008; Decruse *et al.* 2004; Sharma *et al.* 2011), genetic stability after cryopreservation is reported only in *Dioscorea deltoidea* (Dixit-Sharma *et al.* 2005).

Experiments were conducted to test the feasibility of a previously developed method of cryopreservation (Sharma *et al.* 2011) on three more accessions of *B. monnieri* (BCM, BCJ, and BC7) collected from different ecogeographic regions. In all the four accessions tested (including the earlier reported BC1), shoot tips isolated from 24-wk-old cultures and precultured on 0.3-M sucrose medium exhibited 0 to 20% post-thaw regeneration following PVS2 dehydration at 4°C (Fig. 1A, B). As evident from Fig. 1A, shoot tips failed to exhibit post-thaw survival in BC7, one of the accessions tested. The non-frozen controls exhibited 80 to 100% regrowth. Upon transfer to SM medium, multiple shoots were produced and normal growth was observed (Fig. 1C). The morphology of the cryopreserved shoots was comparable to that of non-frozen *in vitro* shoots. Subsequent multiplication of cryopreserved shoots resulted in shoot multiplication with an average of 18 to 22 shoots explant⁻¹ in 8 wk. Plantlets regenerated from cryopreserved shoot tips were successfully transferred to pots with 100% plant survival (Fig. 1D).

Studies have shown that genotype-dependent differences with respect to post-thaw recovery are common (Agrawal *et al.* 2011; Senula and Kellar 2011). In the present study, regrowth of cryopreserved shoot tips occurred without transitory callus formation (Fig. 1B), which is similar to earlier

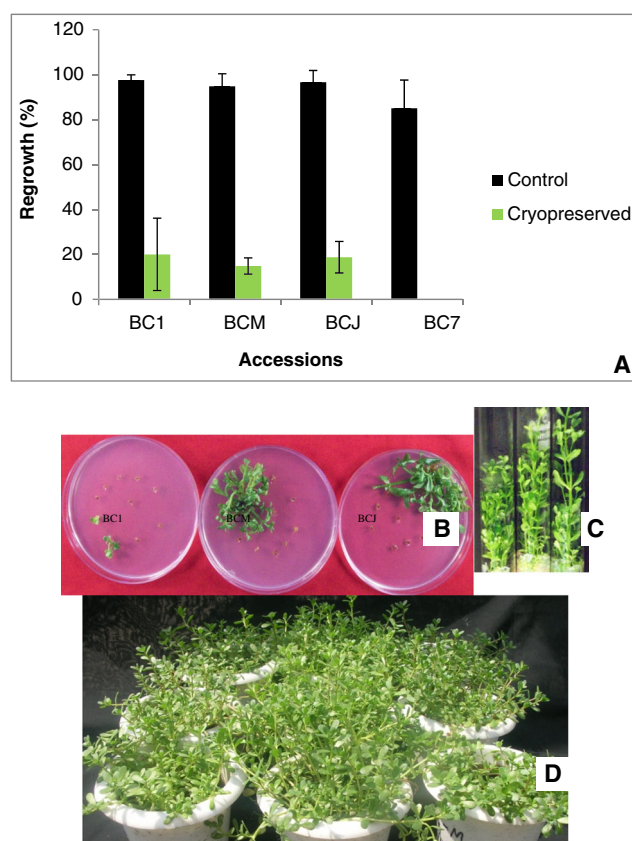


Figure 1. Cryopreservation of shoot tips in four accessions of *Bacopa monnieri* (A–D). (A, B) Post-thaw regrowth, (C) shoot multiplication of cryopreserved shoot tips, and (D) *ex vitro* establishment of regenerated shoot tips.

results (Sharma *et al.* 2011). It is an important observation while developing a cryopreservation method as direct regeneration suggests a low risk of genetic instability. All the regenerated plants appeared similar to the control plants. To further confirm the genetic stability of the conserved plants, molecular and biochemical analyses were also conducted.

On the basis of RAPD analysis, there was no variation among controls and plants regenerated from cryopreserved shoot tips in all the three accessions tested (BC1, BCM, and BCJ). Representative RAPD profiles of plantlets derived from cryopreserved shoot tips and *ex vitro* transferred plants after cryopreservation and controls are shown in Fig. 2. On the basis of RAPD analyses, there was no variation observed among controls and plants regenerated from cultures. A minimum number of five bands were amplified by primers OPA-8 (Fig. 2) and OPA-07, and a maximum number of seven bands were amplified by primer OPA-03. The average number of products amplified per primer was 5.3, and out of the total amplicons generated (53 bands), 43 were found to be monomorphic. The size of products varied from 200 to 1200 bp. A total of 1316 amplification products were produced by RAPD markers computed on the basis of (the number of plants) × (the number of bands produced by ten primers). Of these 1316

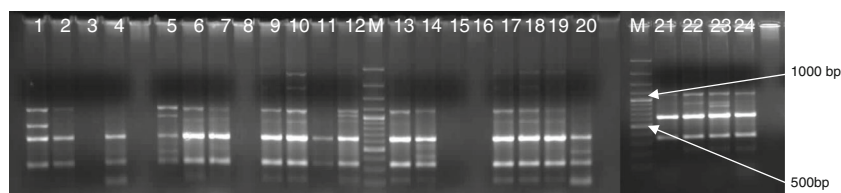


Figure 2. Representative RAPD profiles generated with OPA-08 primer generated from cultures and *ex vitro* transferred plantlets regenerated from controls and cryopreserved shoot tips in three accessions of *Bacopa monnieri*. Cultures—BC1 (1–3 control, 5–7 cryopreserved); BCM (9–11

control, 13–15 cryopreserved) and BCJ (17–19 control, 21–23 cryopreserved). *Ex vitro* plants—BC1 (4 control, 8 cryopreserved); BCM (12 control, 16 cryopreserved) and BCJ (20 control, 24 cryopreserved). M denotes Gene Ruler™ 100-bp DNA ladder.

bands, 1204 were monomorphic (91.14%), indicating not a very significant variation was observed. Ten RAPD primers used in this study generated 53 scorable amplification analyses and indicated the maintenance of genetic stability in conserved cultures. RAPD and ISSR markers have been used to assess genetic stability of micropropagated plants (Ceasar *et al.* 2010) and *in vitro* conserved plants of *B. monnieri* (Sharma *et al.* 2012). In the present study, RAPD marker analyses indicated maintenance of genetic stability in cryopreserved cultures.

Retention of biosynthetic ability, especially bacoside A in cryopreserved plants is extremely important in *B. monnieri*, as bacoside A is shown to be responsible for the pharmacological properties including the memory-facilitating action of ‘brahmi’. Analysis of bacoside A content in cultures after cryopreservation showed that it remained similar to respective controls (Fig. 3), thereby indicating maintenance of genetic stability in the cryopreserved cultures in all the three responding accessions. In one of the accessions tested (BCM), *in vitro* cultures exhibited a higher content of bacoside compared to *ex vitro* transferred plants (Fig. 3). This result was similar to the earlier report on *in vitro* conserved cultures (Sharma *et al.* 2012). On the basis of morphological, RAPD markers, and bacoside A analyses, it was clear that the genetic stability of cryopreserved cultures was maintained in all three responding accessions.

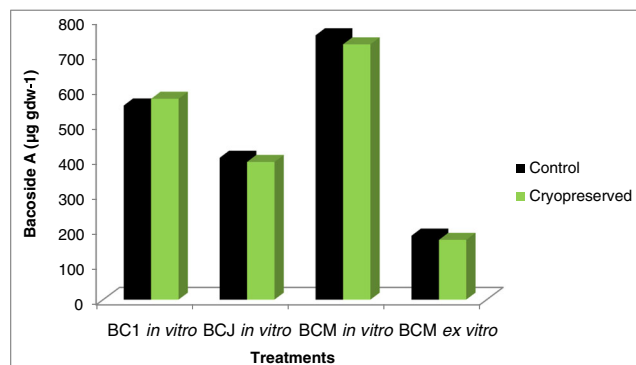


Figure 3. *Bacopa monnieri*: Bacoside A content in plantlets obtained after post-thaw regrowth of shoot tips and their controls in different genotypes and *ex vitro* transferred plantlets (6-mo-old) regenerated from cryopreserved and control shoot tips in genotype BCM.

Conclusions

Due to the low post-thaw recovery, further research is required to standardize various steps of cryopreservation, which generally is genotype-specific. Cryopreservation, though with low recovery, has been proven feasible in three new accessions of *Bacopa*. No difference in RAPD profiles and bacoside A content of control and cryopreserved regenerants confirmed the genetic stability after cryopreservation. Research continues to improve the recovery rates. However, applicability of the developed protocol in three accessions is important research towards reaching a high multiplication rate in surviving shoot tips.

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References

- Agrawal A, Tyagi RK, Goswami R, Uma S, Saraswathi MS, Durai P (2011) Cryobanking of banana (*Musa sp.*) germplasm in India: evaluation of agronomic and molecular traits of cryopreserved plants. *Acta Hortic* 908:129–138
- Anonymous (1988) The wealth of India: raw materials, Vol 2. Council of Scientific and Industrial Research, New Delhi
- Ceasar AS, Maxwell SL, Prasad KB, Karthigan M, Ignacimuthu S (2010) Highly efficient shoot regeneration of *Bacopa monnieri* (L.) using a two stage culture procedure and assessment of genetic integrity of micropropagated plants by RAPD. *Acta Physiol Plant* 32:442–452
- Chandel KPS, Shukla G, Sharma N (1996) Biodiversity in medicinal and aromatic plants in India. National Bureau of Plant Genetic Resources, New Delhi
- Decruze SW, Seeni S, Nair GM (2004) Preparative procedures and culture media effect on the success of cryostorage of *Holostemma annulare* shoot tips. *Plant Cell Tissue Organ Cult* 76:179–182
- Deepak M, Sangli GK, Arun PC, Amit A (2005) Quantitative determination of major saponin mixture Bacoside A in *Bacopa monnieri*. *Phytochem Anal* 16:24–29
- Dixit-Sharma S, Ahuja-Ghosh S, Mandal BB, Srivastava PS (2005) Metabolic stability of plants regenerated from cryopreserved shoot tips of *Dioscorea deltoidea*—an endangered medicinal plant. *Scientia Hortic* 105:513–517
- Jaccard P (1908) Nouvelles recherches sur la distribution florale. *Bull Soc Vaudoise Sci Nat* 44:223–270

- Mandal BB, Dixit S (2000) Cryopreservation of shoot-tips of *Dioscorea deltoidea* Wall.—an endangered medicinal yam, for long-term conservation. *IPGRI Newsl Asia Pac Ocean* 33:23
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Ray A, Bhattacharya S (2008) Cryopreservation of *in vitro* grown nodal segments of *Rauvolfia serpentina* by PVS2 vitrification. *CryoLetters* 29:321–328
- Rohlf FJ (2002) NTSYSpc: numerical taxonomy system, ver. 2.1. Exeter Publishing Ltd., Setauket
- Saghai-Marouf MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proc Natl Acad Sci U S A* 81:8014–8018
- 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
- Sarasan V, Cripps R, Ramsay MM, Atherton C, McMichen M, Prendergast G, Rowntree JK (2006) Conservation *in vitro* of threatened plants—progress in the past decade. *In vitro Cell Dev Biol Plant* 42:206–214
- Senula A, Kellar ERJ (2011) Cryopreservation of mints—routine application in a genebank, experience and problems. *Acta Hort* 908: 467–472
- Sharma N, Pandey R (2013) Conservation of medicinal plants in tropics. In: Normah MN, Chin HF, Reed BM (eds) Conservation of tropical plant species. Springer, New York, pp 437–487
- Sharma N, Satsangi R, Pandey R (2011) Cryopreservation of shoot tips of *Bacopa monnieri* (L.) Wettst. by vitrification technique. *Acta Hort* 908:283–288
- Sharma N, Satsangi R, Pandey R, Singh R, Kaushik N, Tyagi RK (2012) *In vitro* conservation of *Bacopa monnieri* (L.) using mineral oil. *Plant Cell Tissue Organ Cult* 11:291–301
- Sharma N, Satsangi R, Pandey R, Vimala Devi S (2007) *In vitro* clonal propagation and medium term conservation of Brahmi [*Bacopa monnieri* (L.) Wettst.] *J Plant Biochem Biotech* 16:139–144
- Sharma N, Sharma B (2003) Cryopreservation of shoot tips of *Picrorrhiza kurroa* Royle ex Benth., an indigenous endangered medicinal plant through vitrification. *CryoLetters* 24:181–190
- Sharma N, Singh R, Pandey R (2016) *In vitro* propagation and conservation of *Bacopa monnieri* L. In: Jain M (ed) Protocols for *in vitro* cultures and secondary metabolite analysis of medicinal and aromatic plants, second edition, methods in molecular biology, Vol 1391. Springer Science + Business Media, New York, pp 153–171. doi:10.1007/978-1-4939-3332-7_11
- Sneath PHA, Sokal RR (1973) Numerical taxonomy: the principles and practice of numerical classification. WH Freeman, San Francisco