Medicinal and Aromatic Plant Science and Biotechnology ©2009 Global Science Books

Influence of Auxins, Cytokinins and Biotic Elicitors on Accumulation of Memory Enhancer Compound Bacoside-A in Tissue Culture of *Bacopa monniera* (L.) Pennell.

Parale Anuradha Prakash • Nikam Tukaram Dayaram*

Department of Botany, University of Pune, Pune - 411 007, India

Corresponding author: * **tdnikam@unipune.ernet.in**

ABSTRACT

The effects of the auxins [indole-3-acetic acid (IAA), 1-napthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D)], cytokinins [6-benzyladenine (BA), Kinetin (Kin) and thidiazuron (TDZ)] and biotic elicitors were evaluated individually and in combinations, on callus proliferation, shoot growth and accumulation of bacoside-A through liquid and semi-solid nutrient media. Maximum shoot proliferation (19.4 ± 0.91 shoots/explant) was observed in liquid MS medium containing 5 µM BA, which was 1.3 times higher than semi-solid MS medium fortified with same concentration of BA. The callus and shoots showed sustained growth and accumulation of bacoside-A even after two years of initiation of subcultures. The nature of nutrient media did not influence the accumulation of bacoside-A in shoot biomass. Except for *Saccharomyces cerevisiae*-derived elicitors that showed about a 20% increase in shoot biomass production, the growth of callus as well as shoots was inhibited after the addition of other fungal elicitors in MS medium. Among the shoot and callus cultures exposed to *Pencillium notatum*, *Rhizopus stolonifer*, *Coriolus versicolor*, *Mucor* sp. and *S. cerevisiae*-derived elicitors, incorporation of 750 mg/l *S. cerevisiae* or *Mucor* sp. showed about 3.2- and 1.7-fold higher production of bacoside-A in callus and shoot biomass, respectively. This protocol would be helpful in reducing pressure on natural populations of *Bacopa monniera* (L.) Pennell. harvested indiscriminately for bacoside-A.

Keywords: Brahmi, callus culture, multiple shoots, *Saccharomyces cerevisiae*
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **BA**, 6-benzyladenine; **DW**, dry weight; **HPTLC**, high performance thin layer chromatography; **IAA**, indole-3-acetic acid; **Kin**, kinetin; **MS**, Murashige and Skoog (1962) medium; **NAA**, 1-napthaleneacetic acid; **PDA**, potato dextrose agar medium; **TDZ**, thidiazuron

INTRODUCTION

Bacopa monniera (L.) Pennell, (Scrophulariaceae) is an important plant in the Ayurvedic system of medicine, mainly used for treating age-related brain disorders, and for improving cognitive processes. Among the 12 tritepenoids reported from *B. monniera*, bacoside-A is the major constituent believed to be responsible for its cognitive effects (Singh and Dhawan 1997; Mahato *et al*. 2000; Deepak *et al*. 2004). The plant is widely used as a nervine tonic, cardio tonic and diuretic in Indian traditional medicine (Chopra *et al*. 1969). Now, brain tonics available under different trade names consist of *B. monniera* extract. The annual requirement of *Bacopa* biomass during 2004-2005 was 6651.8 tons and demand for it is increasing with an annual growth rate of 7% (Banerjee *et al*. 2008).

B. monniera commonly grows in marshy areas throughout India, Nepal, Sri Lanka, China, Taiwan, and Vietnam. It is also found in Florida and other southern states of USA where it grows in damp conditions alongside ponds or bog gardens. Apart from its memory-enhancing property, it is believed to be an aphrodisiac in certain parts of India. In Sri Lanka it is prescribed for fevers under the name of Loonooweella, whereas in the Philippines, it is used as a diuretic (Russo *et al*. 2005). Though in India it is cultivated in some parts of Tamil Nadu and West Bengal, biomass production is insufficient to meet increasing demands. Its collection from natural wetlands may be ecologically damaging and therefore is controlled by state regulation. Improper harvesting from natural resources, seedling death at the 2-leaf stage and specific habitat requirement have lead to rapid depletion of *B. monniera* from its natural habitat and is now included in the list of endangered plants of India (Tiwari *et al.* 2001). Recently, Prasad *et al*. (2007), the National Medicinal Plant Board (NMPB), Government of India and the Technology Information Forecasting and Assessment Council (TIFAC) have suggested immediate attention for the conservation of this plant species (http://www.nmpb.nic.in/prioritisedmedicinalplants.htm). Plant tissue and cell culture can be used as an alternative source to the whole plant under such circumstances and also for conservation of plant species as well as production of medicinally important compounds. Moreover, these methods allow amendments for enhanced production of specific phytochemicals.

Elicitors of fungal origin have been widely used to enhance production of secondary metabolites in plant cell cultures. This strategy has been effective in stimulating the production of secondary metabolites from various chemical classes (Namdeo 2007). An effective *in-vitro* technique for biomass production is an important prerequisite for the application of such strategy. So far, several reports on tissue culture of *B. monniera* have focused mainly on improving its propagation (Srivastava *et al*. 1999; Tiwari *et al*. 2001; Binita *et al*. 2005; Tiwari *et al*. 2006; Sharath *et al.* 2007). Verghese and Sathyanarayana (2007) obtained variants in *B. monniera* cv. 'Pragyashakti' and 'Calcutta Local' with a high content of bacoside (3.03%) by using *in vitro* techniques and applying gamma irradiation (30-100 Gy). Rahman *et al*. (2002) reported the production of bacoside-A through cell culture. However, information available on factors influencing the production of *in-vitro* biomass and secondary metabolites is meagre.

Received: 29 June, 2009. Accepted: 9 December, 2009.

Original Research Paper

The present study is an attempt to understand the influence of cytokinins and auxins on organogenesis, callus proliferation and accumulation of bacoside-A, and to explore the possibilities of enhanced production of bacoside-A in callus and shoot culture through application of fungal elicitors.

MATERIALS AND METHODS

Plant materials, shoot regeneration and culture conditions

Healthy plants of *B. monniera* procured from the Medicinal Plants Conservation Centre, Pune, were established in the Botanical Garden of Department of Botany, University of Pune, India, and used as an explant source. The branches with 3-4 nodes were excised and washed five times with sterile distilled water. These branches were surface disinfected with 0.1% aqueous mercuric chloride (w/v) for 10 min followed by five thorough rinses with sterile distilled water (SDW). The stem segments consisting of single node and entire leaf were placed on Murashige and Skoog (1962) medium (MS) devoid of plant growth regulators (PGRs) and MS medium containing cytokinins, BA (6-benzyladenine), Kin (kinetin), TDZ (thidiazuron) and auxins, IAA (indole-3-acetic acid), NAA (1-napthaleneacetic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid), individually and in combinations. The cultures were maintained at 25 ± 2°C and illuminated for 8 h with 40 µmol m^{-2} s^{-1} light provided by cool white fluorescent tubes (Champion 40W, Philips Electronics. India Ltd.) followed by a 16-h dark phase.

Maintenance of shoot culture and callus culture

Since maximum shoot multiplication was observed from leaf and stem explants cultured on MS medium containing 5 µM BA, the explants from *in-vitro* raised shoots were subcultured on this medium at 4-week intervals for about 2 years. Callus was maintained on MS medium fortified with auxins and cytokinins, individually and in combinations (**Table 4**). Callus was maintained for about 2 years by subculturing about 500 mg fresh callus on the parental medium at 4-week intervals.

Shoot multiplication in semi-solid and liquid media

The *in-vitro* raised aseptic shoots with 2 to 3 nodes and intact apical meristem were cultured on agar solidified MS medium and liquid MS medium containing BA and Kin individually, and in combination with IAA or NAA (**Table 3**). The data on shoot multiplication and biomass production was recorded after 4 weeks of culture initiation.

Influence of fungal elicitors

The strains of *Pencillium notatum*, *Rhizopus stolonifer*, *Coriolus versicolor*, *Mucor* sp. and *Saccharomyces cerevisiae* were obtained from the National Collection of Industrial Microorganisms, and the National Chemical Laboratory, Pune, India. The fungal cultures were established and maintained in the dark on PDA (Potato Dextrose Agar) medium at 25 ± 2°C. About 10 mg mycelia were inoculated in 1 l sterilised liquid potato dextrose medium and incubated for 4 weeks. The mycelial mat was separated by filtration and washed thoroughly with SDW five times and dried in an oven at 60°C for 72 h. The dried mycelial mass was homogenised to fine powder. Liquid MS medium containing 5 µM BA was used as control for the shoot culture experiment whereas MS medium containing 5 µM NAA in conjunction with 1 µM 2,4-D was selected as control for the callus culture experiment. The mycelial powder was added in the callus and shoot regeneration media at concentrations of 0.0, 250, 500, 750, 1000 mg/l. The data on shoot and callus biomass production and accumulation of bacoside-A was recorded after four weeks of culture initiation.

Estimation of bacoside-A

The control and treated callus and *in-vitro* raised shoots were harvested after 4 weeks of culture initiation and after recording their fresh weight, were dried in an oven at 60°C until constant weight was obtained (Sestak 1971). Bacoside-A was extracted from these samples following the method of Watoo *et al.* (2007). One gram of dried biomass was soaked in 10 ml distilled water. Water was squeezed out of the plant material after 24 h and the residue was extracted with 50 ml 95% ethanol. The ethanolic extract was concentrated and dried under vacuum. The dried residue was redissolved in 1 ml methanol. Bacoside-A was quantified by using HPTLC (Anonymous 1998; Prakash *et al.* 2008). The samples were applied on aluminium sheets of silica gel 60F254 (Merck) using a CAMAG sample applicator. The chromatogram was developed in toluene: ethanol: methanol: glacial acetic acid (4: 3: 3: 1) mobile phase. Methanolic sulphuric acid (5%) was used for detection. The peaks were analyzed by a CAMAG TLC scanner-III at 500 nm. The peak corresponding to bacoside-A was confirmed by spiking the samples with standard (Natural Remedies, Bangalore, India). The bacoside-A content in the crude extract was determined by comparing the peak areas with those of standard (**Figs. 1-3**).

Statistical analysis

All the experiments were arranged in a complete randomised design. Each experiment consisted of four explants per bottle and 10 replicate bottles per treatment. All the experiments were repeated at least three times. The data were subjected to one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) (1955) at the 5% level of significance. Data were collected from three independent experiments on the quantitative analysis of bacoside-A, and presented as mean ± standard error (SE).

RESULTS AND DISCUSSION

Shoot regeneration and accumulation of bacoside-A

The developmental processes ranging from seed germination to root, shoot and flower formation and corresponding accumulation of metabolites are affected by the type of PGR, its concentrations and environmental conditions (Mulabagal *et al*. 2004). Shoot formation was the common response of all the explants in the present study. Stem and leaf explants cultured on PGR-free medium produced shoots as well as roots. In a previous study, the petiole proximal leaf segments and nodal and internodal segments of *B. monniera* were observed to regenerate roots and shoots on solid MS medium lacking PGRs (Mathur *et al*. 1998). These results indicate that the endogenous concentration of shoot and root inducing PGRs is sufficiently in the aerial portion of *B. monniera* plants. However, root explants failed to respond to shoot regeneration on PGR-free and PGR-containing medium (data not shown). Though the shoot proliferation response of the explant was not dependent on its position along the stem (apical, basal or intermediate), it was influenced by the type of cytokinin and its concentration in the medium. Similar results were recorded on the position of leaf explants (**Table 8**). Thus, for shoot multiplication in *B. monniera*, explants from apical, basal or intermediate parts of the stem can be used.

Incorporation of BA and Kin in the medium improved the shoot regeneration response in the explants. Direct shoot formation occurred from the entire surface of the explants on medium containing 1 to 10 µM BA or Kin. No callusing was observed on explants cultured on medium with these concentrations of BA or Kin. Inclusions of 5 µM BA induced maximum number (11.0 ± 0.9) of shoots per leaf explant (**Table 1**). Comparatively fewer shoots per explant were produced when the same concentration of Kin was added (**Table 1**). Srivastava and Rajani (1999) noted that the entire surface area of leaf explants were covered by shoot buds on MS media fortified with 2 µM BA, which made it impossible to count the number of shoot buds. Similarly, adventitious shoot buds induced form leaf explants cultured on media containing TDZ (6.8 µM) or BA (8.9 µM) and a higher number (129.1) of shoot buds was

Table 1 Effect of cytokinins on leaf explants for shoot induction and bacoside-A production in shoots derived from leaf explants of *Bacopa monniera.*

Cytokinins (µM)			Explants forming shoots (%)	No. of shoots per explant	Bacoside-A (mg/g DW)
BA	Kin	TDZ			
-	-	-	80.9 ± 1.8 b	2.5 ± 0.9 h	1.3 ± 0.2 k
1	-	-	95.6 ± 2.7 a	7.1 ± 0.2 e	3.3 ± 0.3 i
2.5	-	-	99.9 ± 0.0 a	7.5 ± 1.0 d	5.3 ± 0.1 e
5	-	-	100 ± 0.0 a	11.0 ± 0.9 a	7.7 ± 1.0 a
7.5	-	-	100 ± 0.0 a	9.8 ± 0.9 b	6.4 ± 0.1 c
10	-	-	100 ± 0.0 a	7.0 ± 0.8 e	5.3 ± 0.1 f
-	1	-	98 ± 1.2 a	4.0 ± 0.2 g	5.2 ± 1.0 g
-	2.5	-	100 ± 0.0 a	6.2 ± 0.9 f	6.3 ± 0.1 d
	5	-	100 ± 0.0 a	9.7 ± 0.4 bc	7.0 ± 0.5 b
-	7.5	-	100 ± 0.0 a	9.6 ± 0.3 c	4.3 ± 0.7 h
-	10	-	100 ± 0.0 a	9.6 ± 0.2 c	3.2 ± 0.2 j
-	-	1	39.3 ± 0.8 c	0.8 ± 0.04* i	1.0 ± 0.03 l
-	-	2.5	38.6 ± 1.3 c	0.5 ± 0.03* j	0.9 ± 0.02 m
-	-	5	30.1 ± 1.7 d	0.2 ± 0.05* k	0.8 ± 0.02 n
-	-	7.5	-	-	-
-	-	10	-	-	-

Data scored after 4 weeks of culture incubation
*: Callus formation, DW: dry weight
Mean values within a column followed by the same letters did not differ at 5% level of probability by DMRT.

Table 2 Effect of cytokinins on stem explants for shoot multiplication and bacoside-A production in shoot derived from stem explants of *Bacopa monniera*.

Cytokinins (µM)			Explants forming shoots (%)	No. of shoots per explant	Bacoside-A (mg/g DW)
BA	Kin	TDZ			
-	-	-	99.5 ± 2.7 a	1.9 ± 0.2 i	1.2 ± 0.3 i
1	-	-	99.7 ± 0.0 a	6.0 ± 0.3 e	2.2 ± 0.3 h
2.5	-	-	100 ± 0.0 a	7.1 ± 1.0 c	3.3 ± 0.4 f
5	-	-	100 ± 0.0 a	8.3 ± 0.2 a	6.4 ± 0.8 b
7.5	-	-	100 ± 0.0 a	7.5 ± 0.6 b	5.4 ± 1.0 c
10	-	-	99.5 ± 0.5 a	5.9 ± 0.8 e	3.3 ± 0.2 f
-	1	-	98.8 ± 1.2 a	2.7 ± 0.2 h	2.2 ± 0.2 h
-	2.5	-	100 ± 0.0 a	4.9 ± 0.1 g	3.2 ± 0.3 g
	5	-	100 ± 0.0 a	6.8 ± 0.3 d	5.3 ± 0.5 d
-	7.5	-	100 ± 0.0 a	6.0 ± 1.1 e	5.2 ± 0.8 e
-	10	-	99.7 ± 0.0 a	5.4 ± 0.2 f	1.2 ± 0.3 i
-	-	1	65.2 ± 0.9 b	1.0 ± 0.02 j	1.0 ± 0.1 j
-	-	2.5	60.3 ± 1.1 c	0.6 ± 0.04 k	1.0 ± 0.1 k
-	-	5	61.7 ± 1.4 c	0.5 ± 0.04 k	0.9 ± 0.1 l
-	-	7.5	-	- *	0.8 ± 0.02 n
-	-	10	-	-*	0.7 ± 0.01 n

Data scored after 4 weeks of culture incubation.
*: Callus formation, DW: dry weight
Mean values within a column followed by the same letters did not differ at 5% level of probability by DMRT.

Table 3 Effect of cytokinins in conjunction with auxins on leaf explants for *in-vitro* shoot multiplication and accumulation of bacoside-A in shoot cultures of *Bacopa monniera*.

Cytokinins (µM)			Auxins (µM)			Cultures showing shoot formation (%)	Shoot per explants	Bacoside-A (mg/g DW)
BA	Kin	TDZ	IAA	NAA	2,4-D			
5	-	-	-	-		100.0 ± 0.0 a	11.0 ± 0.1 a	7.7 ± 1.2 a
	-	-	1	-	-	93.7 ± 1.7 ab	6.9 ± 0.7 e	7.8 ± 1.1 b
	-	-	5	-	-	92.6 ± 1.1 ab	7.0 ± 0.2 e	4.9 ± 1.3 h
	-	-	-	1	-	93.2 ± 2.3 ab	10.4 ± 1.0* b	6.7 ± 1.4 e
	-	-	-	5	-	92.8 ± 1.1 ab	6.6 ± 0.2* f	5.8 ± 1.2 f
	-	-	-	-	1	65.3 ± 2.8 c	3.0 ± 1.1* i	6.8 ± 1.1 d
	-	-	-	-	5	64.8 ± 2.3 c	1.3 ± 0.2* m	4.2 ± 1.3 k
-	5	-	-	-	-	100 ± 0.0 a	8.7 ± 0.3 d	7.3 ± 1.4 c
-		-	1	-	-	96.8 ± 2.5 a	5.8 ± 1.5* h	4.5 ± .98 i
-		-	5	-	-	98.3 ± 1.1 a	9.3 ± 1.0* c	3.5 ± 1.0 m
-		-	-	1	-	93.6 ± 1.7 ab	2.7 ± 0.8* j	5.6 ± 1.1 g
-		-	-	5	-	93.8 ± 1.5 ab	1.0 ± 0.1* n	4.5 ± 1.0 i
-		-	-	-	1	68.9 ± 2.5 b	1.8 ± 0.02* l	4.4 ± 1.3 j
-		-	-	-	5	97.0 ± 1.1 a	1.0 ± 0.1* n	2.4 ± 1.2 n
5	5	-	-	5	-	98.6 ± 1.1 a	2.0 ± 0.1* k	3.7 ± 1.3 l
		-	-	-	-	98.9 ± 0.6 a	6.0 ± 0.1* g	3.7 ± 1.2 l

Data scored after 4 weeks of culture incubation.
DW: dry weight, *: callus formation.
Mean values within a column followed by the same letters did not differ at 5% level of probability by DMRT.

recorded by the end of the third subculture on media fortified with 2.2 µM BA (Tiwari *et al*. 2001). Also, Binita *et al*. (2005) induced about 110 shoot buds per leaf explant on medium containing 1.1 µM BA + 0.2 µM IAA. There is also a report on a higher number (98.6) of shoot bud formation in the absence of PGRs on media into which 300 mg/l bavistin (methyl benzimidazole carbonate), a fungicide, was incorporated (**Table 9**). In the present investigation, numer-

Table 4 Effect of cytokinins in conjunction with auxins on stem explants for *in-vitro* shoot multiplication and accumulation of bacoside-A in shoot cultures of *Bacopa monniera*.

Cytokinins (µM)			Auxins (µM)			Cultures showing shoot formation (%)	No. of shoot per explant	Bacoside-A (mg/g DW)
BA	Kin	TDZ	IAA	NAA	2,4-D			
5	-	-	-	-	-	100.0 ± 0.0 a	8.3 ± 0.2 a	6.4 ± 0.3 a
	-	-	1	-	-	89.7 ± 1.1 b	6.9 ± 0.5 c	5.6 ± 0.2 c
	-	-	5	-	-	89.6 ± 0.6 b	6.2 ± 0.5 d	3.6 ± 0.1 j
	-	-	-	1	-	88.4 ± 1.7 b	7.7 ± 0.7 b	5.6 ± 0.1 b
	-	-	-	5	-	88.9 ± 1.1 b	7.4 ± 0.7 b	4.7 ± 0.3 e
	-	-	-	-	1	78.1 ± 0.6 c	2.1 ± 0.4 i	4.5 ± 0.6 g
	-	-	-	-	5	78.5 ± 1.1 c	0.8 ± 0.8 l	3.5 ± 0.5 k
-	5	-	-	-	-	99.7 ± 0.0 a	5.8 ± 0.3 e	5.3 ± 0.8 d
-		-	1	-	-	78.6 ± 0.5 c	5.8 ± 0.8 e	4.5 ± 0.1 g
-		-	5	-	-	86.1 ± 1.1 b	7.0 ± 0.4 c	4.5 ± 0.2 f
-		-	-	1	-	75.2 ± 2.8 c	4.0 ± 0.7 f	4.5 ± 0.3 g
-		-	-	5	-	75.9 ± 1.1 c	2.6 ± 0.7 h	3.5 ± 0.4 k
-		-	-	-	1	70.1 ± 0.6 d	1.1 ± 0.4 k	3.4 ± 0.9 l
-		-	-	-	5	70.8 ± 1.1 d	1.9 ± 0.9 j	3.4 ± 0.9 m
5	5	-	-	5	-	98.4 ± 1.2 a	3.8 ± 0.7* g	3.7 ± 0.2 i
		-	-	-	-	98.6 ± 1.4 a	2.5 ± 0.9* h	3.7 ± 0.3 h

Data scored after 4 weeks of culture incubation.
*: Callus formation, DW: dry weight
Mean values within a column followed by the same letters did not differ at 5% level of probability by DMRT.

Table 5 Effect of liquid and semisolid nature of nutrient medium on shoot multiplication and bacoside-A accumulation in shoot cultures of *Bacopa monniera*.

MS+ PGR (µM)		Liquid medium		Semi-solid medium	
Cytokinins	Auxins	No. of shoot/ explant	Bacoside-A (mg/g. DW)	No. of shoot/ explant	Bacoside-A (mg/g. DW)
BA	IAA				
0	0	6.8 ± 0.8 h	2.4 ± 0.1 f	4.4 ± 0.9 h	2.2 ± 0.1 f
5	0	19.4 ± 0.9 a	7.3 ± 0.1 a	12.5 ± 0.5 a	7.0 ± 0.1 a
5	1	10.1 ± 0.5 e	4.8 ± 0.1 d	8.2 ± 0.6 d	4.5 ± 0.3 d
5	5	9.4 ± 0.6 f	4.6 ± 0.1 e	8.0 ± 0.5 e	4.2 ± 0.2 e
	NAA				
5	1	11.4 ± 0.4 d	5.7 ± 0.2 c	9.4 ± 0.7 c	5.6 ± 0.2 c
5	5	10.1 ± 0.6 e	5.6 ± 0.2 c	7.6 ± 0.8 de	5.6 ± 0.3 c
Kin	IAA				
5	0	14.5 ± 0.5 b	6.8 ± 0.1 b	10.7 ± 0.8 b	6.5 ± 0.2 b
5	1	7.8 ± 0.5 g	5.5 ± 0.2 c	5.8 ± 0.7 g	5.4 ± 0.2 c
5	5	12.8 ± 0.7 c	5.5 ± 0.2 c	10.3 ± 0.5 b	5.5 ± 0.1 c
	NAA				
5	1	7.8 ± 0.5 g	5.5 ± 0.2 c	6.7 ± 0.5 f	5.5 ± 0.2 c
5	5	6.3 ± 0.4 i	5.5 ± 0.1 c	6.1 ± 0.8 g	5.5 ± 0.2 c

Data scored after 4 weeks of culture incubation.
D.W.: dry weight.
Mean values within a column followed by the same letters did not differ at 5% level of probability by DMRT.

ous small shoot buds formed on the entire surface of leaf explants on MS media fortified with 5 µM BA. However, one main difference should be noted: ~11 shoots per explant elongated on the same medium within four weeks of culture. TDZ is a potent bioregulant of *in-vitro* morphogenesis (Murthy *et al*. 1998). It is the most effective cytokinin for shoot regeneration in herbaceous medicinal-plants like *Arnebia euchroma* (Jiang *et al*. 2005) and *Psoralea corylifolia* (Shinde *et al*. 2009). However, inclusion of 1 to 10 µM TDZ was not very effective for shoot multiplication from stem as well as leaf explants of *B. monniera* in the present investigation (**Tables 1, 2**). A similar effect of TDZ on shoot regeneration has been previously documented in other plant species. Incorporation of TDZ caused browning in several shoot buds and was detrimental to shoot regeneration in *Melia azedarach* (Vila *et al*. 2005). According to Tiwari *et al*. (2001), optimum adventitious shoot bud induction in leaf explants of *B. monniera* occurred when 6.8 µM TDZ was included, but the further development of shoots was poor on TDZ-containing media. Further, incorporation of 8.9 µM BA was superior to other treatments for induction and multiplication of shoots. In the present investigation, similar results were recorded and effectiveness of BA in induction and growth of multiple shoots in *B. monniera* is confirmed. Inclusion of auxins, either IAA (1-5 µM) or NAA (1-5 µM) along with optimum concentration of BA or Kin (5 µM) in the culture medium reduced the frequency of shoot multiplication (**Tables 3, 4**). However, these treatments produced scanty callus from the cut end of explants and occasional formation of adventitious roots. These results indicate that addition of auxins counteract the action of cytokinins for shoot regeneration.

When all other parameters were kept at the optimal level, the nature of medium (liquid or agar solidified) influenced the shoot multiplication frequency in *B. monniera*. Extensive shoot proliferation was observed in liquid medium compared to the agar-solidified medium (**Table 5**). These results are in line with the findings of previous studies on *B. monniera* (Tiwari *et al*. 2000; Praveen *et al*. 2009) and the other members of the Scrophulariaceae, *Isoplexiz canariensis* L. (Arrebola *et al*. 1997). It is quite likely that the availability of nutrients is easier in liquid medium than in agar-solidified medium. Since agar is the costliest constituent of the media (US$ 64.44/kg), its elimination would help in reducing the cost of shoot biomass production *in vitro*. Secondly, it would be more appropriate growth condition for *B. monniera* as its most important adaptation is to wetland environments.

There are few methods reported in the literature for quantification of bacosides in *B. monniera* plant extracts and formulations. The spectrophotometric methods developed by Pal and Serin (1992) and Singh *et al*. (1998) were based on the hydrolysis of bacosides to an aglycone that has an absorption maximum at 278 nm. However, these methods

lack specificity and have interference from other hydrolyzed compounds (Murthy *et al*. 2006). Murthy *et al*. (2006) and Watoo *et al* (2007) reported a reverse phase HPLC method for quantification of different saponins in *B. monniera*. But this method is costly and time-consuming for analysis of a large number of samples. On the other hand, HPTLC is becoming a routine analytic technique because of low operating cost, high sample throughput, simplicity, reproducibility, accuracy, reliability and robustness (Agrawal *et al*. 2006). The added advantage of HPTLC is the minimum sample clean up requirement and hence it is highly suitable for herbal extracts (Srinivasa *et al*. 2004). This technique has been successfully used for quantification of active metabolites in different medicinal plants like, *Plantago lanceolata* (Rischer *et al*. 1998), *Linum* spp. (Vasilev *et al*. 2004) and *Nymphaea stellata* (Rakesh *et al*. 2009). A simple sensitive HPTLC method has been developed for the analysis of bacoside-A in the plant *B. monniera* and in its commercial monoherbal capsule formulations (Gupta *et al*. 1998; Prakash *et al*. 2008). A similar method was adapted for quantitative determination of bacoside-A in cell suspension cultures of *B. monniera* (Rahman *et al*. 2002). In the present study, the HPTLC fingerprint analysis of in *in-vitro* raised biomass with the different treatments showed the variation in spot intensity and peak area as per the accumulation of bacoside-A (**Fig. 1, 3**). Maximum bacoside-A content was observed in shoot biomass raised in liquid MS medium containing 5 µM BA together with 750 mg/l *S. cerevisiae* (**Fig. 1**, Lane No. 2; **Fig. 2**).

Variable amounts of bacoside-A were detected in shoot biomass obtained through all the culture treatments (**Tables 1-4**). Maximum growth and biomass production of *B. monniera* shoots occurred in medium containing 5 µM BA (**Table 1**) whereas Kin and TDZ used over the entire range of concentrations were less effective. Likewise, a higher bacoside-A accumulation was observed in shoots biomass obtained on BA-containing medium. Maximum accumulation of bacoside-A (7.7 ± 1.0 mg/g DW) occurred in the shoot biomass obtained on MS medium fortified with 5 µM BA. These results are in line with the findings of Misra *et al*. (2005) in shoot culture of *Hemidesmus indicus* wherein maximum shoot growth with highest lupeol content was observed at similar combination and concentrations of BA with NAA.

The leaf explant-derived shoots accumulated more bacoside-A compared to the shoots produced from stem explants cultured on MS medium with same concentrations of PGRs. Likewise, the yield of lepidine was dependent on source and type of explant used for *in-vitro* regeneration of plantlets in *Lepidium sativum* (Pande *et al*. 2002). Recently, Praveen *et al*. (2009) reported a 2.2-fold higher accumulation of bacoside-A in the shoots of *B. monniera* regenerated in liquid medium containing 2 mg/l Kin compared to shoots grown on semi-solid medium. In the present investigation, although nature of the medium influenced shoot multiplication, growth of shoots, and overall biomass production, accumulation of bacoside-A was not significantly affected (**Table 5**). Nonetheless, liquid MS medium containing 5 µM BA was superior for *in vitro* production of bacoside-A since biomass production was higher and therefore a correspondingly higher production of bacoside-A was possible. However, Piatczak *et al.* (2005) reported the accumulation of almost the same amount of secoiridoid in *Centuaurium erytroea* shoots grown on liquid as well as agar-solidified medium. On the contrary, the use of liquid medium drastically reduced the cardenolide content in *Digitalis minor* shoots raised *in vitro* (Sales *et al.* 2002). The authors suggested that the negative effect of liquid medium was associated with hyperhydricity in the shoots. In the present investigation, hyperhydricity of the cultures was not observed since the species is adopted to wetland conditions and therefore the use of liquid state culture medium was beneficial for accumulation of bacoside-A.

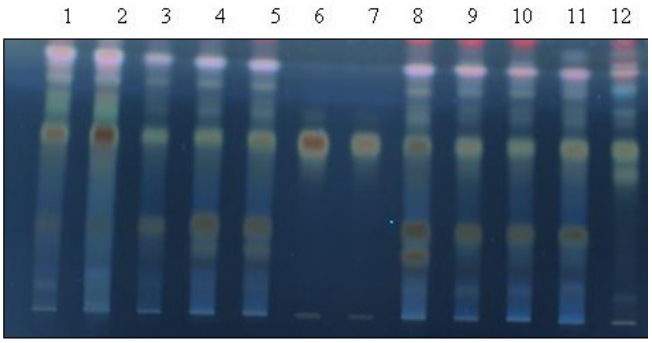

Fig. 1 HPTLC fingerprint profile and densitometric scanning of *in vitro*-raised shoot biomass of *B. monniera.* The sample chromatogram lane from left to right (1-12) were derived on liquid MS + 5 µM BA; liquid MS + 5 µM BA + 750 mg/l *S. cerevisiae*; MS +5 µM BA + 1 µM IAA; MS + 5 µM Kin + 1 µM NAA; MS + 5 µM Kin + 1 µM IAA; standard bacoside-A; standard bacoside-A; MS + 5 µM BA; liquid MS + 5 µM BA + 250 mg/l *C. versicolor*; liquid MS + 5 µM BA + 500 mg/l *Mucor* sp.; liquid MS + 5 µM BA + 750 mg/l *P. notatum*; and liquid MS + 5 µM BA + 500 mg/l *S. cerevisiae*, respectively.

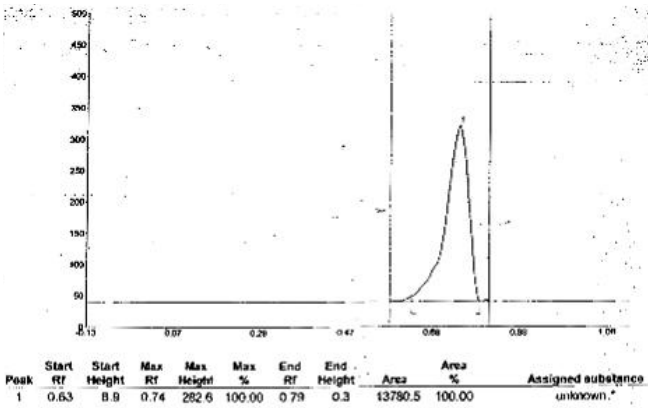

Fig. 2 HPTLC chromatogram showing peak of bacoside-A in shoot biomass obtained on liquid MS + 5 µM BA + 750 mg/l *S. cerevisiae*).

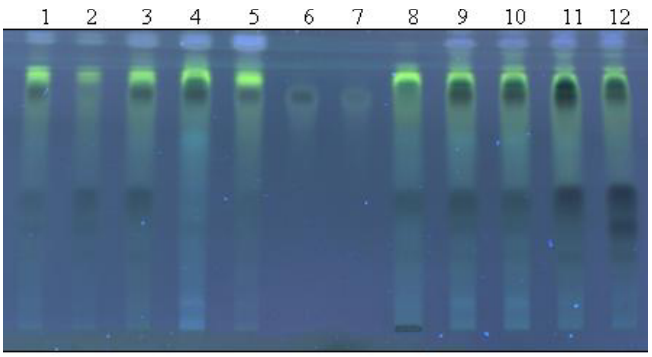

Fig. 3 HPTLC fingerprint profile of leaf derived callus of *B. monniera*. The sample chromatogram lane from left to right (1-12) were derived on 1 µM 2,4-D + 1 µM NAA; 1 µM 2,4-D, 5 µM 2,4-D +1 µM NAA; 1 µM 2,4-D + 5 µM NAA; 10 µM 2,4-D + 10 µM NAA; standard bacoside-A; standard bacoside-A; 5 µM TDZ; 5 µM NAA + 1 µM 2,4-D +1000 mg/l *C. versicolor*; 5 µM NAA + 1 µM 2,4-D + 250 mg/l *Mucor* sp.; 5 µM NAA + 1 µM 2,4-D + 750 mg/l *S. cerevisiae*; and 5 µM NAA + 1 µM 2,4-D + 500 mg/l *S. cerevisiae*, respectively.

Callus culture

Callus cultures were initiated from stem and leaf explants on MS medium supplemented with different concentrations of auxins and cytokinins (**Table 6**). The colour, structure and proliferation of callus varied depending on the PGR used. Among the cytokinins (BA, Kin and TDZ) used, inclusion of 1 to 10 µM TDZ individually in MS medium was effective in induction of callus. The callus produced on such

Table 6 Influence of auxins and cytokinins on callogenesis and bacoside-A production in callus of *Bacopa monniera*.

MS + PGR(s) (µM)						Leaf derived callus		Stem derived callus	
2,4-D	NAA	IAA	BA	Kin	TDZ	DW (mg)	Bacoside-A (mg/g DW)	DW (mg)	Bacoside-A (mg/g DW)
1	-	-	-	-	-	165 ± 2.4 i	1.51 ± 0.4 n	86 ± 1.5 i	0.84 ± 0.1 k
5	-	-	-	-	-	198 ± 3.1 c	1.02 ± 0.5 p	72 ± 1.5 m	0.65 ± 0.02 o
10	-	-	-	-	-	119 ± 3.0 n	0.98 ± 0.01 q	70 ± 1.8 n	0.45 ± 0.04 s
-	1	-	-	-	-	146 ± 2.2 m	2.41 ± 0.2 e	95 ± 2.0 g	0.68 ± 0.01 n
-	5	-	-	-	-	158 ± 2.0 j	2.68 ± 0.6 d	105 ± 1.0 e	0.97 ± 0.05 f
-	10	-	-	-	-	155 ± 2.0 k	1.69 ± 0.6 l	86 ± 1.0 i	0.89 ± 0.06 j
-	-	10	-	-	-	168 ± 1.9 h	1.80 ± 0.0 k	75 ± 1.0 l	0.82 ± 0.06 l
-	-	-	-	-	1	95 ± 3.1 o	0.90 ± 0.03 t	81 ± 2.0 j	0.57 ± 0.05 p
-	-	-	-	-	5	157 ± 3.0 j	0.91 ± 0.03 s	98 ± 3.0 f	0.53 ± 0.05 q
-	-	-	-	-	10	150 ± 3.2 l	0.94 ± 0.01 r	112 ± 2.6 d	0.48 ± 0.01 r
5	-	-	5	-	-	65 ± 2.6 p	1.68 ± 0.5 m	89 ± 2.6 h	0.80 ± 0.04 m
5	-	-	-	5	-	166 ± 1.6 i	2.91 ± 0.2 b	94 ± 1.0 g	0.90 ± 0.04 i
5	-	-	-	-	5	154 ± 1.5 k	1.81 ± 0.1 j	86 ± 1.0 i	0.65 ± 0.01 o
1	1	-	-	-	-	201 ± 1.1 b	2.33 ± 0.1 f	116 ± 1.1 c	0.95 ± 0.02 h
1	5	-	-	-	-	244 ± 1.5 a	3.01 ± 0.6 a	179 ± 1.1 a	1.64 ± 0.1 a
1	10	-	-	-	-	196 ± 1.3 d	2.89 ± 0.8 c	147 ± 1.5 b	1.51 ± 0.1 b
5	1	-	-	-	-	170 ± 1.4 g	1.19 ± 0.5 o	116 ± 2.0 c	1.34 ± 0.1 c
5	5	-	-	-	-	189 ± 1.4 e	2.30 ± 0.5 g	88 ± 2.0 h	1.12 ± 0.5 d
10	10	-	-	-	-	172 ± 1.6 f	2.11 ± 0.6 h	77 ± 1.0 k	0.96 ± 0.05 g
10	1	-	-	-	-	154 ± 3.0k	1.83 ± 0.8 i	75 ± 1.5 l	0.98 ± 0.08 e

Data scored after 4 weeks of culture incubation.
DW: dry weight.
Mean values within a column followed by the same letters did not differ at 5% level of probability by DMRT.

medium was green and compact. The incorporation of BA or Kin induced only shoot regeneration. However, BA or Kin along with 2,4-D had a synergistic effect on callus formation. The callus formed on such medium was greyish and smooth. The use of IAA, NAA and 2,4-D singly resulted in callus formation. Inclusion of 1 µM 2,4-D together with 5 µM NAA was most effective for callus induction in leaf and stem explants. The incorporation of 2,4-D together with NAA was superior to other PGRs for extensive proliferation of callus in leaf as well as stem explants. Such callus was soft and whitish in colour. Maximum dry weight of callus was obtained on MS medium containing 1 µM 2,4-D in conjunction with 5 µM NAA. Considerable callus growth was observed on other combinations of 2,4-D and NAA (**Table 6**). A synergistic effect of auxins in the presence of 2,4-D was reported in cucumber callus culture (Debeaujon and Branchard 1993). Likewise, in *Oryza sativa* unpollinated ovary culture, N6 medium containing 2,4,5-T or 2,4-D with NAA was effective for reasonably good callus induction (Rongbai *et al*. 1998). In the present investigation, incorporation of IAA individually and together with other PGRs was not effective in callus proliferation and was evident from the callus turning brown-blackish after the 2nd and 3rd week of culture initiation on MS medium fortified with IAA.

The effect of PGRs on production of callus biomass and bacoside-A content is shown in **Table 6**. The maximum growth rate in terms of dry weight (244 ± 1.5 mg/culture) of callus and maximum accumulation of bacoside-A (3.0 ± 0.6 mg/g DW) was obtained on MS medium containing 1 µM 2,4-D in conjunction with 5 µM NAA. Roostika *et al*. (2007) reported that the combination of 2,4-D and picloram could produce good quality callus which can be used for secondary metabolite (stigmasterol) production. The bacoside-A content was comparatively less in callus grown on MS medium containing other PGRs (**Table 4**).

Effect of fungal elicitors

It is well known that specific allelochemical interactions between microorganisms and higher plant cells exert stress, which influences the growth and development of plants (Taiz and Zeiger 2006). To overcome stressful conditions, plant cells produce active metabolites which might be medicinally important for humans and animals. In the present investigation the incorporation of mycelial powder of *Penicillium notatum*, *Rhizopus stolonifer*, *Coriolus versicolor*, *Mucor* sp. and *Saccharomyces cerevisiae* might have acted as an allelochemical and created stressful conditions in the callus and shoot cultures of *B. monniera*. The decline in growth of callus and shoot cultures in terms of dry weight is suggestive of the presence of such stressful conditions (**Table 7**). Fungal chemicals in contact with callus or shoots probably induced a defence mechanism which resulted in the additional accumulation of bacoside-A (**Table 7**).

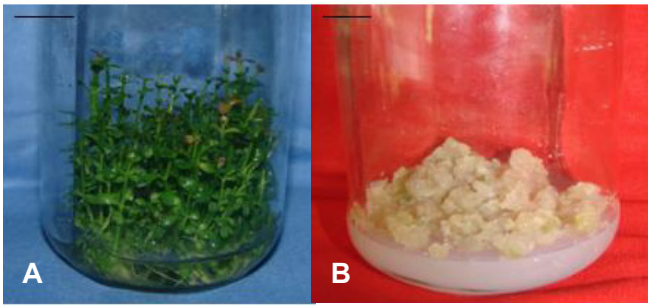

Fig. 4 Callus and shoot cultures of *Bacopa monniera* (L.) Pennell. (**A**) Multiple shoot proliferation from shoot explants on MS + 5 µM BA+ 750 mg/l mycelial biomass of *S. cerevisiae* (bar: 10 mm). (**B**) Leaf derived callus on MS + 5 µM NAA + 1 µM 2,4-D + 750 mg/l *S. cerevisiae* (bar: 10 mm).

The elicited production of bacoside-A was observed when *Mucor* sp. and *S. cerevisiae* were incorporated, whereas the content of bacoside-A was nearer to the control in the callus and shoot cultures treated with mycelial biomass of *P. notatum*, *R. stolonifer* and *C. versicolor* (**Table 7**). Maximum bacoside-A production (14.7 ± 1.1 mg/g DW) was observed in shoots cultured on MS medium containing 750 mg dry biomass of *S. cerevisiae* that resulted in a 1.7-fold higher accumulation of bacoside-A over the control. A 3.2-times higher accumulation of bacoside-A (11.0 ± 0.4 mg/g DW) was also recorded in callus grown on medium containing the same amount of *S. cerevisiae* biomass as compared to the control (3.4 ± 0.1 mg/g DW). However, for higher biomass production and accumulation of bacoside-A, the shoot culture method was superior to the callus culture method (**Table 5, Fig. 4A, 4B**). Concurrent to this, micropropagated plants in association with fungus *Glomus mosseae* and *Trichoderma viride* were superior to non-mycorrhizal micropropagated and normal plants in growth, carbohydrates, protenin, phenolics and bacoside content (Sow-

Table 7 Effect of fungal elicitors on growth and bacoside-A accumulation in shoot and callus cultures of *Bacopa monniera*.

Nutrient medium with fungal elicitors (mg/l)		Shoot culture		Callus culture	
		DW (mg)	Bacoside-A (mg/g DW)	DW (mg)	Bacoside-A (mg/g DW)
C. versicolor	0	426 ± 1.3 def	8.7 ± 1.1 p	270 ± 2.3 a	3.4 ± 0.1 q
	250	451 ± 1.2 cd	8.8 ± 1.1 m	232 ± 1.0 g	3.9 ± 0.1 m
	500	412 ± 1.4 efg	9.2 ± 1.0 i	207 ± 1.2 j	4.3 ± 0.1 i
	750	402 ± 1.3 fghi	8.8 ± 1.2 n	190 ± 1.1 m	5.4 ± 0.0 f
	1000	226 ± 1.1 i	6.7 ± 1.2 r	176 ± 1.2 p	3.2 ± 1.0 r
Mucor sp.	250	391 ± 1.0 ghi	9.0 ± 1.3 k	262 ± 1.1 b	4.6 ± 0.1 h
	500	381 ± 1.9 hij	10.2 ± 1.0 d	244 ± 1.0 de	5.6 ± 0.2 e
	750	340 ± 1.0 jk	13.9 ± 1.0 b	197 ± 1.0 l	10.9 ± 0.2 b
	1000	329 ± 1.0 k	8.7 ± 1.7 o	190 ± 1.2 m	6.0 ± 0.3 d
P. notatum	250	395 ± 1.0 ghi	8.9 ± 1.1 l	184 ± 1.0 n	3.6 ± 1.0 o
	500	372 ± 1.0 ij	9.7 ± 1.0 g	183 ± 0.9 n	4.2 ± 0.1 j
	750	328 ± 1.0 k	5.8 ± 1.0 s	107 ± 1.2 q	3.2 ± 0.1 s
	1000	284 ± 1.1 k	5.5 ± 1.0 t	181 ± 1.0 o	3.0 ± 0.1 t
R. stolonifer	250	389 ± 1.2 hi	8.9 ± 1.3 l	251 ± 1.0 f	4.0 ± 1.0 k
	500	350 ± 1.3 ijk	9.0 ± 1.3 j	245 ± 1.1 e	3.9 ± 0.5 l
	750	316 ± 1.1 kj	9.4 ± 1.1 h	245 ± 1.0 c	3.8 ± 0.5 n
	1000	289 ± 1.0 hi	7.6 ± 1.2 q	243 ± 1.1 d	3.5 ± 0.3 p
S. cerevisiae	250	508 ± 1.0 b	9.5 ± 1.2 f	202 ± 1.2 k	5.0 ± 0.1 g
	500	511 ± 1.9 a	10.4 ± 1.1 c	239 ± 1.2 f	9.9 ± 0.4 c
	750	463 ± 1.3 c	14.7 ± 1.1 a	220 ± 1.1 i	11.0 ± 0.4 a
	100	433 ± 1.1 cde	9.9 ± 1.1 e	225 ± 1.0 h	5.6 ± 1.0 e

Data scored after 4 weeks of culture incubation. DW: dry weight.
Mean values within a column followed by the same letters did not differ at 5% level of probability by DMRT.
Shoot culture medium: MS + 5 µM BA; Callus culture medium: MS + 5 µM NAA+ 1 µM 2, 4 –D

Table 8 Effect of position of explant on shoot regeneration in *Bacopa monniera*.

Position of stem and leaf explant (node no. from shoot apex)	Explants responding for shoot regeneration (%)		No. of shoots per explant	
	Leaf explant	Stem explant	Leaf explant	Stem explant
1- 5	100.0 ± 0.0 a	100.0 ± 0.0 a	11.0 ± 0.9 a	8.3 ± 0.2 a
11 - 15	100.0 ± 0.0 a	100.0 ± 0.0 a	11.0 ± 0.2 a	8.3 ± 0.6 a
21- 25	100.0 ± 0.0 a	100.0 ± 0.0 a	11.0 ± 0.5 a	8.3 ± 0.5 a

Data scored after 4 weeks of culture incubation.
Cultures were incubated on MS + 5 µM BA.
Mean values within a column followed by the same letters did not differ at the 5% level of probability by DMRT.

Table 9 *In vitro* shoot regeneration in *Bacopa monniera*.

Explants used	Range of PGR(s) and additives used	Best explant and media		No. of shoots/explant	Reference
		Shoot induction	Shoot elongation		
Stem, leaf	BA (1-6 µM) Kin (1-6 µM)	Leaf MS + 2 µM BA	Subcultured on MS + 2 µM BA	Large number, impossible to count the number of shoot buds per unit area.	Srivastava and Rajani 1999
Node, internode, leaf	BA (0-22.2 µM) Kin (0-23.2 µM) TDZ (0-22.7 µM) 2-iP (0-24.6 µM)	Leaf MS + 6.8 µM TDZ	MS + 2.2 µM BA	92.7	Tiwari *et al*. 2001
Node, young leaf, internode, shoot tip	BA (0.0-4.4 µM) + IAA (0.2 µM)	Leaf MS + 1.1 µM BA + 0.2 µM IAA	Subcultured on MS + 1.1 µM BA + 0.2 µM IAA	110	Binita *et al*. 2005
Node, internode, leaf	TMP (150-350 mg/l) BVN (150-350 mg/l)	Internode MS + 300 mg/l BVN	MS + 0.1 mg/l BA + 0.2 mg/l IAA	98.6	Tiwari *et al*. 2006
Leaf, stem	BA (0-10 µM) Kin (0-10 µM) TDZ (0-10 µM)	Leaf MS + 5 µM BA	On the same medium without subculture shoot elongation observed.	11.0	Present study

TMP: Trimethoprim; BVN: Bavistin

mya *et al*. 2007). The cell wall of yeast and *Mucor* contain chitoglucan and peptidoglycan, respectively (Wang *et al*. 2006). It is possible that yeast and *Mucor* sp. contain more ingredients advantageous for bacoside-A synthesis. The constructive effect of yeast elicitors on triterpenoid production has been reported in other plant species like *Scutellaria baicalensis* (Yoon *et al*. 2000) and *Unacaria tomentosa* (Feria-Romero *et al.* 2005).

CONCLUSION

The present study demonstrates that *in-vitro* shoot and callus cultures of *B. monniera* can be maintained over a period of 2 years on transfer to fresh parental medium at an interval of 4 weeks and are capable of bacoside-A production. Application of 750 mg/l *S. cerevisiae* in agar-solidified MS medium containing 5 µM NAA together with 1 µM 2,4-D for callus culture and in liquid MS medium fortified with 5 µM BA for shoot culture, significantly enhance the biomass production and accumulation of bacoside-A. This procedure may be adapted for *in-vitro* multiplication and sustainable production of bacoside-A through shoot and callus cultures of *B. monniera* and is comparable or superior to other studies using the same or different media and explants (**Tables 8, 9**).

ACKNOWLEDGEMENTS

The authors are grateful to the Department of Botany, University of Pune, India, for providing the necessary laboratory facilities and to Mr. Dilip Charegaonkar (Managing Director, Anchrom HPTLC Specialists, Mumbai, India) for providing HPTLC facilities.

REFERENCES

Agrawal H, Kaul N, Paradkar AR, Mahadik KR (2006) Separation of bacoside A3 and bacopaside II, major triterpenoid saponins in *Bacopa monnieri*, by HPTLC and SFC. Application of SFC in implementation of uniform design for herbal drug standardization, with thermodynamic study. *Acta Chromatographica* **17**, 125-150

Anonymous (1998) *Indian Herbal Pharmacopoeia* (Vol I), RRL, Jammu Tawi and IDMA, Mumbai, India, 30 pp

Arrebola ML, Socorro O, Verpoorte R (1997) Micropropagation of *Isoplexis canariensis* (L.) G. Don. *Plant Cell, Tissue and Organ Culture* **49**, 117-119

Banerjee M, Shrivastava S (2008) An improved protocol for *in vitro* multiplication of *Bacopa monniera* (L.) *World Journal of Microbial Biotechnology* **24**, 1355-1359

Binita BC, Dave AM, Jasrai YT (2005) *Bacopa monnieri* (L.) Pennell: A rapid, efficient and cost effective micropropagation. *Plant Tissue Culture and Biotechnology* **15 (2)**, 167-175

Chopra RN, Chopra IC, Varma BS (1969) *Glossary of Indian Medicinal Plants*, Council of Scientific and Industrial Research, New Delhi, India, 10 pp

Debeaujon I, Branchard M (1993) Somatic embryogenesis in Cucurbitaceae. *Plant Cell, Tissue and Organ Culture* **34**, 91-100

Deepak M, Amit A (2004) The need for establishing identities of 'bacoside A and B', the putative major bioactive saponins of Indian medicinal plant *Bacopa monniera*. *Phytomedicine* **11**, 264-268

Duncan DB (1955) Multiple range and multiple *F* tests. *Biometrics* **11**, 1-42

Feria-Romero I, Lazo E, Ponce-Noyola T, Cerda-Garcia-Rojas C, Ramos-Valdivia A (2005) Induced accumulation of oleanolic acid and ursolic acid in cell suspension cultures of *Uncaria tomentosa*. *Biotechnology Letters* **27**, 839-843

Gupta AP, Mathur S, Gupta MM, Kumar S (1998) Effect of the method of drying on the bacoside A content of the harvested *B. monniera* shoots revealed using a high performance thin layer chromatography method. *Journal of Medicinal and Aromatic Plant Science* **20**, 1052-1055

Jiang B, Yang Y, Guo Y, Chen Y (2005) Thidiazuron-induced *in-vitro* shoot organogenesis of the medicinal plant *Arnebia euchroma* (Royle) Johnst. *In Vitro Cellular and Developmental Biology – Plant* **41**, 677-681

Mahato SB, Garai S, Chakrvarty AK (2000) Bacosaponins E and F: two jujubogenin bacosides form *Bacopa monniera*. *Phytochemistry* **53**, 711-714

Mathur S, Sushil K (1998) Phytohormone self sufficiency for regeneration in the leaf and stem explants of *Bacopa monnieri*. *Journal of Medicinal and Aromatic Plant Sciences* **20**, 1056-1059

Misra N, Misra P, Datta SK, Melhotra S (2005) *In vitro* biosynthesis of antioxidants from *Hemidesmus indicus* R.Br. cultures. *In Vitro Cellular and Developmental Biology – Plant* **41**, 285-290

Mulabagal V, Tsay HS (2004) Plant cell cultures – an alternative and efficient source for the production of the biologically important secondary metabolites. *International Journal of Applied Science and Engineering* **2**, 29-48

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Murthy BNS, Murch SJ, Saxena PK (1998) Thidiazuron: a potent regulator of *in-vitro* plant morphogenesis. *In Vitro Cellular and Developmental Biology – Plant* **34**, 267-265

Murthy PBS, Raju VR, Ramakrishna T, Chakravarthy MS, Kumar KV, Kannababu S, Subbaraju GV (2006) Estimation of twelve bacopa saponins in *Bacopa monnieri* extracts and formulations by high-performance liquid chromatography. *Chemical and Pharmaceutical Bulletin* **54**, 907-911

Namdeo AG (2007) Plant cell elicitation for production of secondary metabolites: A review. *Pharmacognosy Reviews* **1 (1)**, 69-79

National Medicinal Plant Board (NMPB) (2007) Government of India and Technology Information Forecasting and Assessment Council. Available online: http://www.nmpb.nic.in/prioritisedmedicinalplants.htm

Pal R, Sarin JPS (1992) Quantitative determination of bacosides by UV-spectrometry. *Indian Journal of Pharmaceutical Science* **54**, 17-18

Pande D, Malik S, Bora M, Srivastava SP (2002) A rapid protocol for *in vitro* micropropagation of *Lepidium sativum* Linn. and enhancement in the yield of lepidine. *In Vitro Cellular and Developmental Biology – Plant* **38**, 451-455

Piatczak E, Wielanek M, Wysokinska H (2005) Liquid culture system for shoot multiplication and secoiridoid production in micropropagated plants of *Centaurium erythraea* Rafn. *Plant Science* **168**, 431-437

Prakash O, Singh GN, Sing RM, Mathur SC, Bajpei M, Yadav S (2008) Determination of bacoside A by HPTLC in *Bacopa monnieri* extract. *International Journal of Green Pharmacy* **1**, 173-175

Prasad MNV, Padmalatha K, Jayaram K, Raju NL, Teixeira da Silva JA (2007) Medicinal plants from the Deccan Ecoregion, India: Traditional knowledge, ethnopharmacology, cultivation, utilization, biotechnology and conservation – Opportunities and impediments. *Medicinal and Aromatic Plant Science and Biotechnology* **1**, 155-208

Praveen N, Naik PM, Manohar SH, Nayeem A, Murthy HN (2009) *In vitro* regeneration of brahmi shoots using semisolid and liquid cultures and quantitative analysis of bacoside A. *Acta Physiologia Plantarum* **31**, 723-728

Rahman LU, Verma PC, Singh D, Gupta MM, Banerjee S (2002) Bacoside production by suspension cultures of *Bacopa monniera* (L.) Pennell. *Biotechnology Letters* **24**, 1427-1429

Rakesh SU, Patil PR, Salunkhe VR, Dhabale PN, Burade KB (2009) HPTLC method for quantitative determination of quercetin in hydroalcoholic extract of dried flower of *Nymphaea stellata* Willd. *International Journal of ChemTech Research* **1 (4)**, 931-936

Rischer M, Adamczyk M, Ratz H, Hose S, Marchesan M, Paper DH, Franz G, Wolf-Heuss E, Engel J (1998) Quantitative determination of the iridoid glycosides aucubin and catalpol in *Plantago lanceolata* L. extracts by HPTLC and HPLC. *Journal of Planar Chromatography* **11**, 374-378

Rongbai L, Pandey MP, Garg GK, Pandey SK, Dwivedi DK, Ashima (1998) Development of a technique for *in vitro* unpollinated ovary culture in rice, *Oryza sativa* L. *Euphytica* **104**, 159-166

Roostika I, Purnamaningsih R, Darwati I, Mariska I (2007) *In vitro* culture manipulation on pruatjan for secondary metabolite production. *Journal of AgroBiogen* **3 (2)**, 55-59

Russo A, Borrelli F (2005) *Bacopa monniera*, a reputed nootropic plant: an overview. *Phytomedicine* **12**, 305-317

Sales E, Nebauer SG, Arrilaga I, Segura J (2002) Plant hormones and *Agrobacterium tumefaciens* strain 82.139 induce efficient plant regeneration in the cardenolide-producing plant *Digitalis minor*. *Journal of Plant Physiology* **159**, 9-16

Sestak A, Castky J, Jervis P (1971) Methods of growth analysis. In: Junk W (Ed) *Plant Photosynthtic Production: Manual of Methods*, NV Publishers, The Hague, pp 363-443

Sharath R, Krishna V, Sathyanarayana BN, Prasad MBN, Harish BG (2007) High frequency regeneration through somatic embryogenesis in *Bacopa monnieri* (L.) Wettest, an important medicinal plant. *Medicinal and Aromatic Plant Science and Biotechnology* **1**, 138-141

Shinde A, Malphathal N, Fulzele D (2009) Induced high frequency shoot regeneration and enhanced isoflavones production in *Psoralea corylifolia*. *Records of Natural Products* **3 (1)**, 38-45

Singh HK, Dhawan BN (1997) Neurophychopharmacological effects of the Ayurvedic nootropic *Bacopa monniera* Linn. (Brahmi). *Indian Journal of Pharmacology* **29**, 8359-8365

Singh HK, Rastogi RP, Srimal RC, Dhawan BN (1988) Effect of bacoside A and B on the avoidance response in rats. *Phototherapy Research* **2**, 70-75

Srinivasa H, Bagul MS, Padh H, Rajani R (2004) A rapid densitometric method for the quantification of luteolin in medicinal plants using HPTLC. *Chromatographia* **60**, 131-134

Srivastava N, Rajani M (1999) Multiple shoots regeneration and tissue culture studies on *Bacopa monnieri* (L.) Pennell. *Plant Cell Reports* **18**, 919-923

Sowmya R (2007) Utilization of VA Mycorrhizal fungi and *Trichoderma viride* on plant growth and drug content of micropropagated *Bacopa monnieri* (L.) Pennell. Available online: www.scribd.com/doc/326964/eArticle

Taize L, Zeiger E (2006) Secondary metabolites and plant defense. In: *Plant Physiology* (4th Edn), Sinauer Associates, Massachusetts, USA, pp 315-341

Tiwari V, Tiwari KN, Sing BD (2000) Suitability of liquid cultures for *in vitro* multiplication of *Bacopa monniera* (L.) Wettst. *Phytomorphology* **50 (3-4)**, 337-342

Tiwari V, Tiwari KN, Singh BD (2001) Comparative studies of cytokinins on *in vitro* propagation of *Bacopa monniera*. *Plant Cell, Tissue and Organ Culture* **66**, 9-6

Tiwari V, Tiwari KN (2006) Shoot bud regeneration from different explants of *Bacopa monniera* (L.) Wettst. by trimethoprim and bavistin. *Plant Cell Reports* **25**, 629-635

Varghese DB, Sathyanarayana BN (2007) Induction of variations in two cultivars of *Bacopa monnieri* by gamma irradiation in *in-vitro* cultures. *Medicinal and Aromatic Plant Science and Biotechnology* **1**, 86-89

Vasilev N, Momekov G, Zaharieva M, Konstantinov S, Bremner P, Heinrich M, Ionkova I (2005) Cytotoxic activity of podophyllotoxin-like lignan from *Linum tauricum* Willd. *Neoplasma* **52**, 425-429

Vila S, Gonzalez A, Rey H, Mroginski L (2005) Plant regeneration, origin and development of shoot buds from root segments of *Melia azedarach* L. (Meliaceae) seedlings. *In Vitro Cellular and Developmental Biology – Plant* **41**, 746-751

Wang W, Yu L, Zhou P (2006) Effects of different fungal elicitors on growth, total carotenoids and astaxanthin formation by *Xanthophyllomyces dendrorhous*. *Bioresource Technology* **97**, 26-31

Watoo P, Waraporn P, Hiroyuki T, Kanchalee J, Sakchai W, Kornkanok I (2007) Comparison of various extraction methods of *Bacopa monniera*. *Naresuan University Journal* **15 (1)**, 29-34

Yoon H, Kin H, Ma C, Huh H (2000) Induced accumulation of triterpenoids in *Scutellaria baicalensis* suspension cultures using a yeast elicitor. *Biotechnology Letters* **22**, 1071-1075