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Genetic fidelity assessment of encapsulated in vitro tissues of *Bacopa monnieri* after 6 months of storage by using ISSR and RAPD markers

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Abstract: The present study has investigated the efficiency of regrowth of *Bacopa monnieri* (L.) Pennell after 6 months of storage along with its clonal fidelity checking. Shoot tips and nodal segments of in vitro grown plantlets were encapsulated and stored at different temperatures and the highest regeneration was achieved by storing shoot tips at 4 °C for a period of 6 months. The response of shoot tips was found to be much better when compared to nodal segments in the case of forming healthy shoots and roots. The genetic stability of regrown plantlets was analysed by using RAPD and ISSR markers. Twenty ISSR primers produced 130 clear and reproducible amplicons, of which 125 bands were monomorphic. A total of 115 bands were produced by 25 RAPD markers with 94% monomorphism. This corresponds to a very low genetic variation pertaining to the clonal nature of the regrown plantlets with that of the mother plant. By means of HPLC biochemical analysis, bacoside A content in the regrown plants was also found to be comparable with that of the mother plant. Therefore, the protocol derived can be efficiently utilised for storing the encapsulated shoot tips of *B. monnieri* for about 6 months.

Key words: Encapsulation, storage, regrowth, genetic fidelity, ISSR, RAPD

1. Introduction

Plants have been an important source of medicine as they synthesise and preserve a variety of biochemical products. The widespread reliance on herbal remedies in almost all parts of the world has led to the development of new drugs from plant sources. One such plant having great medicinal uses is *Bacopa monnieri* (L.) Pennell (the water hyssop), a creeping annual plant inhabiting marshy areas throughout India. The herb has been utilised since the very beginnings of Ayurveda, the traditional medical system of India, as a memory-enhancing, antiinflammatory, analgesic, antipyretic, sedative, antioxidant, and antiepileptic agent (Stough et al., 2001). Furthermore, it is recognised as a brain tonic to promote longevity. Researchers have proven its role in providing improved memory, less anxiety, better social adjustment, and less mental fatigue (Singh and Singh, 1980). Due to the arrival of very many pharmaceutical products based on *B. monnieri*, the demand for the plant is increasing (Sharma and Shahzad, 2012) and the plant is now placed in the second position in the priority species list of the most important Indian medicinal plants (Rajani, 2008). In this regard conservation of this elite germplasm is the prime duty of the scientific community at this moment.

Synthetic seed technology has been considered as the most convenient and cost-effective approach for germplasm preservation under in vitro conditions. It renders a way for storing tissues without physical and environmental stress (Hung et al., 2012). Furthermore, alginate encapsulation greatly aids in germplasm exchange programmes and the short- and long-term storage of viable tissues (Germana et al., 2011). It can conserve the cultures in terms of both viability and productivity (Mehrotra et al., 2012) and its efficiency lies in its small size, ease of handling, and requirements of minimum space, time, and care (Mishra et al., 2001). It ensures ready availability of the viable tissues for clonal multiplication (Ray et al., 2008). To date, many medicinally valuable plants have been encapsulated and preserved. Recently the nodal segments of woody climber *Decalepis hamiltonii* Wight & Arn. (Sharma and Shahzad, 2012), shoot tips of *Khaya senegalensis* (Desv.) A.Juss. (Hung et al., 2012) and *Olea europea* L. (Micheli et al., 2007), and microshoots of *Brassica oleracea* L. var. *botrytis* L. (Rihan et al., 2011) have been preserved using this technology. In spite of these advantages, the process will be successful only if the regrown plantlets maintained their genetic nature.

Among the various types of DNA-based molecular techniques utilised to evaluate genetic polymorphism, inter simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) markers are based on

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amplification of single random primers of high GC content. They are considered to be safe and cost-effective due to not needing radioactive probes (Mehrotra et al., 2012). As ISSR covers the whole genome rather than a particular sequence of around 700 bp, it can be considered as good compared to other markers (Doğan et al., 2011). Both of these marker systems have also been intensively used in studying genetic diversity and in population genetics analysis of many plant species like *Papaver* L. (Gürkök et al., 2013) and *Allium* L. (Mukherjee et al., 2013). According to Mehrotra et al. (2012), evaluating genetic stability using 2 types of marker systems gives better analysis of genetic steadiness or differences of the plantlets as they amplify different regions of the genome. Palombi and Damiano (2002) also emphasised the importance of using more than one DNA amplification technique as it is advantageous in evaluating somaclonal variation. In this study, it was planned to evaluate the genetic nature of regrown plantlets using 2 fingerprinting techniques. Furthermore, the quantity of the major chemical constituent of the plant, bacoside A, was estimated in the plantlets regrown after low-temperature storage and compared with the wild-type mother plant. To the best of our knowledge, this is the first report on analysing the genetic stability of *B. monnieri* plantlets regrown after 6 months of storage. This study has also explored the possibility of storing in vitro propagules for about 6 months.

2. Materials and methods

Mother plants of *Bacopa monnieri* were collected from a wild population in the streams near Vellayani Agricultural College, Kerala. In vitro cultures of *B. monnieri* were initiated by culturing the axillary buds on Murashige and Skoog (MS) medium supplemented with 4.44 µM 6-benzylaminopurine (BAP) and 0.53 µM naphthalene acetic acid (NAA) (Ramesh et al., 2006). Encapsulation of nodal segments and shoot tips was carried out using 3% sodium alginate and 80 mM calcium chloride (Ramesh et al., 2009).

2.1. Storage of encapsulated tissues

Encapsulated beads were stored at 3 different temperatures of 4, 8, and 24 °C in autoclaved petri plates for about 1 month. Twenty beads were used for each set and the experiment was repeated 3 times. In order to achieve efficient storage, encapsulation solution was prepared using different sucrose concentrations (1%, 2%, and 3%). After 1 month, synseeds were cultured on plant tissue culture containers containing MS medium supplemented with 4.44 µM BAP + 0.53 µM NAA under suitable culture conditions. Percentage of plant regeneration from synseeds after storage was assessed after 25 days of inoculation. In all cases, nonstored encapsulated beads were treated as the control. After finding out the suitable storage temperature, nodal and shoot tip-derived beads were stored at that particular temperature for a period of 6 months and the percentage of regrowth was evaluated. The experiment was repeated 3 times and the healthy regrown plantlets were slowly acclimatised to the natural environment.

2.2. Assessment of genetic stability

Genomic DNA was extracted from the 25-day-old plants grown after 6 months of storage and the wild-type mother plant by using the HiPur A DNA isolation kit (Hi Media, India). The quality and the quantity of the DNA preparation were checked by gel electrophoresis and spectrometric assay. In order to find out the genetic fidelity of regrown plantlets derived from stored synseeds, ISSR and RAPD fingerprinting analysis was done. Thirty regrown plantlets, i.e. 5 randomly selected plantlets regrown after 6 months of storage from encapsulated shoot tips and nodal segments from 3 independent experiments, were tested for their genetic fidelity.

Genomic DNA was randomly amplified using 20 ISSR (Table 1) and 25 RAPD (Table 2) primers (Sigma, USA). PCR reactions were carried out in an Eppendorf master cycler (Eppendorf, Germany) in a 25-µL PCR mixture consisting of 1X PCR buffer, 1.5 mM $MgCl_2$, 1.25 mM dNTPs, 25 pmol random primer, 0.5 U Taq DNA polymerase (MBI, Fermentas, Lithuania), and 1 µL of 25 ng of template DNA. A total of 42 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min, and extension at 72 °C for 2 min, was performed. The reaction mixture was subjected to 1 cycle of initial denaturation at 94 °C for 5 min and the last cycle was followed by 7 min of final extension at 72 °C. For ISSR analysis, the annealing temperature was 55 °C based on gradient PCR experimentation. The amplified products were separated on 1.5% (w/v) agarose (Sigma) gel in 1X TAE (Tris acetate-EDTA) buffer along with a 1-kb ladder (MBI, Fermentas) as the size marker and were visualised under UV light after staining with ethidium bromide. All the experiments were repeated 3 times to ascertain the reproducibility and were photographed with a gel documentation system (Gel Doc XR+, Bio-Rad, Hong Kong) aided by Quantity One software.

2.3. Estimation of bacoside A

Extraction and high-performance liquid chromatography (HPLC) analysis of bacoside A were carried out following the method of Murthy et al. (2006). One gram of powdered plant material was extracted in 100 mL of 70% methanol by heat refluxing and filtered through 0.45-µM membrane filters. Analysis was performed using a HPLC system equipped with a Phenomenex C18 column (25 cm × 4.6 mm i.d.) with 5-µm particle size, LC 8A preparative pumps, CBM-20A Communication Bus Module system controller, SPD-M20A photodiode array detector (Shimadzu, Japan), and LC solution software Release 1.24. The mobile phase

Table 1. ISSR fingerprinting analysis of encapsulated shoot tips and nodal segments of *Bacopa monnieri* after 6 months of storage at 4 °C.

S. no.	Name of the primer	Nucleotide sequence (5'-3')	Total no. of amplified bands	Amplicon size range (bp)	No. of polymorphic bands
1.	ISSR 33	AGAGAGAGAGAGAGAGA	9	250–1000	0
2.	ISSR 35	AGAGAGAGAGAGAGAGT	5	270–950	1
3.	ISSR 42	ACACACACACACACACCG	6	200–1100	0
4.	ISSR 46	ACACACACACACACACGG	10	250–2000	1
5.	ISSR 67	TCTCTCTCTCTCTCCC	5	200–700	0
6.	ISSR Y5	CTCTCTCTCTCTCTCTAC	3	300–550	1
7.	ISSR Y11	GAGAGAGAGAGAGAGAT	4	200–600	1
8.	ISSR A1	GAGAGAGAGAGAGAGA	5	600–1500	0
9.	ISSR A3	ACACACACACACACACC	5	350–1000	1
10.	ISSR A5	GAGAGAGAGAGAGAGATC	6	400–1000	0
11.	ISSR A6	GACAGACAGACAGACA	8	400–1500	0
12.	ISSR A8	ACACACACACACACACT	7	750–2000	0
13.	ISSR A10	AGAGAGAGAGAGAGAGG	4	250–1000	0
14.	ISSR A11	GAGAGAGAGAGAGAGAGAT	8	400–1500	0
15.	ISSR A12	TCTCTCTCTCTCTCTCG	2	400–500	0
15.	ISSR A13	CCGCCGCCGCCGCCGCCG	5	400–1000	0
16.	ISSR A14	TAGAGAGAGAGAGAGAGAG	12	400–2000	0
17.	ISSR A15	ACACACACACACACACACT	4	250–900	0
18.	ISSR A16	CTCCTCCTCCTCCTCCTC	8	400–1400	0
19.	HB12	CACCACCACGC	7	600–2500	0
20.	HB 15	GTGGTGGTGGC	7	400–1500	0
		Total	130		5

was a mixture of 0.2% phosphoric acid and acetonitrile (65:35, v/v; pH 3) at a flow rate of 1 mL/min and column temperature was maintained at 30 °C. The detection wavelength was set at 205 nm. The injection volume was 20 µL and the chromatography system was equilibrated by the mobile phase. Bacoside A standard was obtained from Natural Remedies Pvt. Ltd., Bangalore, India.

3. Results and discussion

3.1. Plant regeneration after storage

Finding the suitable storage temperature plays a crucial role in cold preservation. Three different temperatures of 4, 8, and 24 °C were tested for storing the encapsulated propagules (Figures 1a and 1b) for about 1 month. Results showed drastic differences in conversion response among the temperature profiles tested. The beads at 24 °C started sprouting after 4 days of storage and thus it was found to be inefficient for storage (Figure 1c). Meanwhile, 100% regrowth was seen in shoot tip-derived seeds stored at 4 °C as compared to the nonstored controls (Figure 2). Previously, 4 °C was reported to be very promising for low-temperature storage in *Rauvolfia serpentina* L. (Ray et al., 2008), *Spilanthes acmella* L. (Singh et al., 2009), and *Eclipta alba* L. (Ray et al., 2010). Kavyashree et al. (2006) also reported maximum conversion response of mulberry synseeds stored at 4 °C. Therefore, further storage of encapsulated shoot tips and nodal segments was done only at 4 °C for about 6 months.

It is worth noting here that the concentration of sucrose used for encapsulation also plays a vital role in plant regeneration after storage. Percentage of regrowth was high with lower or decreased concentrations of

Table 2. RAPD fingerprinting analysis of encapsulated shoot tips and nodal segments of *Bacopa monnieri* after 6 months of storage at 4 °C.

S. no.	Name of the primer	Nucleotide sequence (5'-3')	Total no. of bands	Amplicon size range (bp)	No. of polymorphic bands
1.	OPA 02	TGCCGAGCTC	8	200–1500	0
2.	OPA 03	AGTCAGCCAC	3	500–800	0
3.	OPA 04	AATCGGGCTG	5	100–300	1
4.	OPA 10	GTGATCGCAG	2	400–500	1
5.	OPA 11	CAATCGCCGT	9	450–2000	1
6.	OPB 01	GTTTCGCTCC	2	500–900	0
7.	OPB 04	GGACTGGAGT	5	350–1000	0
8.	OPC 02	GTGAGGCGTC	5	300–1050	1
9.	OPC 07	GTCCCGACGA	2	1000–1050	1
10.	OPC 18	TGAGTGGGTG	6	300–1000	0
11.	OPD 02	GGACCCAACC	5	350–1000	0
12.	OPD 03	GTCGCCGTCA	4	400–2000	1
13.	OPD 08	GTGTGCCCCA	4	100–850	1
14.	OPE 01	CCCAAGGTCC	5	700–2000	0
15.	OPE 03	CCAGATGCAC	5	600–2000	0
16.	OPH 02	TCGGACGTGA	6	700–2200	0
17.	OPH 03	AGACGTCCAC	6	400–900	0
18.	OPH 04	GGAAGTCGCC	6	700–1800	0
19.	OPH 05	AGTCGTCCCC	2	700–1000	0
20.	OPG 01	CTACGGAGGA	3	500–1300	0
21.	OPG 02	GGCACTGAGG	7	600–2000	0
22.	OPG 04	AGCGTGTCTG	5	300–1500	0
23.	OPG 05	CTGAGACGGA	4	500–1500	0
24.	OPF 01	ACGGATCCTG	7	500–2000	0
25.	OPF 02	GAGGATCCCT	5	250–800	0
		TOTAL	115		7

sucrose compared to the normal 3%. While using the normal sucrose concentration for encapsulation, we could regrow 48.3% of encapsulated shoot tips (Figure 3). This has been found to correlate with the results of storing encapsulated shoot tips of *Eclipta alba* in different sucrose concentrations. It has also been demonstrated that sucrose concentrations lower than 3%, i.e. 1% or 2%, had an impact in enhancing storage potential of synseeds up to a certain period (Ray et al., 2010).

A high percentage of regrowth of about 86.6% was obtained from shoot tip-derived synseeds and 60% of regrowth from nodal segments after 6 months of storage (Figure 3). Very high frequencies of shoot regrowth were obtained in *Glycyrrhiza glabra* L. (98%) (Mehrotra et al., 2012) and in *Cineraria maritima* L. (82%) (Srivastava et al., 2009) following 6 months of storage. Hung et al. (2012) also reported 71%–98% regrowth in *Khaya senegalensis* and 92%–100% from *Corymbia torelliana* × *C. citriodora*

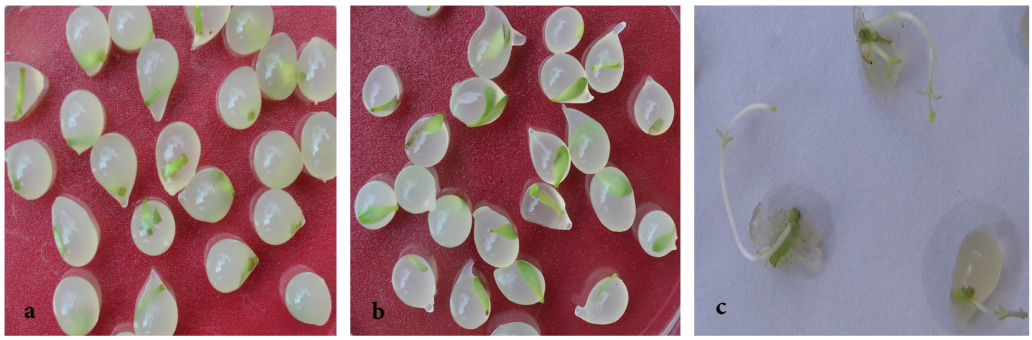

Figure 1. Storage of encapsulated propagules of *Bacopa monnieri*: a- encapsulated nodal segments, b- encapsulated shoot tips, c- emergence of shoots while storing the encapsulated nodal segments of *B. monnieri* at 24 °C.

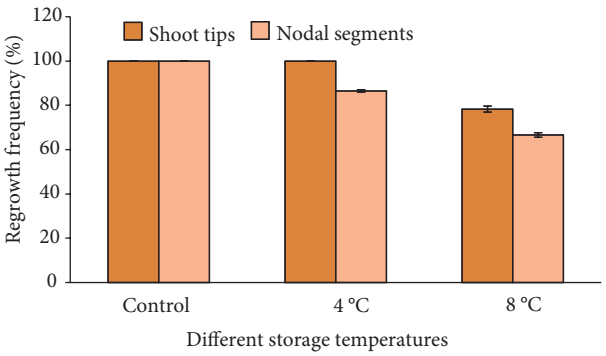

Figure 2. Effects of different storage temperatures on frequency of regrowth of encapsulated in vitro propagules of *B. monnieri* following 1 month of storage.

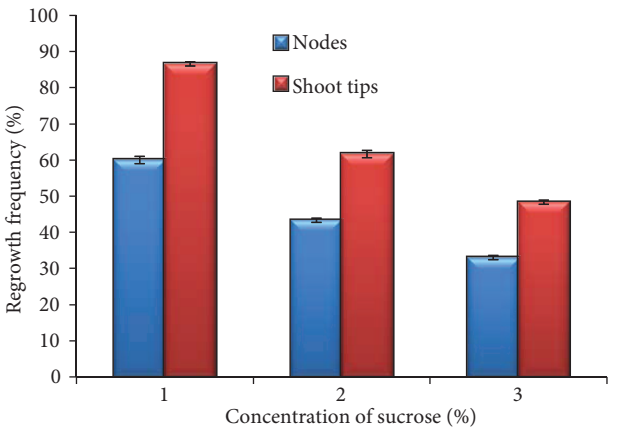

Figure 3. Effects of sucrose concentrations on frequency of regrowth of encapsulated in vitro propagules of *B. monnieri* after 6 months of storage at 4 °C.

after 12 months of storage. In addition, shoot tip-derived beads started rupturing (Figure 4a) within 10 days after transferring them to regeneration medium. Figure 4b shows a closer view of a sprouted synseed with 3–4 shoots. This substantiates the advantages of using shoot tips for encapsulation, like high survival, increased regrowth percentage, and high genetic stability (Paul et al., 2000). Shoot tips have been found to possess reliable regrowth capacity in medicinal plants like *Picrorhiza kurrooa* Kutki (Mishra et al., 2001), *Rouvolfia serpentina* (Ray et al., 2008), *E. alba* (Ray et al., 2011), *Camellia sinensis* L. (Mondal et al., 2002), and *Zingiber officinale* Rosc. (Sundararaj et al., 2010). The robustness of the roots and the multiple shoots (Figure 4c) of the plantlets regrown from encapsulated shoot tips after storage demonstrated the efficiency of the storage potential. Hardening was done after 25 days. Thirty healthy regrown plants were transferred to small pots containing an autoclaved mixture of red soil and vermicompost at a 1:1 ratio. The pots were covered with polythene bags with minute holes to maintain humidity and placed inside the laboratory. After 2 weeks, they were moved to a greenhouse for further growth. Out of 30 plants, 28 survived the acclimatisation process and remained healthy in natural conditions with a hardening rate of 93%. Hence, the protocol devised can be reliably utilised for mass conservation of this potent multipurpose ayurvedic herb under minimal requirements without any subculturing for the stipulated period of 6 months.

3.2. ISSR and RAPD analysis

Twenty ISSR primers created 130 amplified products, out of which 5 bands were polymorphic while the rest were monomorphic. The number of bands for each primer varied from 12 (ISSR A14) to 3 (ISSR Y5) with an average of 6 bands per primer. The size of amplified products ranged from 200 to 2000 bp (Table 1). The ISSR technique detected a polymorphism rate of 4% and the amplification profiles of primers ISSR 35, HB 15, and HB 12 are depicted in Figures 5a–5c. On the other hand, 25 random

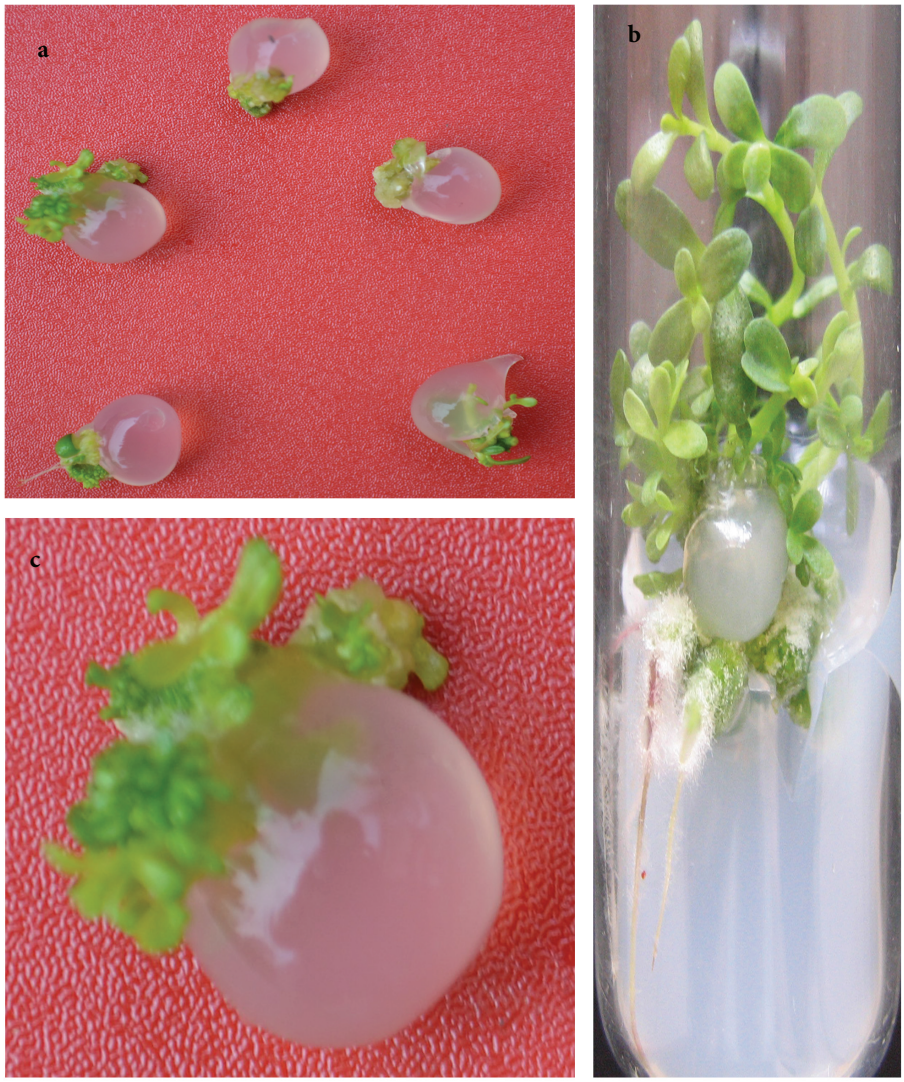

Figure 4. Plant regrowth from encapsulated shoot tips of *Bacopa monnieri* after 6 months of storage at 4 °C: a- sprouting of encapsulated shoot tips of *B. monnieri* after 6 months of storage at 4 °C, b- closer view of the sprouted synseed, c- regrown plantlet with healthy shoots and roots from shoot tip-derived synseed after 6 months of storage at 4 °C.

decamer RAPD primers amplified the genomic DNA of regrown plantlets and produced a sum of 115 scorable and reproducible bands with an average of 4.4 bands per primer. The number of bands ranged from 9 (OPA 11) to 2 (OPA 10, OPC 07, and OPB 01) of about 100–2000 bp in size (Table 2). Seven polymorphic bands were detected from the total of 115 bands, corresponding to 6% polymorphism. The banding patterns of primers OPF 02, OPH 04, and OPB 04 are shown in Figures 5d–5f. It is very important to note here that the variations were detected only in DNA samples of regrown plantlets derived from nodal segments and not from shoot tips. From this it is seen that using shoot tips for encapsulation and storage is wise as it maintains the genetic fidelity.

Both marker systems identified low genetic variation between the control mother plant and the randomly selected regrown plantlets after 6 months of storage. This highlights the genetic stability of meristamatic tissues like shoot tips used for encapsulation. Thus, the clonal nature of the stored plantlets has proven that the process of storage did not affect the genetic makeup of the plantlets. Similar types of studies dealing with low-temperature storage combined with genetic fidelity analysis have been reported for some medicinal plants. Using RAPD primers, the encapsulated microshoots of *Picrorhiza kurrooa* Kutki (Mishra et al., 2001), *Eclipta alba* (Ray et al., 2010), and in vitro regenerants of *Dioscorea bulbifera* L. (Narula et al., 2007) were analysed for genetic

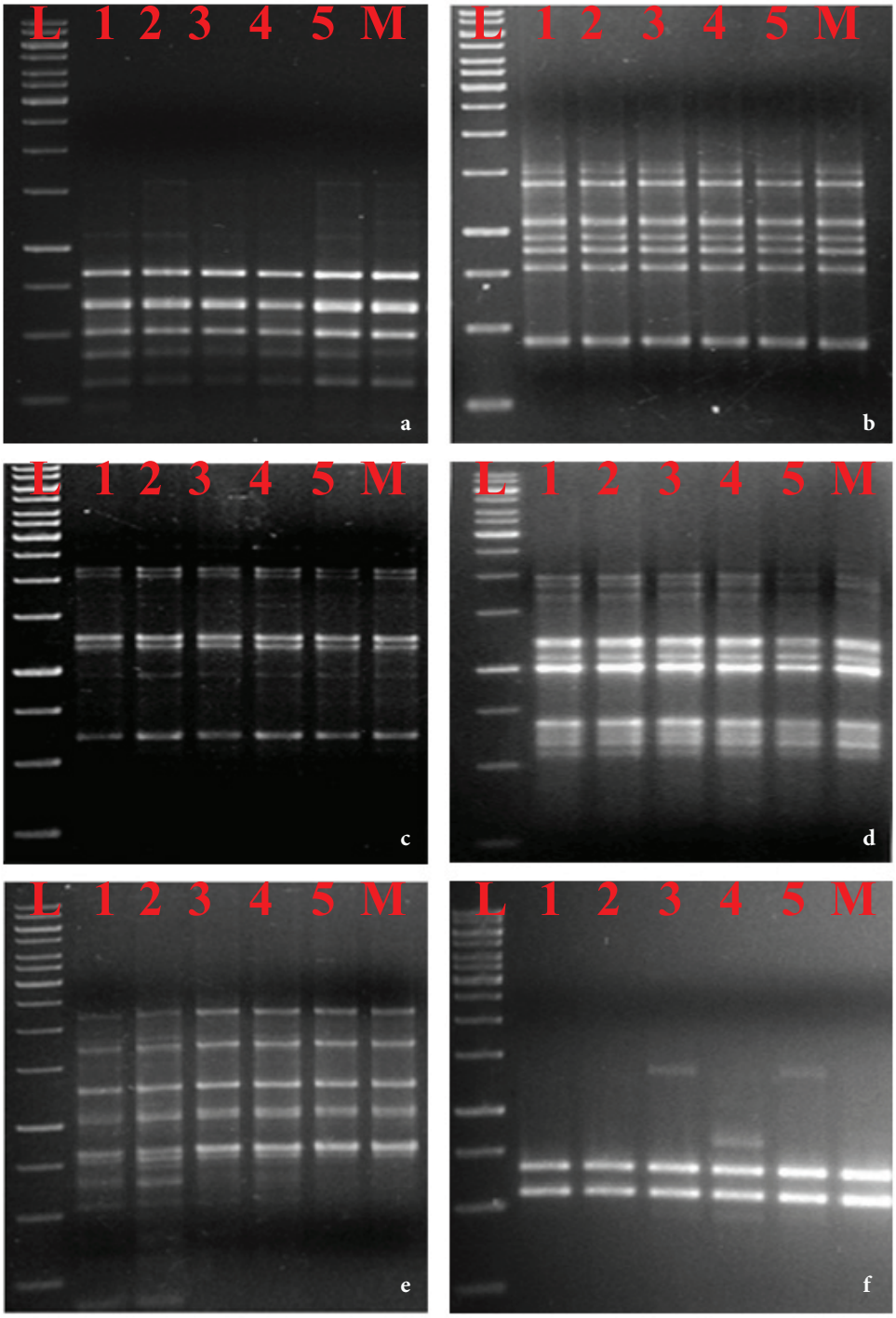

Figure 5. ISSR and RAPD banding profiles of the regrown plantlets after 6 months of storage at 4 °C. ISSR banding pattern obtained on using the following primers: a- ISSR 35, b- HB 15, c- HB 12; RAPD amplification obtained on using the following primers: d- OPF 02, e- OPH 04, f- OPB 04. L: 1-kb ladder; 1 to 5: randomly selected regrown plantlets after 6 months of storage at 4 °C; M: mother plant.

variation after low-temperature storage. ISSR markers were utilised to check the genetic stability of encapsulated nodal segments of *Cannabis sativa* L. stored for 6 months (Lata et al., 2011), along with micropropagated guava (Liu and Yang, 2012) and gerbera plants (Bhatia et al., 2009). The RAPD technique was utilised to assess the genetic

fidelity of *Viola patrinii* DC. regenerated via petiole callus (Chalageri and Babu, 2012). Genetic stability of long-term micropropagated shoots of banana, *Vanilla planifolia* Andrews, and almonds were assessed using RAPD and ISSR markers by Lakshmanan et al. and Sreedhar et al. in 2007 and Martins et al. in 2004, respectively. Devarumath et al. (2002) tested the genetic nature of in vitro grown tea clones with the help of RAPD, ISSR, and RFLP techniques. Recently in *Glycyrrhiza glabra* L., a low level of genetic polymorphism was found by using RAPD and ISSR markers after 6 months of storage (Mehrotra et al., 2012).

3.3. HPLC analysis

As bacoside A is the most important secondary metabolite of the plant used commercially in large quantities, we have estimated its quantity in mother and regrown plants after the storage process. HPLC analysis revealed the homogeneity in bacoside profiles and contents in mother and regrown plants. The quantity of the 4 different components of bacoside A (bacoside A_3, bacopaside II, jujubogenin isomer of bacopasaponin C, and bacopasaponin C) in the mother and regrown plants is shown in Table 3. Results showed that the storage process did not affect the chemical nature of the regrown plants. Quantification of phytochemicals in plants regrown after low temperature is inevitable so that it is confirmed that the storage process has maintained the chemical stability of the plant. However, only a very few reports are available in this regard. Lata et al. (2011) estimated the cannabinoids content of a *Cannabis sativa* mother plant and randomly selected clones propagated through synthetic seeds following storage for 6 months. They showed that the level of Δ^9-tetrahydrocannabinol, the major psychoactive compound, in the mature buds of the synthetic seeds' raised clones of *C. sativa* plants was comparable to that of the mother plant. Similarly, we have also estimated the quantity of 4 compounds of bacoside A in mother and regrown plantlets. As the storage process maintained the quantity of bacoside A, stored plants can be efficiently utilised commercially for extraction of the same.

Therefore, in order to obtain genetically and chemically stable regrown *B. monnieri* plants after the process of storage at 4 °C for 6 months, shoot tips should be utilised as explants for encapsulation. The simple and cost-effective technique followed here has much importance in the context of germplasm exchange. This has also opened new horizons for further research in this plant to preserve its elite tissues permanently at ultralow temperatures.

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Table 3. Estimation of 4 components of bacoside A in mother and regrown plants after the storage process by means of HPLC.

S. no.	Name of the subcompound of bacoside A	Quantity in mother plant (%)	Quantity in regrown plants after the storage process (%)
1.	Bacoside A3	0.60	0.62
2.	Bacopaside II	0.65	0.64
3.	Jujubogenin isomer of Bacopasaponin C	0.97	1.01
4.	Bacopasaponin C	0.89	0.84

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