Int J Pharm Bio Sci 2015 July; 6(3): (B) 746 - 756

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

REGENERATIVE POTENTIAL AND PHYTOCHEMICAL DIVERSITY AMONG FIVE ACCESSIONS OF *BACOPA MONNIERI* (L.) WETTST

A. KOUL[1], M. KUSHWAHA[2], A.P. GUPTA[2†] AND S. MALLUBHOTLA[1*]

[1]School of Biotechnology, Shri Mata Vaishno Devi University, Katra-182320, J&K, India
[2]QC-QA, Indian Institute of Integrative Medicine, CSIR Jammu-180001, India

ABSTRACT

Distant geographical niches have shown a pronounced effect on variability in organoleptic characteristics as well as biochemical constituents between accessions of a given plant species, keeping this in view the present study has been designed using five accessions of memory enhancing herb *Bacopa monnieri*. Maximum friable callus was regenerated in accession BM003 (GI: 54.61 ± 0.11) on B_5 media supplemented with 2,4-D (1.0 mg l^{-1}) while, MS media with NAA (1.0 mg l^{-1}) + BAP (0.5 mg l^{-1}) resulted in maximum indirect organogenesis in BM003 (GI: 99.58 ± 0.06). Biochemical profiling proved the qualitative differences in the carbohydrate, alkaloid, saponin and terpenoid contents in leaf, shoot and root samples of all accessions. LC-ESI-MS analysis revealed that the shoot extract of accession BM002 (7236.38 µg gm^{-1}) contains highest Bacoside content as compared to that of its root extract (1015.32 µg gm^{-1}) and the shoots had higher Bacoside contents as compared to the roots.

KEYWORDS: Brahmi, micropropagation, biochemical profiling, Liquid chromatography-Electro spray ionization-Mass spectrometry.

S. MALLUBHOTLA
School of Biotechnology, Shri Mata Vaishno Devi University, Katra-182320, J&K, India

***Corresponding author**

†Co-Corresponding author

A.P. GUPTA
QC-QA, Indian Institute of Integrative Medicine CSIR-Jammu-180001, J&K, India

INTRODUCTION

Bacopa monnieri (L.) Wettst is an elite nootropic perennial herb found throughout the Indian subcontinent[2]. The herb is reported to have an extensive range of pharmacological properties viz. antioxidant, antiinflammatory, cardiotonic and cognitive enhancing which are attributed due to the presence of triterpenoid saponins, bacosides[9, 10, 19, 25]. Researchers have developed many techniques for conservation of this threatened species[22, 24] through *in vitro* regeneration using varied types of explants[3, 13, 14, 15]. Extensive work has been reported on the effect of media manipulations and elicitation for secondary metabolite production[5, 20, 24].These reports have used a single accession of the plant species. The present study was conducted using five accessions of a *B. monnieri* collected from different ecological niches (Table: 1), to evaluate the differences in structural, morphological, phytochemical and regenerative capabilities. It is a proven fact that the pathway of *in vitro* regeneration depends not only on the type of explants used, but also on the media type and concentration and combinations of plant growth regulators used. Apart from these studies, many detailed communications are available in developing analytical methods for the detection of the active constituents of *B. monnieri* using spectrophotometry, spectroflurometry, HPTLC and HPLC [4, 6, 8, 19, 21, 23]. Till date, studies on evaluation of morphological features, chlorophyll content and biochemical profiling of leaf, shoot and root explants for different accessions of the herb has not been attempted. The present study attempts to evaluate these by analyzing plant parts to evaluate and estimate the contents of Bacoside A3, Bacoside A II, Bacopasaponin C and Bacoside X in shoot and root extracts. The role of two types of nutrient media along with various plant growth regulators on *in vitro* regeneration from leaf explants of these accessions is also reported.

MATERIALS AND METHODS

Collection of Plant material

Five different accessions of *Bacopa monnieri* were collected from different geographical locations of India (Table: 1) and habituated in the Herbal Garden of SMVDU, Katra, J&K (Latitude = 28°66′ North, Longitude = 77°21' East and Altitude = 754m) [12].

Macroscopic evaluation

Macroscopic characters for fresh leaves, shoots and roots like size, shape, color, surface, venation, apex, margin, base, and texture in all five accessions were evaluated to determine differences in phenotypic characters between accessions.

Sample preparation

1. Chlorophyll estimation

Fresh leaves (100 mg) from all the accessions were collected and dissolved individually in 2 ml of 80% Acetone. The extract so obtained was centrifuged at 500 rpm for 10 min at room temperature and optical density (OD) of supernatant was read at 663 nm and 645 nm. The chlorophyll (a), chlorophyll (b) and total chlorophyll content were calculated through Arnon's equation[2].

2. Biochemical analysis

Dried leaf, shoot and root samples of five accessions of the herb were accurately weighed (10 mg ml^{-1}) and dissolved in distilled water and kept overnight on a shaker at 80 rpm at room temperature to prepare an aqueous extract for detection of various organic compounds according to the standard methods available for biochemical analysis[11, 24, 26].

3. Quantification of Bacoside A3, Bacoside II, Bacopasaponin C and Bacoside X by LC-ESI- MS

Dried leaf, shoot and root samples of all the accessions were accurately weighed (100 mg), air dried for 4-5 days and extracted with methanol for 5 hours using a Soxhlet extraction

apparatus at 60°C. The sample extracts were filtered through 0.45µm (Millipore) filtes. LC-MS analysis was performed on an Agilent 6410 LC-MS (Agilent Technologies, USA) triple-stage quadrupole mass spectrometer equipped with electrospray ionization (ESI) interface. Liquid chromatography analysis were carried out using an Agilent 1260 Infinity (Agilent, USA) quaternary pump equipped with an auto sampler, column heater and online degasser. Analytical chromatographic separations of crude sample extracts were carried out on a chromolith performance RP-18e column (50 x 4.6 mm, E. Merck, Germany) protected by a chromolith guard column. The column temperature was maintained at 30^0C. The flow rate was optimized to 0.6 ml/min with a sample injection volume of 5 µL. The mobile phase consisted of solvent A (water and 0.1% formic Acid) and solvent B (methanol). Isocratic elution was performed at the ratio of A and B (57:43 v/v).

4. Culture establishment and explant regeneration

Shoot tips having 4-6 leaves were collected from 2 month old green house grown plants and sterilized according to the method previously standardized[12].Leaves of *Bacopa monnieri* were reported to have the highest bacoside content as compared to individual plant parts[22]. Leaf explants (1-1.2 cm length, 0.4-0.5 cm width) were excised, washed, blotted dry and inoculated onto agar gelled (0.7% w/v) media in culture tubes containing separate series of Murashige and Skoog (MS)[16] and Gamborg's B_5 (B_5) [7] media supplemented with different concentrations (0, 0.5, 1.0 mg l^{-1}) of auxins [Naphthalene acetic acid (NAA), 2, 4-Dichlorophenoxy acetic acid (2,4-D)] and cytokinins [6-Benzyl aminopurine (BAP), Kinetin (KN)] either singly or in combinations. The basal media containing no plant growth regulator (PGR) were considered as the control for all the experiments. The cultures were incubated for 28 days at 25±2°C under controlled photoperiod (16 hour) conditions provided by cool white fluorescent tubes (3000 lux) in the culture room throughout the experiment. The experiment was repeated thrice in duplicates with six replicates per treatment and observed every week for data collection until the end of four weeks and photographed. The mean of Growth Index (GI) and P value were calculated using the SPSS 17 software.

RESULTS AND DISCUSSION

The present study aims to address the morphological, biochemical, phytochemical and regenerative potential of five accessions of *Bacopa monnieri*. A new LC-ESI-MS method through single ion monitoring (SIM) was developed for the simultaneous identification and quantification of Bacoside A3, Bacoside II, Bacopasaponin C and Bacoside X in crude extracts of shoot and root samples of the herb.

Plant Morphology

Bacopa monnieri plants are fleshy, succulent with ascending branches, sessile leaves and rooting at nodes but noticeable differences were observed between the morphological characteristics and chlorophyll content in the different accessions (Table: 1). The differences may be attributed to the geographical origin of the plant[11,]. Accession BM005 showed a maximum total chlorophyll (40.488) content amongst all the accessions tested (Table: 1).

Biochemical Analysis

Qualitative biochemical tests were performed for the aqueous extracts of leaf, shoot and root parts of all the accessions, which revealed the presence of medicinally active constituents. Phytochemically active compounds of *B. monnieri* were qualitatively analyzed in leaves, shoots and roots separately. During the screening process different plant parts showed varied profiles for carbohydrates, alkaloids, saponins, and terpenoids as presented in Table: 2.

Quantification of Bacoside A3, Bacoside II, Bacopasaponin C and Bacoside X by LC-ESI-MS: A LC-ESI-MS assay was developed for selective identification as well as accurate and precise quantification of Bacoside A3, Bacoside II, Bacopasaponin C and Bacoside X in the crude extract of plant parts. Electrospray ionization (ESI) mode was preferred over the

atmospheric pressure chemical ionization (APCI) mode because it was found to be more sensitive during optimization of the LC-MS method. The standard curve was evaluated for the further quantification in extract samples (Fig 1a, 1b). The presence of Bacoside A3, Bacoside II, Bacopasaponin C and Bacoside X were successfully quantified in the crude extracts of *B. monnieri* accessions (Fig. II).

In vitro regeneration

Presently, an attempt has been undertaken to study the regenerative potential of leaf explants for five different accessions of *B. monnieri* using MS and B_5 media supplemented with varying concentrations and combinations of PGRs. Leaf explants of all the accessions responded differently to the treatments and it was possible to establish shoot, root and callus culture lines. It was found that the culture medium composition had a profound effect on morphological explant competence and biomass production (Table: 3 and 4). Reports are available that indicate biomass and callus induction is greatly influenced by growth hormones in MS media[1, 14, 17, 18] but there are no available reports on callusing in B_5 media using leaf explants in different accessions of *B. monnieri*. The explants cultured in control treatments were found suitable for *de novo* plantlet regeneration[12].

The accession which showed the best results for generation of friable calli was BM003 followed by BM004 > BM001 > BM002 > BM005 cultured on B_5 media. Indirect organogenesis was observed in MS media in BM003 > BM002 > BM005 > BM004 > BM001 (Table: 5). The present study reveals the fact that the composition of the nutrient media supplemented with different PGRs directly influences morphogenetic response and growth behavior of leaf explants *in vitro* regeneration.

Profuse differences have also been noted in the morphological, biochemical and phytochemical characteristics between accessions. The differential responses observed in the five accessions could probably be attributed to the environmental and genotypic differences in their origin of collection.

Table 1
Comparative morphological, organoleptic characters and Statistical differences in area and chlorophyll content of five accessions of Bacopa monnieri

Accession number	Location of collection	Leaf morphology	Shoot morphology	Root morphology	Mean of leaf area ± SE	Total Chlorophyll content (µg/ml)	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)
BM001	Indian Institute of Integrative Medicines, Jammu, J&K	Sessile, green, oblong in shape, non venous, light mid rib, succulent	Brown to light green, lateral growth, less glabrous	Brown , lateral growth, tap root system, less root hairs and tubular fleshy root	0.64±0.05	39.056	21.481	17.586
BM002	Rajinder Agricultural University, Pusa Samistipur, Bihar	Sessile, light green, obtuse form, non venous, light mid rib, less succulent	Green, lateral growth, glabrous	Brown, lateral growth, tap root system, abundant root hairs and fleshy root	0.66±0.05	33.933	21.173	12.771
BM003	Forest Institute, Dehradun, Uttarakhand	Sessile, dark green, obtuse form, non venous, light mid rib, succulent	Green to light green, lateral growth, glabrous	Dark brown, lateral growth, tap root system, root hairs	0.51±0.03	29.853	20.71	9.15
BM004	Institute of Himalayan Biodiversity and Technology, Palampur, Himachal Pradesh	Sessile, light green, obvate form, non venous, mid rib not visible,non venous and less succulent	Brown to light green, lateral growth and less glabrous	Light to purple brown, cluster forming, lateral growth, single tap root system, devoid of root hairs	0.43±0.04	36.446	21.649	14.807
BM005	Wild Collection, Jajarkotli, Reasi, J&K	Sessile, green in, oblate form, non venous, light mid rib and succulent	Green to light green, lateral growth, glabrous	Brown, lateral growth, minute root hairs, fleshy root	0.41±0.03	40.488	21.497	19.004

SE is the Standard Error for six replicates and P value = 0.000 (Anova SPSS17) & {Chlorophyll (a) (ug/ml) = 12.7 (A663) -2.69 (A645)
Chlorophyll (b) (ug/ml) = 22.9 (A645) - 4.68 (A663)
Total Chlorophyll content (ug/ml) = 20.2 (A645) + 8.02(A663)}

Table 2
Biochemical analysis of plant parts for five accessions of Bacopa monnieri

PLANT ACCESSIONS																
Biomolecules Analysed	Standard tests performed	BM001			BM002			BM003			BM004			BM005		
		Leaf	Shoot	Root	Leaf	Shoot	Root	Leaf	Shoot	Root	Leaf	Shoot	Root	Leaf	Shoot	Root
Glycosides	Fehling's solution	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Anthraquinones	Borntrager's Test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alkaloids	Mayer's reagent Test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Saponins	Froth Test	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-
Flavonoids	Conc. HCl Test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Steroids	Chloroform Test	+	+	-	-	-	-	-	-	-	+	-	-	+	-	-
Tannins	Ferric chloride Test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrates	Mollsch's Test	+	+	-	+	+	-	+	-	-	+	+	+	+	+	+
Polyuronides	Mucilage Test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Terpenoids	Conc. H_2SO_4 Test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(+ indicates Presence & - indicates Absence)

Table 3
Morphogenetic responses obtained for five accessions of Bacopa monnieri in MS media

Treatments	BM001				BM002				BM003				BM004				BM005			
	%age of Response	Deg. of Response	Nature of Response	Growth Index± SE	% age of Response	Deg. of Response	Nature of Response	Growth Index± SE	%age of Response	Deg. of Response	Nature of response	Growth Index± SE	%age of Response	Deg. of Response	Nature of Response	Growth Index± SE	%age of Response	Deg. of Response	Nature of Response	Growth Index± SE
Control	54	++	R + St	5.49±0.14	58	+	R + St	3.77±0.04	64	+++	R + St	15.53±0.03	52	+	R	2.05±0.05	48	++	R + St	1.54±0.02
2,4 D (0.5mg/l)	49	--	NC	22.40±0.11	47	--	NC	20.46±0.03	62	+	NC	44.87±0.02	58	+	NC	62.27±0.05	41	--	NC	3.14±0.03
2,4 D (1mg/l)	34	+	NC	24.34±0.11	66	--	NC	21.35±0.02	85	--	NC	58.16±0.03	40	+	NC	26.69±0.02	52	--	NC	8.15±0.02
NAA (0.5mg/l)	58	+	OC +R	12.38±0.05	68	++	OC +R	42.46±0.02	50	+++	OC +R	99.42±0.04	46	+ +	OC + R	14.69±0.02	64	++	OC + R	26.53±0.02
NAA (1mg/l)	56	+	OC +R	10.28±0.03	38	++	OC +R	71.28±0.05	58	+	OC +R	15.35±0.18	44	+	OC + R	1.19±0.03	32	+	OC + R	21.61±0.16
2,4D+BAP (0.5mg/le)	44	--	NC	41.69±0.16	48	--	NC	27.16±0.04	82	+	NC	79.71±0.08	56	+	OC + R	74.01±0.03	34	+	OC + R	5.92±0.03
2,4D+BAP (1+0.5mg/l)	46	--	NC	23.24±0.14	40	++	NC	27.24±0.02	86	+++	NC	80.24±0.05	50	--	NC	27.91±0.02	42	--	NC	8.23±0.02
NAA+BAP (0.5mg/le)	54	--	NC	42.08±0.11	58	+	OC +R	48.14±0.03	40	+	OC +R	10.03±0.03	38	+	OC + R	12.71±0.03	32	--	NC	8.65±0.02
NAA+BAP (1+0.5mg/l)	72	+	OC	62.16±0.03	70	++	OC +R	63.69±0.04	92	+++	OC +R	99.58±0.06	46	--	NC	12.57±0.02	44	--	NC	11.15±0.03
2,4 D+ KN (0.5mg/l)	52	++	OC	44.07±0.03	62	--	NC	23.62±0.03	60	+	NC	46.62±0.07	48	--	NC	38.43±0.01	30	+	OC + R	5.75±0.01

[-- : No response, + : Poor, ++ : Good, +++ : Excellent, R : Root, St : Shooting, OC : Organogenetic calli, NC : Non-organogenetic calli]
Growth Index: $\frac{\text{Final dry weight} - \text{Initial dry weight}}{\text{Initial dry weight}}$; SE is the Standard error for 2 experiments and P value : 0.000 calculated from SPSS 17 software

Table 4
Morphogenetic responses obtained for five accessions of Bacopa monnieri in B_5 media

Treatments	BM001				BM002				BM003				BM004				BM005			
	%age of Response	Deg. of Response	Nature of Response	Growth Index± SE	% age of Response	Deg. of Response	Nature of Response	Growth Index± SE	%age of Response	Deg. of Response	Nature of response	Growth Index± SE	%age of Response	Deg. of Response	Nature of Response	Growth Index± SE	%age of Response	Deg. of Response	Nature of Response	Growth Index± SE
Control	46	++	R+St	1.60± 0.02	42	+	R	0.69± 0.01	62	++	R+St	1.65± 0.07	56	+	R	1.69± 0.07	38	+	R	1.15± 0.04
2,4 D (0.5mg/l)	48	+	NC	35.34 ±0.16	46	++	NC	22.12 ±0.07	52	++	NC	30.58 ±0.11	56	+	NC	37.63 ±0.11	44	+	NC	6.34± 0.07
2,4 D (1mg/l)	46	+	NC	20.73 ±0.17	44	+	NC	19.74 ±0.09	86	++ +	NC	54.61 ±0.16	58	+	NC	29.32 ±0.15	54	+	NC	18.74 ±0.08
NAA (0.5mg/l)	56	++	OC + R	50.98 ±0.17	52	+	OC + R+ St	25.73 ±0.07	58	++	OC + R	53.74 ±0.09	62	+ +	OC	67.70 ±0.07	52	++	OC + R	23.30 ±0.07
NAA (1mg/l)	60	+	OC + R	39.88 ±0.17	56	+	OC + R	22.58 ±0.16	64	++	OC + R	50.75 ±0.12	66	+	OC + R	70.33 ±0.12	58	++	OC + R	23.28 ±0.08
2,4D+BAP (0.5mg/le)	44	--	NC	13.64 ±0.10	54	--	NC	10.21 ±0.10	56	+	NC	19.77 ±0.18	56	+	OC + R	24.23 ±0.11	42	--	NC	4.01± 0.01
2,4D+BAP (1+0.5mg/l)	46	--	NC	17.69 ±0.15	46	++	NC	18.92 ±0.11	74	++	NC	44.92 ±0.05	48	--	NC	27.48 ±0.13	40	--	NC	5.91± 0.05
NAA+BAP (0.5mg/le)	54	+	OC + R	29.84 ±0.09	48	+	OC + R	20.86 ±0.10	65	+	OC +R+ St	31.68 ±0.08	78	+	OC + R	45.71 ±0.14	42	--	NC	9.65± 0.11
NAA+BAP (1+0.5mg/l)	44	+	OC + R	17.11 ±0.06	46	++	OC + R	24.96 ±0.10	48	++	OC + R	27.75 ±0.07	56	--	NC	31.20 ±0.11	40	--	NC	11.42 ±0.15
2,4 D+ KN (0.5mg/l)	62	++	NC	32.4± 0.09	56	++	NC	22.64 ±0.10	68	++	NC	36.44 ±0.13	64	--	NC	35.83 ±0.07	38	--	NC	8.36± 0.15

[-- : No response, + : Poor, ++ : Good, +++ : Excellent, R : Root, St : Shooting, OC : Organogenetic calli, NC : Non organogenetic calli]

Growth Index: $\frac{\text{Final dry weight} - \text{Initial dry weight}}{\text{Initial dry weight}}$ SE is the Standard error for 2 experiments and P value : 0.000 calculated from SPSS 17 software

Figure Ia and Ib

LC-MS chromatogram of Bacoside A3, Bacoside A II, Bacopasaponin C and Bacoside X in the standard mixture and crude root extract of Bacopa monnieri (BM003)

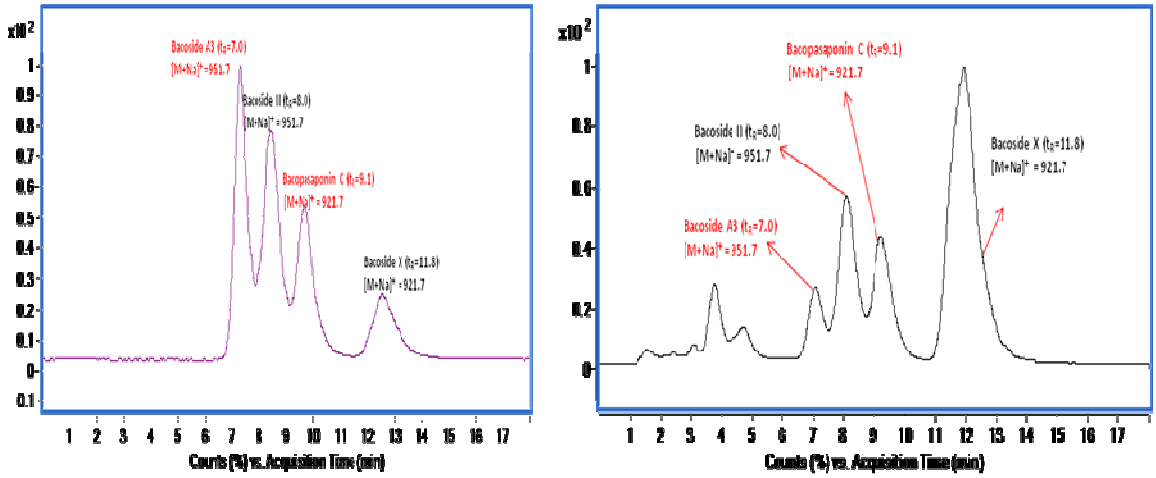
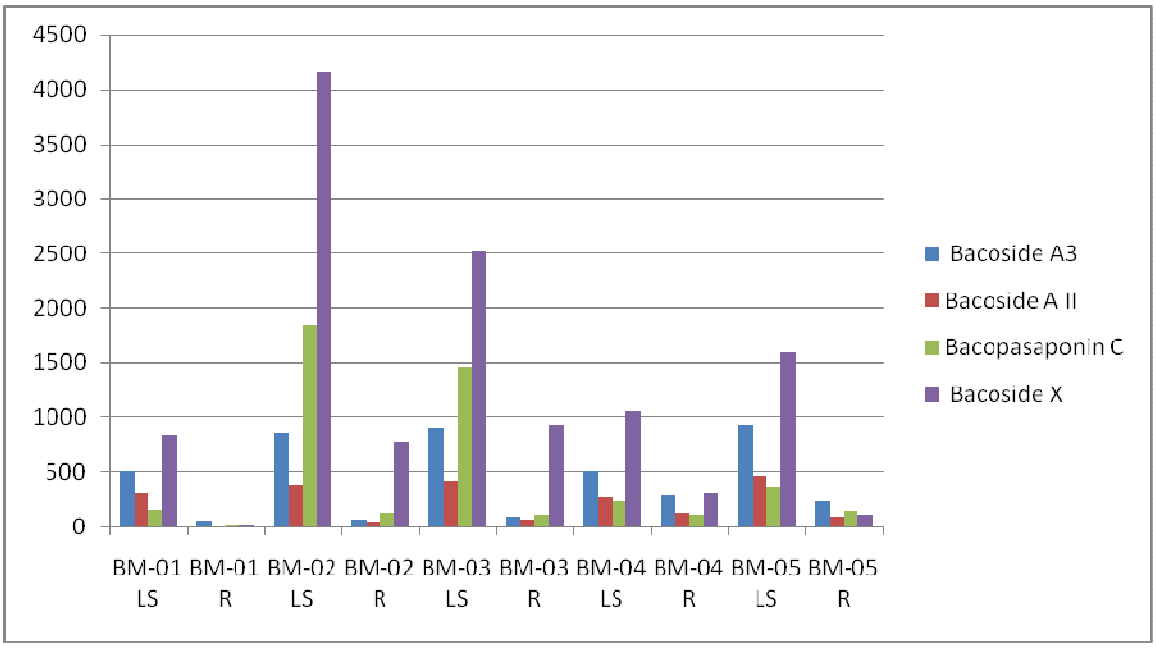


Figure Ia **Figure Ib**

Figure II

Bacoside A3, Bacoside A II, Bacopasaponin C and Bacoside X content in crude extracts of five accessions of Bacopa monnieri, LS-Leaf and shoot, R-Root.



CONCLUSION

In conclusion, the present work has paved way for methods of *in vitro* propagation of and highlights a two step protocol for efficient and reproducible mass multiplication of this endangered and commercially valuable plant species. Biochemical analysis also revealed the presence of bio-active compounds and an array of bacosides which confirm the therapeutic properties of *Bacopa monnieri*.

ACKNOWLEDGEMENT

The authors thank Director, Indian Institute of Integrative Medicine (CSIR), Jammu; for providing the necessary facilities for chemical analysis of the samples. Financial assistance received from University Grants Commission, Govt. of India under Major Research Project Scheme F. No. 41-546/2012 (SR) to SM and Fellowship to AK are acknowledged.

CONFLICT OF INTEREST

Conflicts of interest declared none.

REFERENCES

1. Ahire ML, Laxmi B, Walunj P R, Kishor K and Nikam TD, Effect of potassium chloride and calcium chloride induced stress on *in vitro* cultures of *Bacopa monnieri* (L.) Penell and accumulation of medicinally important Bacoside A, Journal of Plant Biochemistry and Biotechnology, 23(4): 366-378, (2014).
2. Arnon DI, Copper enzymes in isolated chloroplasts, polyphenoxidase in *Beta vulgaris*, Plant physiology, 24: 1-15, (1949).
3. Azad AK, Awang M and Rahman MM, Phytochemical and Microbiological Evaluation of a Local Medicinal Plant *Bacopa monnieri* (l.) Penn., International Journal of Current Pharmaceutical Review and Research, 3(3): 66-78, (2012).
4. Bhandari P, Kumar N, Gupta AP, Singh B and Kaul VK, Micro LC determination of Swertiamarin in Swertia species and Bacoside A in Bacopa monnieri, Chromatographia, 64: 599-602, (2006).
5. Bhusari S, Wanjari R and Khobragade P, Cost effective *in vitro* clonal propagation of *Bacopa monnieri* L. Pennel, International Journal of Indigenous Medicinal Plants, 46(2): 1239-1244, (2013).
6. Deepak M, Sangli G, Arun P and Amit A, Quantitative determination of the major saponin mixture bacoside A in *Bacopa monnieria*, Phytochem Anal, 16: 24-29, (2005).
7. Gamborg OL, Murashige T, Thorpe TA and Vasil IK, Plant tissue culture media, *In vitro*, 12: 473-478, (1976).
8. Ganzerra M, Gampenrieder J, Pawar R, Khan I and Stuppner H, Separation of the major triterpenoid saponins in *Bacopa monnieira* by high-performance liquid chromatography, Anal Chim Acta, 516: 149-154, (2004).
9. Garai S, Mahato S, Ohtani K and Yamasaki K, Dammarane-type triterpenoid saponins from *Bacopa monniera*, Phytochemistry, 42(3): 815-820, (1996).
10. Jain P and Kulshreshtha D, Bacoside A-1 a minor saponin from *Bacopa monnieira*, Phytochemistry, 33(2): 449-451, (1993).
11. Gubbannavar JS, Chandola HM, Harisha CR, Khanpara K and Shukla VJ, A comparative pharmacognostical and preliminary physic- chemical analysis of stem and leaf of *Bacopa monnieri* (L.) Pennel and *Bacopa Floribunda* (R.BR.) Wettst, AYU, 34(1): 95-102, (2013).
12. Koul A, Sharma A, Gupta S and Mallubhotla S, Cost effective protocol for micropropagation of *Bacopa monnieri* using leaf explants, International Journal of Science and Research, 3(4): 210-212, (2014).

13. Kumari S, Starlin MN and Huxley JA, *In vitro* propagation of *Bacopa monnieri* (L.)- A wetland medicinal plant, Journal of Basic & Applied Biology, 4: 138-142, (2010).
14. Mehta J, Ansari R, Syedy M, Khan S, Sharma S, Gupta N, Rathore R and Vaishnav K, An effective method for high frequency multiple shoots regeneration and callus induction in *Bacopa monnieri* (L.) Pennell: An important medicinal plant, Asian Journal of Plant Science and Research, 2 (5): 620-626, (2012).
15. Mohapatra H, Bark DP and Rath SP, *In vitro* regeneration of medicinal plant *Centella asiatica*, Biologia Plantarum, 52(2): 339-342, (2008).
16. Murashige T and Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue culture, Physiol. of Plants, 15: 473-497, (1962).
17. Naik PM, Manohar SH and Murthy HN, Effects of macroelements and nitrogen source on biomass accumulation and bacoside A production from Adventitious shoot cultures of *Bacopa monnieri* (L.), Acta Physiol Plant, 33: 1553-1557, (2011).
18. Parale A, Barmukh R and Nikam T, Influence of organic supplements on production of shoot and callus biomass and accumulation of bacoside in *Bacopa monnieri* (L.) Pennell, Physiol. Mol. Biol. Plants, 16(2): 167-175, (2010).
19. Rastogi S, Pal R and Kulshreshtha D, Bacoside A3-a triterpenoid saponin from *Bacopa monnieria*, Phytochemistry, 36(1): 133-137, (1994).
20. Sarkar M, Tiwari L, Rai N, Sharma S and Sharma KR, Development of Analytical Profiles on *Bacopa monnieri* (L.) Wettst. (Brahmi) Resourced form Different Ecological Habitats, A Journal of Ecology, 1(1): 1-8, (2012).
21. Sharma M, Khajuria RK and Mallubhotla S, Annual Variation in Bacoside content of *Bacopa monnieri* (L.) Wettst plants, International Journal of Pharma and Bio Sciences, 4(4): 266- 271, (2013).
22. Sharma N, Satsangi R, Pandey R and Devi SV, *In vitro* clonal propagation and medium term conservation of Brahmi (*Bacopa monnieri* (L) Wettst), Journal of Plant Biochemistry and Biotechnology, 16(2): 139-143, (2007).
23. Subashri B and Pillai YJK, Phytochemical analysis and *in vitro* antioxidant activity from the extract of *Bacopa monnieri* (l.) Pennel – a multipurpose medicinal plant, International Journal of Pharma and Bio sciences, 3(2): 698- 702, (2012).
24. Tanveer A, Khan M and Shah F, *In vitro* micropropagation of Brahmi- *Bacopa monnieri* (L.) Pennel- A step for conservation, Nanobiotechnica Universale, 1: 139-150, (2010).
25. Tiwari KN, Tiwari V, Singh BD and Ahuja P, Synergistic effect of trimethoprim and bavistin for micropropogation of *Bacopa monnieri*, Biologia Plantarum, 56(1): 177-180, (2012).
26. Trivedi MN, Khemani A, Vachhani UD, Shah CP and Santani DD, Comparative pharmacognostic and phytochemical investigation of two plant species valued as medhya rasayanas, International Journal of Applied Biology and Pharmaceutical Technology, 2(3): 28-36, (2011).