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ORIGINAL PAPER

Diversity among wild accessions of *Bacopa monnieri* (L.) Wettst. and their morphogenetic potential

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Abstract Biochemical and molecular diversity among 14 accessions of *Bacopa monnieri* (L.) Wettst. collected from various locations of India was investigated. A significant variation was recorded in bacoside A contents of these accessions. A scatter plot of principle component analysis based on bacoside A contents clubbed these populations into two major groups and accession BM14 was placed separately. Similarly, about 35 % variations were detected in these populations based on combined data of random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR). Individually, ISSR markers detected higher variation (44.9 %) as compared to RAPD markers (23 %). Clustering based on molecular marker data grouped these accessions into two major groups and also placed accession BM14 as an out group. The shoot organogenic potential of leaf explants taken from microshoots and rooting of microshoots also varied among accessions. Maximum shoot organogenic potential was observed in accession BM5 and maximum rooting potential was observed in accessions BM1, BM2, BM7, BM10 and BM14. Present study is an important step for developing long-term strategy for conservation of this important medicinal herb.

Keywords Conservation · Micropropagation · Molecular markers · Shoot organogenesis

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Introduction

Bacopa monnieri (L.) Wettst., family Scrophulariaceae, is popularly known as 'Brahmi'. It grows in wet, damp and marshy areas and on the banks of slow flowing rivers and lakes (Anonymous 1988), ascending up to an altitude of 1,320 m amsl (National Medicinal Plants Board, NMPB 2008). It is used in traditional Ayurvedic medicine for improving memory (Russo and Borrelli 2005), treatment of anxiety and to prepare popular Ayurvedic preparations like 'Brahmirasayanam' and 'Brahmighritam' (Govindrajan et al. 2005; Prasad et al. 2008). In addition to memory boosting activity, it is also used for treatment of cardiac, respiratory and neuropharmacological disorders like insomnia, insanity, depression, psychosis, epilepsy and stress (Russo and Borrelli 2005; Nadkarni 1976). The pharmacological properties of brahmi are mainly attributed to the presence of saponins called 'bacosides' (Singh and Dhawan 1997). Bacosides are complex mixture of closely related compounds, glycosides of either jujubogenin or pseudojujubogenin (Rastogi et al. 1994).

Due to various pharmacological properties, *B. monnieri* is placed second in a priority list of the most important medicinal plants (Rajani 2008) and is one among 32 medicinal plant species identified for cultivation and conservation by the NMPB (National Medicinal Plants Board 2004). *B. monnieri* is also one among the seven medicinal plants recommended for immediate attention by NMPB and Technology Information Forecasting and Assessment Council (TIFAC). According to NMPB (National Medicinal Plants Board (NMPB) Technology Information Forecasting and Assessment Council (TIFAC), Department of Science and Technology, Government of India 2007) market demand for 'brahmi' is estimated around 1,000 tones per year and is likely to increase due to its

multipurpose therapeutic uses. The entire demand of raw material is met from the wild populations. Quality of raw material of medicinal plants especially collected from wild populations is likely to be affected by genotype (Nadeem et al. 2002). Recently, there are many reports indication the status of *B. monnieri* as an endangered plant (Tripathi et al. 2012; Karthikeyan et al. 2011; Ramesh et al. 2011). Therefore, there is a need for identification of elite accessions (based on active principle content) and molecular characterization of wild population for diversity documentation on one hand and optimization of in vitro propagation protocol (applicable for range of populations) for conservation on the other hand.

Polymerase chain reaction (PCR)-based DNA markers such as random amplified polymorphic DNA (RAPD; Williams et al. 1990), and inter-simple sequence repeats (ISSR; Bornet and Branchard 2001) have been widely used to study the genetic diversity within the various populations of different plant species including medicinal plants (Jayanthi and Mandal 2001; Rout and Das 2002; Chatterjee and Pradeep 2003; Tripathi et al. 2012). The RAPD marker has been used to study the genetic variations among plants of *B. monnieri* collected from different locations of Southern India (Karthikeyan et al. 2011) and Central India (Tripathi et al. 2012).

Micropropagation of *B. monnieri* through apical shoot multiplication (Tiwari et al. 2001), de novo shoot regeneration (Aggarwal et al. 2012; Tiwari et al. 1998, 2001, 2006; Ceasar et al. 2010; Shrivastava and Rajani 1999) and somatic embryogenesis (Tiwari et al. 1998) has been reported. However, plants are known to exhibit clonal variations in different morphogenetic responses in culture (Aggarwal et al. 2012). Therefore, the present study was designed to investigate the variations in the levels of bacoside A (important active principle) and genetic diversity among 14 accessions of *B. monnieri* collected from different locations of India using RAPD and ISSR markers. Variation in the levels of bacoside A was also studied in these accessions to select the elites. Further, shoot organogenic potential of leaf explants and rooting of microshoots was also investigated. These studies will be helpful in developing the strategies for the conservation of this medicinally important herb.

Materials and methods

Plant material, chemicals culture conditions

Plants of *B. monnieri* (L.) Wettst. were collected from 14 different locations of India (Table 1). These were multiplied by vegetative propagation and maintained in nursery at TIFAC-CORE, Thapar University, Patiala, India. All routine chemicals were purchased from HiMedia Laboratories (Mumbai, India), and plant growth regulators and bacoside A were purchased from Sigma Chemical Co. (St Louis, MO, USA). Unless otherwise mentioned, all tissue culture experiments were conducted in 300-ml glass culture bottles (Kasablanka, Mumbai) with 50 ml of Murashige and Skoog medium (Murashige and Skoog 1962) containing 58 mM sucrose, 0.7 % (w/v) agar (MS medium), and variously supplemented with plant growth regulators. The pH of medium was adjusted to 5.8 before autoclaving (121 °C, 20 min). Unless otherwise mentioned, cultures were incubated at 25 ± 1 °C under cool white fluorescent lights (Philips India Ltd, Mumbai) with light intensity of 42.0 µmol m^{-2} s^{-1} (inside culture vessels) and a 16 h light/8 h dark cycle.

Estimation of bacoside A

Samples (aerial parts of plants) were harvested during September and dried in shade. These were grounded to fine powder using blender and used for estimation of various components of bacoside A, namely bacoside A3, bacopaside II and bacosaponin C.

Extraction of bacoside A

The samples were extracted according to Phrompittayarat et al. (2007) with minor modification. In brief, samples (1.0 g dried powder in triplicate) were soaked in 10.0 ml water for 24 h. These were filtered through glass wool and filtrates were discarded. Residues were extracted with 20.0 ml of aqueous ethanol (95 %, v/v) for 3 days. These were then filtered. The extraction was repeated three times (×20 ml) with 95 % (v/v) ethanol. Filtrate from three extractions was pooled and dried in vacuo. Residues were reconstituted 1.0 ml methanol and filtered through 0.45 µm filters (Millipore-Carrigtwohill, Ireland) prior to quantification using high-performance liquid chromatography (HPLC).

Quantification of bacoside A

Bacoside A contents in purified extracts were estimated using reverse phase HPLC (Waters Corporation, USA) equipped with high-pressure binary pump system (515), PDA detector (2998) and Rheodyne injector with 20-µl sample loop. Samples (20 µl) were injected through injector into Sunfire™ C18 column, 250 mm × 4.6 mm i.d. particle size 5.0 µm (Waters, Ireland) and elution was carried out in an isocratic mode with a mobile phase consisted of aqueous acetonitrile (65:35 v/v) containing phosphoric acid (0.2 %, v/v; pH 3.0) at a flow rate of 1.0 ml min^{-1}. Column eluates were monitored with online

Table 1 Accessions of *B. monnieri* collected from various locations and their Bacoside A contents (mg g^{-1} D. Wt.)

S. no.	Accession code	Place of collection	Bacoside A3	Bacopaside II	Bacopasaponin C	Bacoside A
1	BM1	Kolkota	0.83[a]	0.63[c]	2.48[a]	3.95[a]
2	BM2	Solan	0.67[e]	0.68[b]	1.47[e]	2.83[e]
3	BM3	New Delhi area	0.53[f]	0.58[d]	1.47[e]	2.60[h]
4	BM4	Yamunanagar	0.75[d]	0.70[a]	1.47[e]	2.93[d]
5	BM5	Chandigarh	0.76[e]	0.53[e]	2.37[c]	3.66[c]
6	BM6	Patanjali yogpeeth, Haridwar	0.67[e]	0.63[c]	1.38[f]	2.69[f]
7	BM7	FRI, Dehradun	0.83[a]	0.63[c]	2.48[a]	3.95[a]
8	BM8	Kolher Pani, Dehradun	0.39[g]	0.58[d]	1.24[g]	2.23[k]
9	BM9	Manakpur	0.67[e]	0.58[d]	1.38[f]	2.64[g]
10	BM10	Ambala	0.78[b]	0.14[f]	1.61[d]	2.54[i]
11	BM11	Varanasi	0.83[a]	0.58[d]	2.47[b]	3.90[b]
12	BM12	Saharanpur	0.39[g]	0.58[d]	1.47[e]	2.46[j]
13	BM13	Rohtak	0.65[f]	0.09[g]	1.20[h]	1.94[l]
14	BM14	Joginder Nagar (H.P.)	0.13[h]	0.58[d]	0.54[i]	1.26[m]

Values are average of three replicates. Means followed by the same letter are not significantly different at 0.05 % by Duncan's multiple range test

PDA detector set at 205 nm. Quantifications were carried out using external standard curve plotted by taking known quantities of standard compounds (individually bacoside A3, bacopaside II, bacosaponin C). Plants from different populations were grouped based on bacosides content by principle component analysis using loading plots (SPSS 16, IBM, Chicago, USA).

Genetic diversity

PCR-based markers (RAPD and ISSR) were used to study the genetic diversity among various accessions collected from different locations. Genomic DNA was isolated from actively growing shoots (2.0 g each sample) using the modified CTAB method (Doyle and Doyle 1990). Quality of DNA was checked on 0.8 % (w/v) agarose gel and quantified using Nanodrop 1000 Spectrophotometer (Thermo Scientific). All the samples were adjusted to uniform concentration (20 ng μl^{-1}).

PCR amplifications were performed in 20-µl volume with 40 RAPD decamer (OPD1–OPD20, OPA1–OPA20; Operon Technologies, Alameda, CA) and 27 ISSR primers (Table 2) primers using Gene Amp 9700 thermal cycler (Applied Biosystems, Foster City, CA). The reaction mixture consisted of 20.0 ng genomic DNA, 1.0 U *Taq* DNA polymerase (Larova, Teltow, Germany), 100 µM dNTPs mixture, 2.0 µl reaction buffer (10×) and 10.0 nmol primer and Milli-Q water (Millipore India, Bangalore, India) was added to make up the volume to 20 µl. Amplification conditions were an initial denaturation at 94 °C for 4 min, 41 cycles of 94 °C for 60 s, 36 °C (55 °C in the case of ISSR) for 45 s and 72 °C for 90 s, with a final extension at 72 °C for 5 min. Amplified products were separated on a 1.2 % (w/v) agarose gel and viewed under the UV trans-illuminator (Gel Doc Mega; Biosystematica, USA) following ethidium bromide staining.

Phylogenetic analysis

The size and number of the amplicons were determined from gel photographs by comparing with molecular weight markers. Each band of amplified DNA fragment was transformed into discrete variables or binary characters matrix, '1' (to mark presence) and '0' (to mark absence). The binary data matrixes were used to estimate the level of polymorphism by dividing the number of polymorphic bands (not present in all samples) by the total number of scored bands. Amplified fragments in the size range of 250–3,000 bp, were included in the analyses. Data were analyzed by Jaccard's coefficient to generate matrix and the values were used to construct dendrograms of unweighted pair group method with arithmetic means (UPGMA) using Multivariate Statistical Package 3.2.1 (MVSP; Kovach Computing Services, Anglesey, Wales). A scatter plot of these accessions was drawn by PCA using the RAPD and ISSR data (SPSS 16) to reveal pattern of relatedness within a matrix coordinates in two dimensions.

Shoot regeneration

Establishment of cultures

Cultures were established using terminal portions of actively growing shoots according to the procedure described earlier (Aggarwal et al. 2012). Following surface

Table 2 Sequence of various ISSR primers used for amplifications and subsequent amplification parameters observed

S. no.	Primer code	Sequence of primer	Amplicon size range (bp)	No amplified bands	No. of polymorphic bands	Percent polymorphism
1	ISSR2	$(GA)_8CG$	450–2,000	14	8	57.14
2	ISSR3	$(GA)_8TC$	250–2,500	16	8	50
3	ISSR4	$(AC)_8GC$ GC	250–2,000	13	5	38.46
4	ISSR5	$(AC)_{10}$	250–2,000	14	6	42.85
5	ISSR7	$(CA)_8GC$	250–2,000	8	4	50
6	ISSR9	$(GC)_8T$	450–2,000	12	7	58.33
7	ISSR10	$(GC)_8A$	250–2,000	14	8	57.14
8	ISSR12	$(CT)_8G$	300–2,500	13	9	69.2
9	ISSR16	$(GT)_8TC$	250–2,000	9	5	55.55
10	ISSR17	$(AT)_8C$	250–2,000	12	9	75
11	ISSR18	$(AT)_8G$	250–2,000	4	1	25
12	ISSR19	$(AT)_8GC$	250–2,000	8	4	50
13	ISSR20	$(AT)_8$	250–2,000	11	9	81.81
14	ISSR21	$(GA)_8TG$	250–2,000	12	5	41.66
15	ISSR22	$(GA)_8C$	250–2,000	11	8	72.72
16	ISSR23	$(GA)_8CT$	250–2,500	13	4	30.76
17	ISSR24	$(GA)_8CA$	250–2,000	7	2	28.57
18	ISSR25	$(GA)_8CC$	250–2,000	9	2	22.22
19	ISSR26	$(GA)_8$ T	250–2,000	7	3	42.85
20	ISSR27	$(CT)_8T$	250–2,000	9	5	55.55
				209 (total no. of bands)	94 (total no. of polymorphic bands)	44.9 (average polymorphism)

disinfection, nodal segments were trimmed from the cut ends and cultured on MS medium supplemented with 2.5 μM benzyladenine (BA). The cultures were sub-cultured on the same medium at 14-day interval. The shoots sprouted from the axillary buds were excised and cultured on same medium. The leaves taken from these microshoots were used for further experimentation.

Shoot organogenesis

Shoot organogenic potential of leaf segments taken from microshoots of various accessions was studied following protocol reported earlier (Aggarwal et al. 2012). Leaves from actively growing microshoots were excised, these were given transverse cuts through midrib (3–4 mm thick) and cultured on MS medium supplemented with 12.5 μM BA and 1.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D). These explants were sub-cultured on the same medium at 4-week interval. Data for the shoot organogenesis were recorded after 8 weeks of culture.

Rooting of microshoots

Microshoots (3–4 cm) from each accession were excised and inoculated on basal MS medium (15 shoots per culture vessel). Data for percent rooting of microshoots, number of roots per shoot and root length were recorded after 2 weeks of culture.

Statistical analysis

Percentage explants showing shoot organogenesis and number of shoots per explant were determined. Each treatment consisted of five culture vessels (three explants in each culture vessel) and experiments were repeated four times. Data were analyzed by analysis of variance (ANOVA). Means were compared Duncan's multiple range test at $P \leq 0.05$.

Results

Bacoside A composition in various accessions of *B. monnieri*

The plants raised from the various accessions of *B. monnieri* collected from various locations of India (Table 1) were propagated in nursery under identical conditions and actively growing shoots were harvested and used for the measurement of bacosides A contents. The major

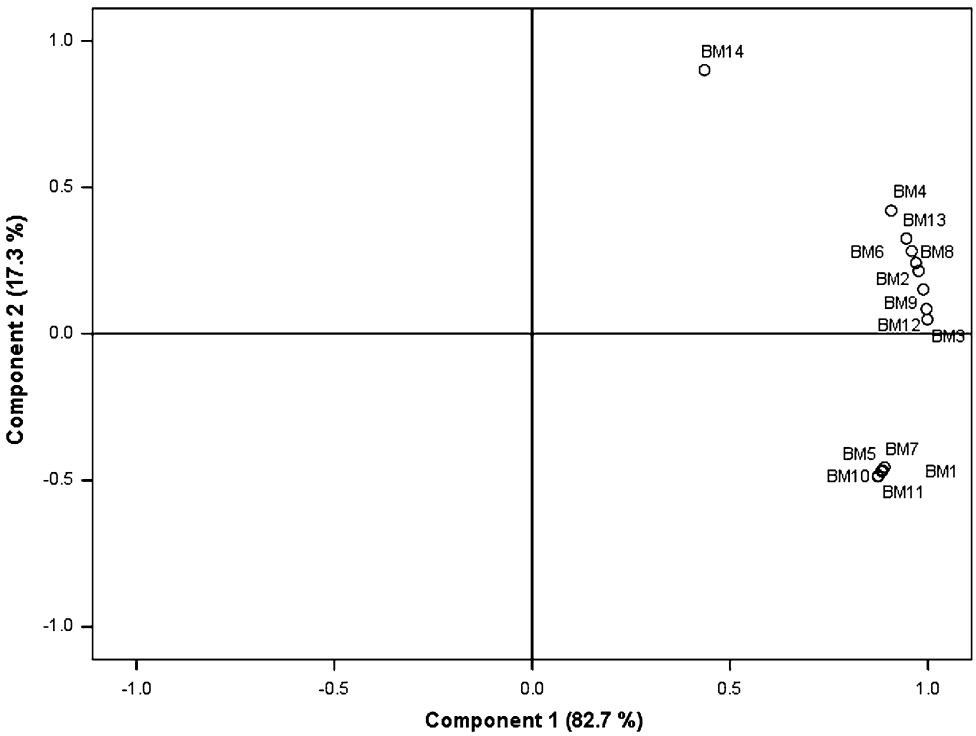

Fig. 1 *Scatter plot* of various accessions of *B. monnieri* by principle component analysis (PCA) of components of 'bacoside A' contents

components of bacoside A namely, bacoside A3, bacopaside II and bacopasaponin C were estimated in these accessions and showed significant variations (Table 1). Maximum level of bacoside A3 was recorded in samples of accessions BM1, BM7 and BM11 (0.83 mg g^{-1} D.Wt.) and minimum level was detected in samples of accession BM14 (0.13 mg g^{-1} D.Wt.). However, bacopaside II contents were higher in accession BM4 (0.7 mg g^{-1} D.Wt.) and lowest level of bacopaside II was recorded in samples of accession BM13 (0.09 mg g^{-1} D.Wt.). Level of bacopasaponin C was much higher as compared to bacoside A3 and bacopaside II (Table 1). Maximum level of bacopasaponin C was recorded in accession BM1 and BM7 (2.48 mg g^{-1} D.Wt.) and lowest level of bacopasaponin C was recorded in samples of accession BM14 (0.54 mg g^{-1} D.Wt.).

Two-dimensional scatter plot of various accessions generated by PCA based on various contents bacoside A revealed that overall variation represented on two components were 82.7 and 17.3 %, respectively. All the accessions fell into two bigger clusters and accession BM14 separated out independently (Fig. 1). All the populations were positively correlated to component 1, whereas on component 2 one group was positively correlated, whereas other group showed negative correlation.

Genetic variations

Genetic variations among these accessions were studied using 40 RAPD and 27 ISSR primers. Out of these, 35 RAPD and 20 ISSR primers produced reproducible and scorable bands and hence used in this study. A total of 422 markers were scored (213 for RAPD primers and 209 for ISSR primers). Among 422 markers scored 143 (33.9 %) were polymorphic. Polymorphic bands recorded for RAPD markers were 49 (23 %) and ISSR markers were 96 (44.9 %). The size of amplified fragment ranged from 250 to 2,500 bp (Table 2; Fig. 2a–d). In case of RAPD, maximum number of markers (13) was scored for primer OPD14 and maximum polymorphic markers (6) were scored with primers OPD14 and OPA2 individually. In case of ISSR primers, average number of markers produced per primer was higher (10.45) as compared to RAPD (6.08). Maximum number of ISSR markers (16) was amplified with primers ISSR2, ISSR5 and ISSR10 and maximum polymorphic markers (9) were scored for primers ISSR12, ISSR17 and ISSR20 (Table 2).

The Jaccard's similarity coefficient of 14 accessions of *B. monnieri* based on RAPD and ISSR revealed that similarity value among accessions ranged from 0.758 to 0.871, indicating moderate levels of genetic similarity (Table 3). Maximum similarity value of 0.871 was recorded among BM9 and BM13. Dendrogram constructed using Jaccard's similarity coefficient is shown in Fig. 3. All the accessions were grouped into two major clusters that got subdivided into smaller groups (Fig. 3). Accession BM14 was placed separately as an out group as also in case of PCA cluster analysis using bacoside A contents (Fig. 1).

Two-component PCA analysis showed 55.0 and 8.7 % variation on component 1 and component 2, respectively

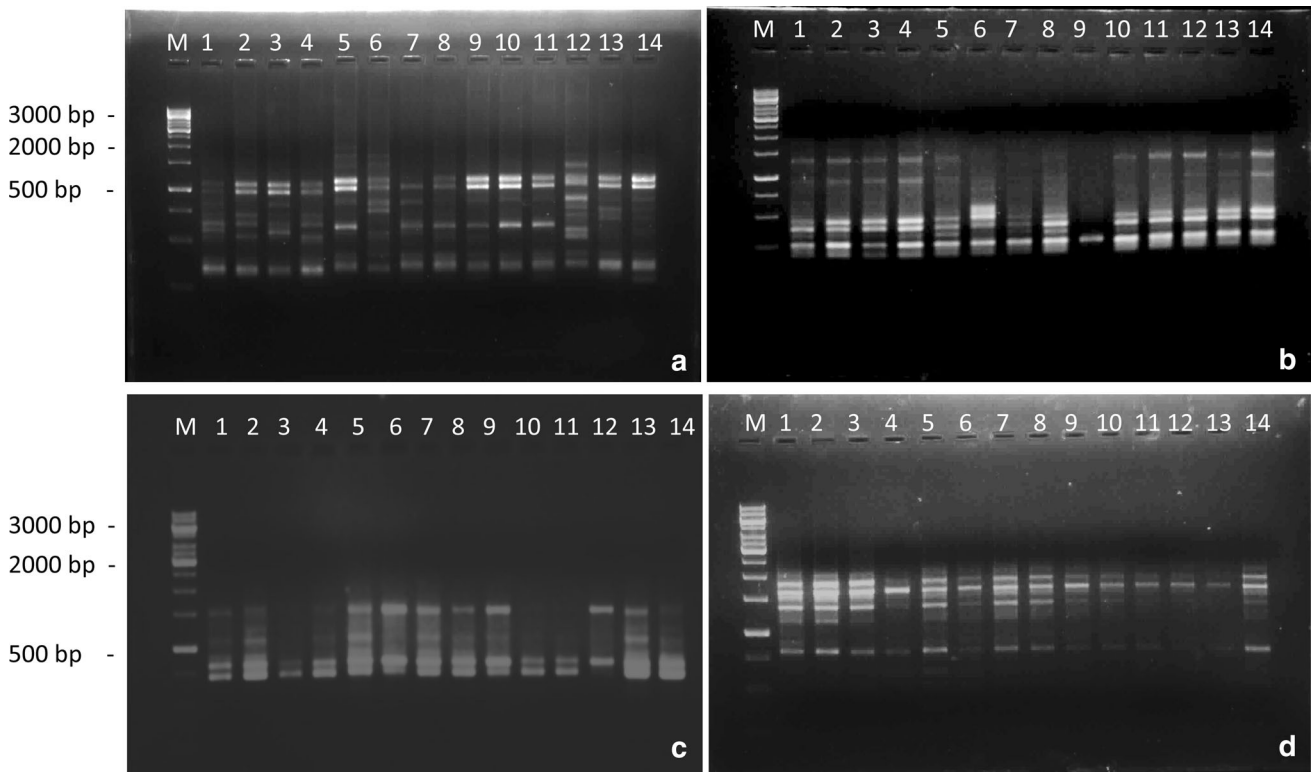

Fig. 2 RAPD (A & B) and ISSR profile of 14 accessions of *B. monnieri* showing the banding pattern. **a** ISSR profile using primer ISSR23; **b** ISSR profile using primer ISSR 26; **c** RAPD profile using primer OPA18; **d** RAPD profile using primer OPA5. *Lane M*: 1 kbp ladder; *lanes 1–14*: accession BM1–BM14, respectively

Table 3 Jaccard's coefficient of various accessions of *B. monnieri* using combined ISSR and RAPD data

	BM1	BM2	BM3	BM4	BM5	BM6	BM7	BM8	BM9	BM10	BM11	BM12	BM13	BM14
BM1	1.000													
BM2	0.871	1.000												
BM3	0.839	0.859	1.000											
BM4	0.822	0.852	0.849	1.000										
BM5	0.841	0.861	0.848	0.847	1.000									
BM6	0.828	0.805	0.826	0.800	0.823	1.000								
BM7	0.829	0.825	0.798	0.835	0.814	0.821	1.000							
BM8	0.824	0.820	0.817	0.806	0.800	0.807	0.850	1.000						
BM9	0.814	0.800	0.802	0.830	0.818	0.816	0.822	0.812	1.000					
BM10	0.798	0.818	0.825	0.828	0.817	0.786	0.830	0.802	0.835	1.000				
BM11	0.820	0.820	0.789	0.773	0.819	0.817	0.822	0.817	0.832	0.841	1.000			
BM12	0.777	0.787	0.785	0.792	0.781	0.808	0.828	0.823	0.818	0.857	0.843	1.000		
BM13	0.780	0.781	0.792	0.799	0.793	0.820	0.825	0.835	0.871	0.854	0.861	0.867	1.000	
BM14	0.804	0.794	0.802	0.815	0.830	0.833	0.802	0.758	0.856	0.837	0.823	0.840	0.816	1.000

(Fig. 4). All the accessions were positively correlated on component 1, whereas on component 2 both positive and negative correlations were observed. In this two-dimensional PCA scatter plot also accession BM14 was again separated out.

Shoot organogenesis

The shoot regeneration potential of various accessions was studied using leaf explants on MS medium supplemented with 12.5 µM BA and 1.0 µM 2,4-D (Table 4). Significant

variation with respect to shoot regeneration was recorded among various accessions. Maximum regeneration potential was recorded in case of accession BM6, where 96 % explants showed shoot organogenesis with an average of 39.8 shoots per explant. In case of accession BM3, only 36.3 % explants showed shoot organogenesis, whereas in case of accession BM14 minimum number of shoots regenerated per explant (Table 4).

Rooting of microshoots

A high rooting efficiency of microshoots was observed on basal MS medium (Table 5). A minimum rooting efficiency of microshoots (93 %) was recorded for accession BM5 and 100 % rooting of shoots was recorded in accessions BM 1, BM2, BM7, BM10 and BM14. Number of roots per shoot varied from 1.3 in accession BM8 and a maximum of 4.0 was recorded in many accessions (Table 5).

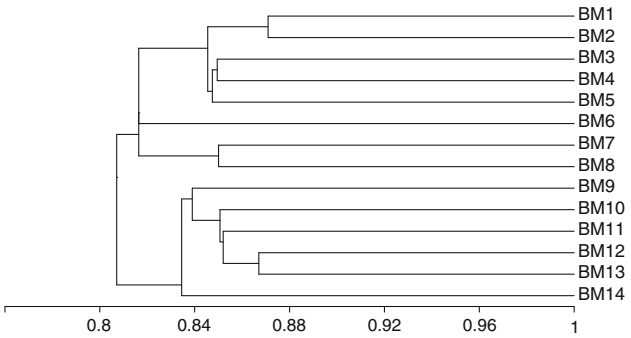

Fig. 3 Unweighted pair group method with average (UPGMA) cluster based on Jaccard's coefficient calculated from combined ISSR and RAPD data various accessions of *B. monnieri*

Discussion

In this study, relationship among 14 accessions of *B. monnieri* collected from various locations of India was studied on the basis of active principle content (bacoside A) and PCR-based molecular markers (RAPD and ISSR). Subsequently, shoot organogenic potential of leaf explants and rooting efficiency of microshoots of these accessions were also investigated. These studies will be helpful in developing strategy for long-term sustainable utilization of this medicinally important plant and a step towards effective conservation. Earlier, efforts have been made for in vitro conservation of this important plant through slow growth under mineral oil (Sharma et al. 2012). The present study was focussed to identify elite genotypes, which can be used for commercial propagation to provide quality raw material and maintain assured supply for various Ayurvedic formulations. The shoot organogenic potential of these accessions was investigated, which could be utilized for in vitro conservation of this important herb.

Various components of bacoside A namely, bacoside A3, bacopasideII and bacosaponin C were detected in all

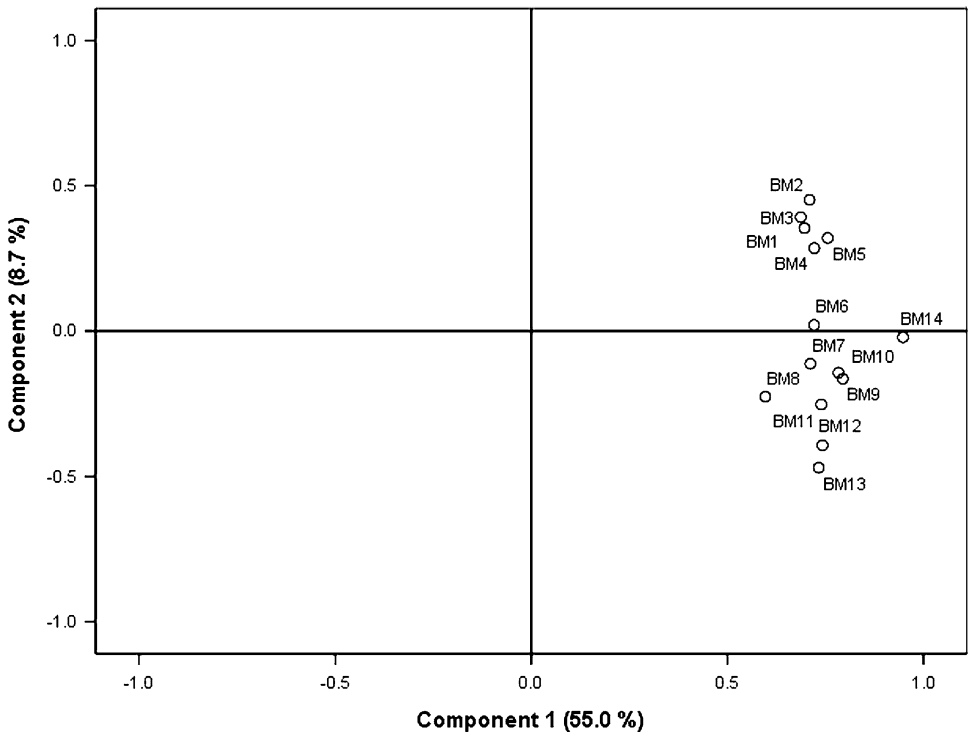

Fig. 4 *Scatter plot* of principle component analysis (PCA) of combined data of RAPD and ISSR of various accessions of *B. monnieri*

Table 4 Comparison of shoot organogenic potential of leaf explants of various accessions of *B. monnieri* on MS medium supplemented with 12.5 μM BAP and 1.0 μM 2,4-D

Populations	Percent explants showing shoot regeneration	No. of shoots per explant
BM1	77.5^{d}	20.4 ± 2.1^{c}
BM 2	75.2^{f}	16.3 ± 1.6^{d}
BM 3	36.3^{m}	14.1 ± 1.3^{g}
BM 4	86.3^{c}	15.3 ± 1.4^{f}
BM 5	95.4^{b}	24.6 ± 2.3^{b}
BM 6	96.5^{a}	39.8 ± 4.8^{a}
BM 7	63.4^{j}	13.8 ± 1.2^{h}
BM 8	73.7^{g}	12.6 ± 0.7^{i}
BM 9	52.1^{l}	11.9 ± 1.6^{j}
BM 10	72.8^{h}	15.8 ± 0.7^{e}
BM 11	53.6^{k}	5.7 ± 0.2^{k}
BM 12	63.9^{i}	3.7 ± 0.02^{m}
BM 13	76.4^{e}	3.9 ± 0.01^{l}
BM 14	72.6^{h}	2.8 ± 0.01^{n}

Data were recorded after 8 weeks of culture. Means followed by the same letter are not significantly different at 0.05 % level by Duncan's multiple range test

Table 5 Comparison of rooting efficiency of shoots of various accessions of *B. monnieri* on MS medium

Accessions	Percent shoots rooting	Number of roots/explants	Root length (cm)
BM1	100	3.6 ± 0.3^{b}	0.3 ± 0.08^{e}
BM2	100	4 ± 0.5^{a}	0.5 ± 0.05^{de}
BM3	97	4 ± 0.0^{a}	0.6 ± 0.05^{de}
BM4	98	4 ± 1.0^{a}	0.56 ± 0.21^{de}
BM5	93	3.6 ± 0.6^{b}	0.5 ± 0.05^{de}
BM6	95	4 ± 0.5^{a}	0.85 ± 0.02^{d}
BM7	100	2.6 ± 0.8^{d}	0.7 ± 0.03^{d}
BM8	99	1.3 ± 0.3^{e}	1.1 ± 0.11^{c}
BM9	95	3.6 ± 0.6^{b}	1.9 ± 0.14^{a}
BM10	100	3.3 ± 0.3^{b}	0.6 ± 0.05^{de}
BM11	99	3.6 ± 0.3^{b}	0.67 ± 0.4^{de}
BM12	98	4 ± 0.0^{a}	0.76 ± 0.06^{d}
BM13	98	3 ± 0.5^{c}	1.5 ± 0.12^{b}
BM14	100	4 ± 0.5^{a}	1.4 ± 0.05^{b}

Data were recorded after 2 weeks of inoculation. Means followed by the same letter are not significantly different at 0.05 % level by Duncan's multiple range test

the accessions. The relative contents of these compounds varied significantly among accessions (Table 1). Earlier, there is only one preliminary report investigating the variation in bacoside A content of accessions of *B. monnieri* (Darokar et al. 2001) and reported a total a 14-fold variation was in bacoside A content of different accessions. In present study, bacoside content varied from 1.26 to 3.95 mg g^{-1} D.Wt, thus showing about threefold variations. Earlier, variation in active principle contents in wild populations of medicinal plants has also been reported (Nadeem et al. 2002, 2007). Further, it has also been suggested that variations in secondary metabolites contents is the function of stress and changing environment (Fernie 2007). Similar findings with respect to secondary metabolite variability have been reported earlier in many plant species (Lim et al. 2005; Cirak et al. 2007; Sanaa et al. 2012). Scatter plot of PCA carried out using bacoside content grouped these accessions into two major groups and accession BM14 was placed separately. It was also important to note that accession BM14 was also separated out when these accessions were grouped based on the molecular marker data (RAPD and ISSR). Thus, biochemical data generated were also in agreement with the molecular marker data.

Documentation and preservation of genetic diversity of natural populations is important for conservation of medicinal plants (Karthikeyan et al. 2011). Genetic diversity was assessed among various accessions of *B. monnieri* using RAPD and ISSR markers. The utility of these markers in studying genetic diversity among wild populations has been highlighted earlier (Pharmawati et al. 2004; Mohapatra and Rout 2005; Barik et al. 2006; Karthikeyan et al. 2011). In the present investigation, 35 RAPD and 20 ISSR primers produced 143 polymorphic bands that unambiguously grouped 14 *B. monnieri* accessions collected from different locations into two major clusters. The level of diversity detected by ISSR (44.9 %) was much higher as compared to the diversity detected by RAPD markers (23 %). This discrimination could be due to the fact that these markers target different parts of the genome (Gajera et al. 2010). The present study reflected higher genetic variations than the earlier reports in *B. monnieri* using RAPD markers (Ramesh et al. 2011; Karthikeyan et al. 2011). In this study, significant polymorphism (more than 35 %) was observed among the accessions, indicating diverse genetic base of these accessions of *B. monnieri* collected from different wild populations. The higher diversity recorded could be due to intraspecific variations reported earlier (Nayak et al. 2006). These results are also in agreement with earlier report (Tripathi et al. 2012). Several studies on assessment of genetic diversity of plants using molecular markers have established the correlation between geographical distance and genetic similarity between individuals (Islam 2004). However, no such correlation could be established in this study. The possible reasons for these differences could be a limited number of markers scored in the earlier studies (Ceasar et al. 2010). Earlier, Cirak et al. (2012) have also reported similar results in *Hypericum orientale*, where no such relations

based on geographical area of collection could be established. This seems to be the first study to investigate genetic variation on the larger group of populations spreading over larger geographical area. Two-dimensional PCA scatter plot of accessions using RAPD and ISSR data also showed nearly same pattern of grouping as UPGMA clustering. It was interesting to note that accession BM 14 was separated in UPGMA dendrogram and two-dimensional PCA analysis performed using biochemical and also molecular data. However, this was not true in case of all accessions. The grouping based on bacoside A content is also in agreement with the molecular marker data.

The shoot organogenic potential of leaf explants varied significantly among different accessions (Table 4). This could be due to genetic variations among these accessions as detected by RAPD and ISSR. It was important to note that such differences were not specific to climatic conditions of the place of collection ruling out the possibility of sole role of environmental factors in these variations as observed by Tripathi et al. (2012). This hypothesis is supported by data on active principle (bacoside A) and also RAPD- and ISSR-based molecular marker. The 96.5 % of explants taken from accession BM6 showed shoot organogenesis with 39.8 shoots per explant, whereas 36.3 % explants from accession BM3 showed shoot organogenesis with 11.9 shoots per explant. The variation in organogenic potential of genetically diverse groups has also been reported in other plants (Guo and Cao 1982; Khanna and Raina 1988; Pandey et al. 1994; Hsia and Korban 1996).

Rooting is one of the most important parameters for the vegetative and in vitro propagation of important plants with an aim to preserve the genetic diversity in such genetically diverse populations. Similarly, rooting potential of these accessions also varied significantly. Although, many factors are known to influence the rooting of microshoots, genetic makeup of plant has been reported as one of the most important factors (Aggarwal et al. 2011). These results are in line with the earlier findings on other plant species (Dick et al. 1996; Vahdati et al. 2004). This difference in potential of rooting of different accessions could be due to the difference in the levels of endogenous auxins and/or differential sensitivity of the tissue towards the endogenous auxins. Further work is required to be taken up to arrive at the logical conclusion.

From this study, it can be concluded that there is a significant variation in bacoside A contents of the various accessions collected from different locations. The RAPD and ISSR markers also documented considerable variations among these accessions and thus both biochemical and molecular data were in line with each other. Further, level of variations recorded with ISSR data was higher as compared to RAPD data. The shoot organogenic potential of the leaf explants and rooting potential of microshoots also varied significantly from accession to accession, thus highlighting a need to optimize clone-specific micropropagation protocol for effective in vitro conservation.

Author contribution Ms. Mahima Bansal (Project Fellow), She has been involved in performing the various experiments, data analysis and writing the initial draft of manuscript. Dr. Anil Kumar, He has been involved in the designing and execution of various experiments and finalized the manuscript for submission to journal. Prof. M. Sudhakara Reddy, He has been involved in designing and data analysis of experiments related to the molecular markers. Corrected the final version of manuscript submitted to journal.

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