Biological Forum — An International Journal, **2**(1): 18-22(2010) **ISSN : 0975-1130**

Effective organogenesis from different explants of *Bacopa monniera* L.(Wettst.)-An important medicinal plant

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ABSTRACT : *Bacopa monniera* commonly known in India as 'Brahmi', it is an important ancient ayurvedic medicinal plant. In the traditional system of medicine Brahmi is used as a nervine tonic. It is also used to treat asthma, epilepsy, enlargement of spleen, rheumatism. It possesses anti-inflammatory, analgesic and antipyretic activity. It contains several alkaloids e.g., nicotine, brahmine, herpestine and saponins such as bacosides A,B,C and D. Propagation of *B. monniera* through seeds is slow due to short viability and frequent seedling death. Its vegetative propagation is slow due to poor performance of propagules. Therefore, development of a rapid clonal multiplication of this medicinally important herb has become imperative in order to reduce the pressure on natural populations and constant supply of plant materials for pharmaceutical industries. The morphogenic potential of node, internode and leaf explants was investigated to develop reliable protocol for shoot regeneration. A range of cytokinins tested (6-benzylaminio purinre, BAP; kinetin, KIN; thidiazuron, TDZ; and 2-isopenteny adenine,2-iP) for multiple shoot induction. Of the four cytokinins tested TDZ(6.8µM) and BAP(8.9µM) proved superior to other treatments. A mass *in vitro* propagation system devoid of growth regulators has been developed. MS medium supplemented with an antibiotic (trimethoprim) or a fungicide (bavistin) supports direct shoot bud induction on internode and leaf explants. Bavistin showed a marked cytokinin like activity thus optimum adventitious shoot buds induction occurred on MS medium supplemented with 1569.14µM bavistin. MS medium supplemented with 0.44µM BAP and 1.14µM IAA found most suitable for shoot elongation. *In vitro* derived shoots exhibited better rooting response on MS medium containing 4.9µM IBA. Regenerated rooted plantlets of *B. monniera* were successfully acclimatized in soilrite. The *in vitro* raised plants were grown normally in the field without showing any morphological variation.

Keywords : Brahmi, *Bacopa monniera*, Shoot regeneration

Abbreviations : BVN-bavistin {Methyl benzimidozole carbamate}; BAP-6-benzylaminopurine; GR-growth regulator; IAA-indole-3-acectic acid; IBA-indole-3-butyric acid; KIN-kinetin; MS medium-Murashige and Skoog (1962) medium; NAA-α naphthaleneacetic acid; TDZ-thidiazuron (N-phenyl-N'-1, 2, 3-thidiazol-5-ylurea); TMP-trimethoprim {2,4 di-amino-5-{3,4,5-trimethoxybenzyl} pyrimidine.

INTRODUCTION

Bacopa monniera (L.) wettst., commonly knowm as 'Brahmi', is an important ancient medicinal plant. It is belong to family Scrophulariaceae. It is a renowned medicinal plant with legendasy reputation as amemory vitalizer (Anonymous, 1998). In the traditional system of Indian medicine (Ayurveda), 'Brahmi' is classified as medhya rasayan, *i.e.*, a drug that is supposed to counteract the effect of mental stress and improve intelligence and memory function. It is used to treat asthma, insanity, epilepsy, enlarge ment of the spleen, rheumatism, leprosy and eczema (Singh *et. al.*, 1979). It possess anti-inflamatory, analgesic, antipyretic and diuretic activity (Vohora *et. al.*, 1997; Stough *et. al.*, 2001). *Bacopa monniera* was placed in a priority list of the most important Indian medicinal plants evaluated on the basis of medicinal importance, commercial value and potential for further research and development (Karke and Williams, 1999). The medicinal properties of *B.monniera* have been attributed to the presence of different types of saponins, *e.g.*, bacosides *A*, *B*, *C* and *D* (Rastogi *et. al.*, 1994).

The requirement of brahmi is met solely from the natural populations, leading to their gradual depletion. Seed of *B.monniera* are poor propagule due to their short viability and frequent seedling death, which makes it difficult to raise plants from seeds. Vegetative propagation is also slow (Shah, 1965). It is now listed as a threatened species by International Union for Conservation of Nature and Natural Resources (Pandey *et. al.*, 1993). Therefore, development of a rapid clonal multiplication protocol of this medicinally important herb has become imperative in order to reduce the existing pressure on natural populations and constant supply of plant material for pharmaceutical industry. In previous study shoots induction from different explant of *B.monniera* (Thakur and Ganpathy, 1978; Tiwari *et. al.*, 1998; Shrivastava and Rajani, 1999) were reported. In present study different cytokinins evaluated for its multiple shoot induction so that a suitable concentration of cytokinin recommended for high yield of biomass and continuous propagation of this medicinal plant. In present study an antibiotic-trimethoprim (TMP) and fungicide-

bavistin (BVN) are also evaluated for its regeneration potential from different explants of *B. monniera*.

MATERIALS AND METHODS

Explants preparation and culture conditions. Node (0.8-1.0 cm), internode (1.0 cm) and leaf (0.6 cm) explants were excised from 4-week old shoots raised from axillary buds of nodal explants cultured on GR-free MS (Murashige and Skoog 1962) medium. The shoot regeneration potential of different explants was assessed on MS medium containing BAP(0.0-22.2µM), kinetin(0.0-23.2µM), TDZ(0.0-22.7µM), 2iP(0.0-24.6µM) Table1. BVN(0.0-1830.5µM) and TMP(0.0-1205.4 µM) were added separately in MS medium (Table 2) to evaluate its potential for organogenesis. TMP (Hi-Media, Mumbai, India) and BVN (BASF, Mumbai, India) were added in the media before autoclaving. All the media were supplemented with 3% sucrose. The media were solidified with 0.8% agar (w/v) (Himedia, Mumbai, India). The pH of the medium was adjusted to 5.8 prior to autoclaving. Media sterlized by autoclving at 15 p.s.i. (1.04 kg/cm^2) pressure for 20 minutes. The cultures were incubated at 24 ± 2°C under 16-h photoperiod with white cool florescent tubes (Philips, India) at a unit of irradiance of 50 µmolm^{-2}s^{-1}.

Table : 1. Response of different explants of *Bacopa monniera* cultured on MS medium supplemented with various cytokinins.

Cytokinins	Concentration (µM)	Explant		
		Node	Internode	Leaf
BAP	0.0	0.0^{a} (0)*	8.3^{a} (75)	6.1^{a} (100)
	0.44	8.1^{b} (100)	16.1^{b} (100)	14.2^{b} (100)
	2.2	8.5^{b} (100)	17.8^{c} (100)	22.6^{c} (100)
	4.4	17.6^{d} (100)	23.2^{e} (100)	28.5^{e} (100)
	6.6	21.6^{e} (100)	26.8^{g} (100)	35.7^{d} (100)
	8.9	25.2^{e} (100)	30.5^{f} (100)	82.4^{g} (100)
	22.2	12.2^{c} (100)	22.3^{d} (100)	70.5^{f} (100)
KIN	0.0	0.0^{a} (0)	6.9^{a} (80)	3.2^{ab} (95)
	0.46	3.4^{b} (100)	8.4^{b} (100)	5.2^{a} (100)
	2.3	3.8^{c} (100)	17.2^{c} (100)	7.8^{b} (100)
	4.6	10.2^{d} (100)	19.4^{d} (100)	22.4^{f} (100)
	6.9	20.4^{f} (100)	22.5^{e} (100)	15.2^{e} (100)
	9.3	12.5^{e} (100)	17.3^{c} (100)	13.2^{d} (100)
	23.2	7.4^{d} (100)	15.8^{c} (100)	11.4^{c} (100)
TDZ	0.0	0.0^{a} (100)	7.2^{b} (100)	5.8^{a} (100)
	0.45	3.2^{b} (100)	12.4^{c} (100)	20.2^{c} (100)
	2.2	10.6^{c} (100)	16.2^{d} (100)	22.7^{c} (100)
	4.5	15.7^{e} (100)	18.1^{e} (100)	30.8^{d} (100)
	6.8	50.2^{g} (100)	30.1^{f} (100)	120.8^{f} (100)
	9.0	25.1^{f} (100)	5.2^{b} (100)	52.2^{e} (100)
	22.7	9.2^{d} (100)	2.2^{a} (100)	18.2^{b} (100)
2-iP	0.0	0.0^{a} (100)	4.2^{bc} (100)	5.8^{b} (100)
	0.49	3.4^{b} (100)	6.8^{bc} (100)	3.9^{a} (100)
	2.4	5.2^{c} (100)	4.5^{b} (100)	4.7^{ab} (100)
	4.9	6.2^{c} (100)	6.2^{bc} (100)	8.1^{c} (100)
	7.3	8.8^{d} (100)	7.8^{c} (100)	13.4^{e} (100)
	9.3	11.2^{e} (100)	15.8^{d} (100)	14.1^{e} (100)
	24.6	6.4^{d} (100)	2.8^{a} (100)	12.5^{d} (100)

Each mean is based on two replicates, each of which consisted 20 culture tubes (Culture age; 4 weeks). Mean having different letters in superscript are significantly different from each other ($p < 0.05$) according to ANOVA and Duncan multiple range test.

* Figures within parentheses represent responding frequency in percent.

Table : 2. Effect of different concentrations of additives; Trimethoprim (TMP) and Bavistin (BVN) on shoot bud induction from different explants of *Bacopa monniera*.

Additives (µM)		Number of shoots produced		
TMP	BVN	Node	Internode	Leaf
0.0	0.0	0.0(0)^{a}	0.0(0)^{a}	0.0(0)^{a}
172.2	0.0	1.2(40)^{b}	5.4(30)^{b}	7.2(25)^{b}
344.4	0.0	2.2(55)^{bc}	20.4(65)^{d}	15.0(62)^{c}
516.0	0.0	4.2(75)^{c}	30.5(80)^{f}	25.0(75)^{cd}
688.8	0.0	8.9(90)^{cde}	40.2(100)^{g}	32.0(95)^{d}
861.0	0.0	5.4(82)^{cd}	25.0(75)^{e}	22.4(90)^{cd}
1033.2	0.0	3.1(40)^{bc}	15.6(40)^{cd}	14.8(50)^{c}
1205.4	0.0	2.0(10)^{b}	10.2(20)^{c}	8.6(15)^{b}
0.0	261.5	5.6(50)^{cd}	25.2(60)^{e}	15.2(55)^{c}
0.0	523.0	12.2(56)^{e}	40.1(70)^{g}	25.5(70)^{cd}
0.0	784.0	14.4(75)^{ef}	65.6(82)^{h}	52.4(78)^{e}
0.0	1046.0	20.5(80)^{f}	12.4(90)^{cd}	65.8(85)^{f}
0.0	1307.5	25.2(85)^{fg}	95.8(90)^{i}	85.5(90)^{g}
0.0	1569.1	40.4(100)^{h}	125.1(100)^{j}	95.4(100)^{h}
0.0	1830.5	15.2(60)^{ef}	25.0(60)^{e}	22.2(55)^{cd}

Each mean is based on two replicates, each of which consisted of 20 culture tubes (culture age: 4 weeks). Mean having different letters in superscript are significantly different from each other ($p < 0.05$) according to ANOVA and Duncan multiple range test.

Elongation, rooting and acclimatization. For elongation and growth of shoots, MS medium supplemented with GA_3 (0.29-2.89 µM), BAP(0.04-0.44 µM) and BAP(0.04-0.44 µM) plus IAA(1.14 µM) Table 3. For root induction elongated shoots 5cm were harvested and cultured on MS media supplemented with IBA (0.49-4.90 µM) Table 4. Plantlets were removed from the culture vials, washed gently under running tap water, placed in a 250 ml beaker containing tap water for 15 min, and finally transferred to cups filled with sterilized soilrite. To achieve high humidity the cups were covered with polythene bags and irrigated daily with 1-2 ml tap water; the plants were maintained in culture room at 24 ± 2ºC and 16-h daily illumination of 20 µmol m^{-2} s^{-1} provided by the cool white fluorescent tubes for another 2 weeks before the plants were transferred to the plastic cup containing mixture of garden soil and sand before shifting into the field.

Table : 3. Elongation of shoots of *B. monniera* cultured on MS medium supplemented with different growth regulators.

Growth regulators (µM)			Shoot length (cm)	Number of nodes/shoot
GA3	BAP	IAA		
0.0	0.0	0.0	0.0^{a}	0.0^{a}
0.29			2.1^{b}	2.4^{b}
1.44			3.2^{c}	2.8^{b}
2.89			3.8^{cd}	3.1^{bc}
	0.04		3.4^{c}	3.2^{bc}
	0.22		4.2^{d}	3.9^{c}
	0.44		5.1^{e}	4.1^{c}
	0.04	1.14	4.3^{d}	4.0^{c}
	0.22	1.14	5.4^{e}	5.8^{d}
	0.44	1.14	7.8^{f}	6.2^{d}

(Contd...)

Each mean is based on two replicates, each of which consisted of 20 culture tubes (culture age: 4 weeks). Mean having different letters in superscript are significantly different from each other ($p < 0.05$) according to ANOVA and Duncan multiple range test.

Table 4 : Evaluation of different concentrations of IBA for *in vitro* root regeneration from shoots of *B. monniera* cultured on MS medium.

Concentation of IBA (µM)	Number of roots/shoots	Mean root length (cm)
0.49	1.2^{a}	1.1^{a}
0.98	3.2^{b}	2.5^{b}
1.97	4.8^{bc}	3.8^{c}
3.44	6.7^{c}	4.5^{cd}
4.90	8.2^{d}	5.2^{d}

Each mean is based on two replicates, each of which consisted of 20 culture tubes (culture age: 4 weeks). Mean having different letters in superscript are significantly different from each other ($p < 0.05$) according to ANOVA and Duncan multiple range test.

Statistical analysis of the data. Experiments were set up in a randomized block design and each experiments usually had two replicates; each of which consisted of 20 culture tubes. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means. The treatment means were compared using Duncan's multiple range test (DMRT) at the 5% probability level according to Gomez and Gomez (1984).

RESULTS AND DISCUSSION

The four cytokinins (BAP, KIN, TDZ and 2-iP) were evaluated for multiple shoot induction on node, internode and leaf explants. Shoot buds were induced after 4 weeks of culture initiation. Analysis of variance revealed a significant effect ($P < 0.05$) of explant, cytokinin within each explant and cytokinin concentration within cytokinins within explant for a number of adventitious shoot buds produced per explant. A comparison of the different explant showed that maximum average number of adventitious shoot buds per explant was observed in leaf (Fig.1C) followed by internode (Fig.1B) and node (Fig.1A), Table 1.

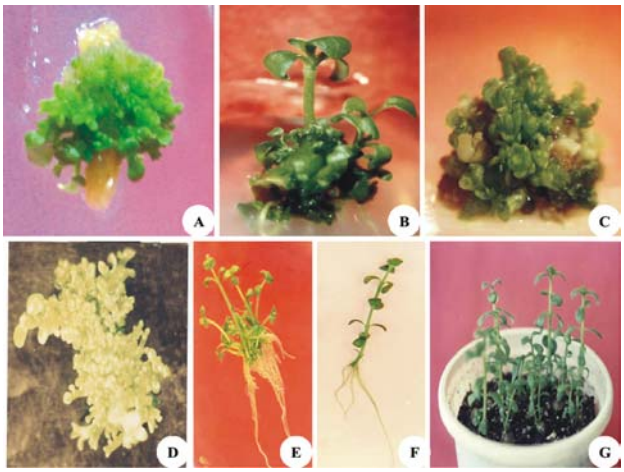

Fig. 1A. Shoot induction from node on MS+8.9 µM BAP, **B.** Adventitious shoot bud induction from internode cultured on MS+8.9 µM BAP, **C.** Adventitious shoot bud induction from leaf cultured on MS+8.9 µM BAP, **D.** High frequency adventitious shoot bud induction from internode cultured on MS+1569.14 µM bavistin, **E.** Shoot elongation on MS+0.44 µM BAP+1.14 µM IAA, **F.** Rooting of elongated shoot cultured on MS+ 4.9 µM IBA, **G.** Acclimatized plantlets of *B. monniera* transferred into plastic cup containing the mixture of garden soil and sand.

In general, BAP was more effective than other cytokinins. The stimulating effect of BAP on multiple shoot formation has been reported for several medicinal plant species (Wang *et. al.*, 2004; Espimosa *et. al.*, 2006).

TMP and BVN concentration had a stimulatory effect on shoot bud induction from different explants of *Bacopa monniera* Table 2. In general, the number of shoot buds per explant increased up to certain concentrations depending upon the additives TMP (688.8 µM) and BVN (1569.1 µM) .In absence of additives, shoot buds were not produced. BVN is superior than TMP for shoot induction. The explants cultured on MS medium containing 1569.1 µM BVN were the most effective both in terms of frequency and number of shoot bud regeneration Table 2. Internode was the most responsive explant (Fig.1D), producing the highest number of adventitious shoot buds (125.1) followed by leaf (95.4) and node (40.4) explants. BVN is a systemic fungicide that belongs to benzimidazole family. Benzimidazoles are a group of organic fungicides with systemic action. Its molecular structure shows resemblance with cytokinins (Tripathi and Ram, 1982). Benzimidazoles have a cytokinin activity in soy and radish (Skene, 1972; Thomas, 1974). It needs further investigation to explain the mechanism by which BVN triggers shoot regeneration in *Bacopa monniera* cultures.

Shoots developed from different explants they did not elongate further. Therefore, young shoots were transferred to MS medium supplemented with GA_3 (0.29-2.89 µM), BAP (0.04-0.44 µM) and BAP (0.04-0.44 µM)+IAA (1.14 µM) Table 3. Optimum shoot growth in terms of shoot length (7.8 cm) and number of nodes per shoots(6.2) was recorded on MS medium supplemented with BAP (0.44 µM) and IAA (1.14 µM) (Fig.1E).

Elongated shoots of 5-6 cm in length were separated and culture on MS medium supplemented with IBA (0.49-4.90 µM) Table 4. Analysis of variance revealed a significant effect ($p < 0.05$) on the number of roots/shoots and mean root length. A comparison by DMRT revealed optimum number of roots/shoot (8.2) and root length (5.2 cm) (Fig.1F). IBA is highly effective auxin for rooting of *in vitro* regenerated shoots in several plants species (Gururaj *et. al.*, 2007).

The acclimatized plantlets (Fig.1G) were successfully established in the field with 95% survival. There was no variation observed among field transferred plants with respect to morphology and growth characteristics.

This study established the very high frequency *in vitro* regeneration protocol for mass multiplication of *B. monniera*. The present study demonstrates the novel role of BVN in inducing high-frequency adventitious shoot bud formation.

ACKNOWLEDGEMENT

The financial support to JS in the form of SRF by UGC, New Delhi is gratefully acknowledged.

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