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Bacopa monnieri (L.) Pennell: A Rapid, Efficient and Cost Effective Micropropagation

B. Chaplot Binita, M. Dave Ashok and T. Jasrai Yogesh[1*]

Agribiotechnology Laboratory, GSFC Science Foundation, Vigyan Bhavan, Vadodara -391750 Gujarat, India

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Abstract

An efficient and cost effective protocol is described for rapid and large scale *in vitro* propagation of the valuable medicinal herb, *Bacopa monnieri* (L.) Pennell by bud proliferation on nodal segments, young leaves, internodes and shoot tips isolated from field-grown mature plants. This was achieved on MS solid and liquid medium with 1.1 μM BA and 0.2 μM IAA within three weeks of inoculation. Normally, the axillary nodes gave rise to seven - eight shoots. In addition to this, each leaf gave rise to a large number of shoot buds (110 shoots) from all over the surface, while internodes gave rise to a clump of shoots (28 shoots). The solid medium was more effective for bud proliferation from the leaf while the liquid medium proved more suitable for axillary nodes and internode explants. Axillary buds located at middle level nodes (4 - 7 from shoot tip) were found to be more promising and resulted in direct multiplication of about eight shoots. Elongation of shoots and subsequent root induction were achieved on the same proliferation medium only. On an average, within a period of three subcultures, different explants like leaf-, node- and internode explants generated 12100, 49, 784 shoots, respectively thereby favoring the economics of the cost of the materials and time factors. The regenerated plants resembled the mother plants in general habit without any morphological variation. HPLC analysis of the regenerated shoots revealed a phytochemical profile similar to that of the market sample and mother plants. A reproducible, very simple - one step procedure for *in vitro* propagation of *Bacopa monnieri* has been established. This protocol can be used to generate foundation stocks of elite planting material for large scale cultivation.

Introduction

Medicinal plants are of great interest to the researchers in the field of biotechnology as most of the drug industries depend, in part, on plants for the production of pharmaceutical compounds (Chand et al. 1997). Among the World's 25 best selling pharmaceutical medicines, 12 are plant derived (O'Neill

*Present address: Department of Botany, School of Sciences, Gujarat University, Ahmedabad-380009, India. [1]Department of Botany, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara-390002, Gujarat, India.E-mail: yjasrai@yahoo.com

and Lewis 1993). *Bacopa monnieri* (L.) Pennell belonging to the family Scrophulariaceae is an amphibious plant of the tropics and normally found growing on the banks of rivers and lakes. It is commonly known in India as *brahmi* or *jala-brahmi*. It is a small creeping, glabrous and succulent herb with thick, soft, ascending branches and sessile, obovate-oblong or spatulate leaves; flowers are whitish blue with purple veins on long pedicels. It has a great market demand due to its high medicinal values. Moreover, because of the heavy demand and short supply, it is the most adulterated species in *Ayurvedic* formulations. So there is a need to mass-propagate the selected clones. Furthermore, their natural regeneration is hampered by death at two leaf stage and specific habitat requirement. The submerged shoots of *B. monnieri* can hardly ramify to attain the required growth and multiplication. Therefore, it is necessary to develop and standardize the large-scale multiplication through micropropagation.

Brahmi is also known as "Medhya Rasayana" in Ayurveda as it increases mental clarity and brain stimulating action (Bhattacharya and Ghosal 1998). It also possessess anti-inflammatory, analgesic, antipytretic, epilepsy, insanity, anticancer and antioxidant activities (Satyavati et al. 1976; Jain et al. 1994; Elangovan et al. 1995; Tripathi et al. 1996; Vohora et al. 1997). It is also used in the treatment of asthma, hoarseness, water retention and blood cleaning. Moreover, leaf juice of brahmi is given to children for relief in bronchitis and diarrhoea.

The medicinal properties of *Bacopa monnieri* responsible for improving memory-related functions have been attributed to the presence of different types of saponins such as bacosides A, B, C and D which are the active triterpenoid principles and known as "memory chemicals" (Rastogi et al. 1994). These compounds are attributed with the capability to enhance the transmission efficiency of nerve impulses, thereby strengthening memory and cognition (Singh et al. 1997). Two new dammarane type jujubogenin bisdesmosides, bacosaponins E and F of biological interest have also been isolated from this herb (Mahato et al. 2000). The present communication reports an effective, efficient, rapid, cost-effective protocol for large-scale *in vitro* multiplication of *brahmi.*

Materials and Methods

Juvenile shoots were obtained from three-month-old mature plants of *Bacopa monnieri* (L.) Pennell growing in the Botanical Garden of the Maharaja Sayajirao University of Baroda. Axillary nodes, young leaves and internodes were used as explants.

The explants were thoroughly washed under running tap water (30 min) and treated with 0.2% (v/v) aqueous surfactant Teepol (BDH, India) for 15 min

followed by repeated rinsing with distilled water. Subsequently, explants were treated (20 min) with 0.1% (w/v) carbendenzim (BASF, India). Further sterilization was done under aseptic conditions inside a laminar Airflow Hood (Lab Services, India).

Explants were surface sterilized with 50% (v/v) ethanol (1 min) followed by a 3 min treatment with 0.01% (w/v) $HgCl_2$. Finally, the explants were washed thoroughly (4 - 5 times) with sterilized distilled water. Throughout the experiments, MS medium with 3% (w/v) sucrose and gelled with 0.8% (w/v) agar (Qualigens, India) was used. The pH of all media was adjusted to 5.8 before autoclaving at 121°C (15 min). The cultures were incubated in a culture room at 25 ± 1°C under 16 h photoperiod (50 $\mu Em^{-2}s^{-1}$) provided by cool white fluorescent tubes (Phillips, India).

For initiation, various explants as described above were inoculated on both agar based semi-solid and liquid MS medium supplemented with different concentrations of BA (0.5 - 4.4 µM) alone and with IAA (0.1 - 0.2 µM). The regenerated shoots were subcultured every three weeks in the same medium. Experiments were also carried out to check the effect of different nodes (2 - 7 nodes) from *in vitro* developed shoots on MS media separately with different concentrations of BA (0.5 - 4.4 µM) alone and in combination with IAA (0.1 - 0.2 µM).

The experiments were performed in replicates of ten for each type of explants and all experiments were repeated three times. The growth responses of the explants were studied at weekly intervals in terms of the initiation and distribution sites of shoots and root regeneration.

Phytochemical evaluation was carried out by HPLC for six-month-old micropropagated plants, market samples and field-grown plants. All the samples were air-dried and crushed to a fine powder form. Optimal extraction was achieved by heating 1 g of fine powdered drug with 20 ml methanol on a hot water-bath under reflux for 5 h. The extract was cooled, transferred to a separating funnel and further extracted with chloroform (30 ml; three times). The combined chloroform layer was collected through sodium sulfate into a beaker. The chloroform was evaporated on a warm water-bath and residue was dissolved in methanol (100 ml).

Separation and determination of Bacoside was performed with HPLC column (250 mm × 4.6 mm) that contained ODS (18) packing (Sigma-Aldrich Hypersil ODs 5mm). The solvent flow-rate was 0.5 ml/min and separated components were monitored by UV (240 nm).

For acclimation, the regenerated plantlets were transferred to small plastic pots containing sand, soil and farmyard manure in the ratio of 1 : 1 : 1. Initially,

high humidity was maintained with water spray at regular intervals (Jasrai et al. 1999) and then transferred to the Botanical Garden of GSFC Science Foundation for further growth.

Results and Discussion

Earlier reports available on *Bacopa monnieri* demonstrated plant regeneration through axillary nodes, internodes and young leaves on media with high concentrations of cytokinin (Tiwari et al. 2001; Shrivastava and Rajni 1999). However, we report here a one-step medium with low concentrations of cytokinin and auxin that were found suitable in all the types of explants for a rapid and large scale multiplication at a cost-effective level.

The nodal segments (Fig. 1A) implanted on MS medium supplemented with only BA (1.1 μM) showed multiple shoot (3 - 4) within two weeks of incubation. Several workers have reported multiple shoot induction with cytokinins in the growth medium (Clog et al. 1990; Stamp et al. 1990). Addition of IAA (0.2 μM) with BA (1.1 μM) enhanced the number of shoots (7 - 8 shoots) from the node (Fig. 1B) and emergence of shoot buds at the base of internodes which later differentiated into shoots in both liquid and solid MS medium. Shoot regeneration potential of IAA has also been reported by Tejavathi and Shailaja (1999) in *Bacopa monneria* with stem and flower buds as explants.

Proliferation of shoot buds and elongation growth of shoots was comparatively higher in the liquid medium than agar-solidified medium. In the liquid medium regeneration response was uniform; a higher biomass with eight shoots from the nodal explants was recorded compared to that in the solid medium with five shoots (Table 1). Further, increase in shoot length was faster in the liquid medium, 5 - 6 cm within 15 - 18 days than that observed in the solid medium (20 - 25 days). This might be due to better uptake of nutrients as large surface areas of explants were in contact with the liquid medium, thereby increasing the growth and multiplication. Furthermore, the liquid medium helps in maintaining $O_2 : CO_2$ balance (Biondi and Thorpe 1981). The slow growth and fewer shoots on agar solidified medium might be partly due to: (a) the specific habitat needs of this medicinal plant species, (b) a lower diffusion rate of molecules passing through the medium to the regenerant , (c) growth inhibition by an undefined agar-borne inhibitor and (d) reduced availability of water to tissues growing on agar solidified medium (Stevenson and Haris 1980; Kohlenbach and Wernicke 1978; Stoltz 1971). Earlier, suitability of the liquid medium for this plant species has been reported (Shrivastava et al. 1999; Tewari et al. 2000).

Performance of the nodal explants with reference to their position on *in vitro* developed shoots was evaluated in optimal liquid medium. It was observed that

upper nodes (2 - 3) were slower in growth, while lower nodes (4 - 7) were found to be more promising and resulted in multiplication of greater number of shoots (8 shoots). Shoot tip explants were also used; however proliferation response was found to be poor with only a single shoot.

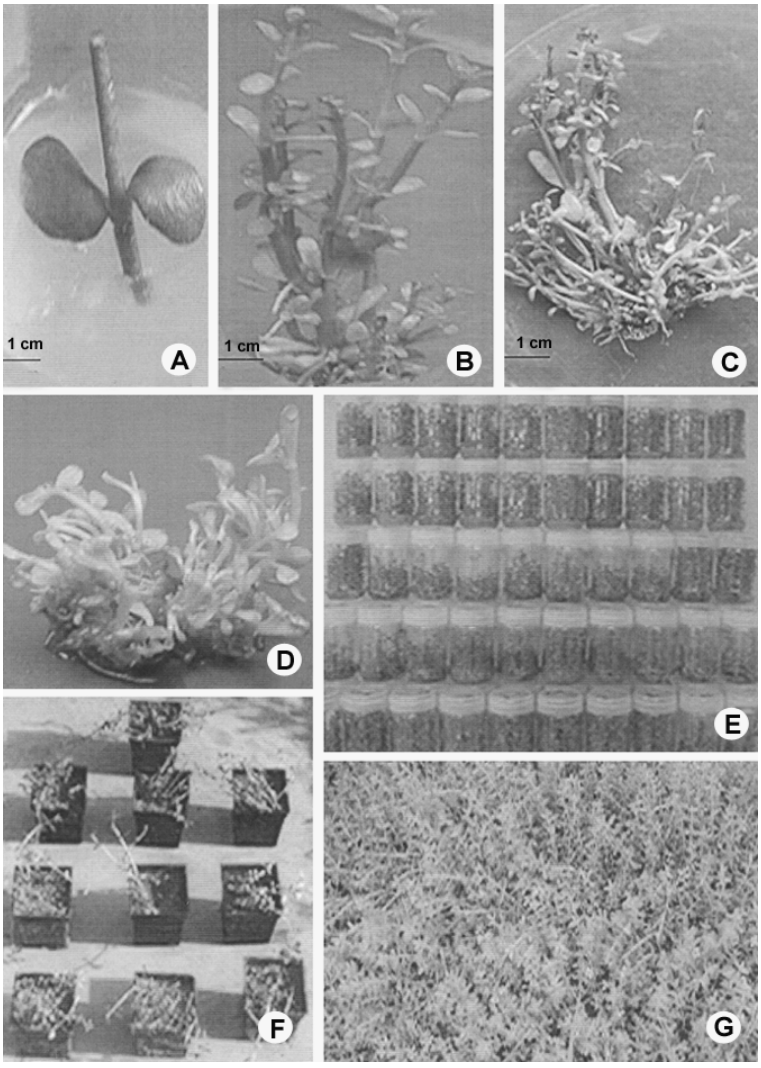

Fig. 1. A. Bud break from nodal explants of *Bacopa monnieri*. B. Multiple shoot formation from nodal explants on MS containing BA (1.1 µM) and IAA (0.2 µM). C. Large number of shoot formation on the internode explants on MS containing BA (1.1µM) and IAA (0.2 µM). D. Multiple shoot regeneration from the leaf surface on optimal medium. E. Shoot regeneration from all the explants during third subculture. F. Hardened regenerated plants maintained in net-house and ready for transplantation. G. A large population of acclimated plants growing in open field (Horizontal bar in the figures indicate increase/decrease in magnification of 1 cm).

The internode explants, when cultured on the optimal solid medium, yielded a large number of shoot (28) regenerants within a period of two and a

half weeks (Fig. 1C). This is in agreement with the results reported by Tiwari et al. (1998) with (23) shoots on a higher concentration of BA (4.4 µM). In contrast, Shrotri and Mukundan (2004) observed fewer shoots (8) from internode explants on a higher concentration of BA (4.44 µM) and IAA (5.71 µM), while Mathur and Kumar (1998) recorded 15 shoots from internode explants after a six-week incubation period but without any growth hormone supplement.

Table 1. Effect of different combinations of BA and IAA in MS medium on shoot formation through nodal explants of *B. monnieri* and % survival rate in the field.

Concentrations (µM)		Number of shoots/explant*	Response (%)	Shoot length (cm)*	Field survival (%)
BA	IAA	Agar based solid medium			
0.0	0.0	3.5 ± 0.26	75	3.1± 0.06	80
0.5	0.2	3.2 ± 2.43	84	2.1 ± 0.29	91
1.1	**0.2**	**6.9 ± 1.15**	**100**	**5.4 ± 0.14**	**100**
2.2	0.2	4.1± 1.20	95	4.1 ± 0.21	98
4.4	0.2	3.1± 0.26	95	3.2± 1.3	98
		Liquid medium (stationary)			
0.0	0.0	1.1± 0.3	76	2.1± 0.6	
0.5	0.2	1.1 ± 0.23	91	3.2 ± 0.32	100
1.1	**0.2**	**7.8 ± 1.13**	**100**	**5.6 ± 1.19**	**100**
2.2	0.2	3.9 ± 0.12	100	3.5 ± 0.12	100
4.4	0.2	3.4 ± 0.43	97	2.1 ± 0.35	100

*Values are mean ± standard error of three replicates with ten cultures per replicate; data scored after three weeks.

Table 2. Effect of different combinations of BA and IAA on direct organogenesis from leaf explants and % survival in the field.

Concentrations (µM)		Number of shoots/explant*	Shoot length (cm)*	Response (%)	Field survival (%)
BA	IAA				
0.0	0.0	1.2 ± 0.45	0.5 ± 1.4	55	64
0.5	0.1	5.4 ± 0.69	2.1 ± 0.22	67	69
0.5	0.2	25 ± 1.32	2.2 ± 0.61	74	81
1.1	**0.2**	**110 ± 2.31**	**3.2 ± 0.25**	**100**	**98**
2.2	0.2	35 ± 0.12	2.9 ± 0.24	**86**	92

*Values are mean ± standard error of three replicates with ten cultures per replicate; data scored after three weeks.

A large number of shoot buds were also observed on *in vitro* leaf explants on liquid and agar based MS media with BA (1.1 µM) and IAA (0.2 µM) without the intervention of callus. Shoot buds that developed on the leaf did not correlate to somatic embryos. Shoot bud proliferation was observed initially from the base which subsequently extended all over the surface. The results are in agreement

with those reported earlier (Mathur and Kumar 1998). A maximum of 110 shoots was observed (Fig. 1D) within three weeks of incubation in the first sub-cycle (Table 2).

Research is said to be more successful if it is cost effective. The number of shoots per subculture and media quantity per subculture was standardized from the commercial point of view. Within a period of three subcultures, the number of shoots at each subculture generated from young leaves, axillary nodes and internodes were: 12100, 49, 784 shoots, respectively (Fig. E). The system demonstrated a continuous supply of shoots up to ten cycles without any decline in their

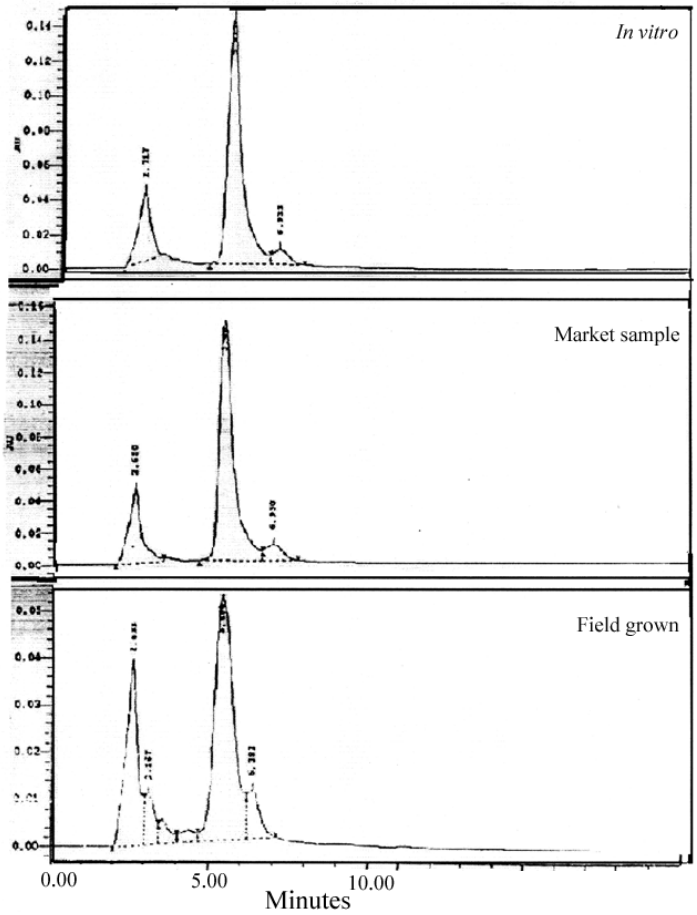

Fig. 2. Separation of bacoside - A present in the methanol extracts of *Bacopa monnieri* by HPLC using mobile phase MeOH-Water (80 : 20) of *in vitro* generated plants, market sample and field-grown plants.

number in subsequent subcultures. Subcultures were performed frequently (3 weeks), as delayed subcultures (more than four weeks) in the liquid medium were found to cause vitrification of shoots similar to that observed in the tissue-

culture-raised carnation plantlets (Ziv et al. 1983). Furthermore, 20 ml of basal media for leaf and 40 ml liquid media for nodal and internode explants with ten explants were found to be optimal.

Well-grown shoots (3 - 4 cm) were isolated and transferred to the basal and the optimal medium for root induction. Root induction was found to be better in the optimal medium as compared to the basal medium. The approximately 5 - 6 cm long shoots with 3 - 4 cm roots were transferred to trays containing sand, soil and farmyard manure in the ratio of 1 : 1 : 1 and kept under shade for hardening. All plants regenerated from different explants were hardened directly in the net-house skipping the greenhouse stage. Initially high humidity was maintained by five sprays of water a day at 5 - 6 h interval. The plantlets so hardened for two weeks in net-house (Fig. 1F) were subsequently transferred to open beds (Fig. 1G) with 100 and 98% survival rate for node/internode and leaf based explants, respectively. No morphological variation of any nature was observed among the *in vitro* raised plants when compared with the mother stock.

For HPLC, different solvent strengths of mobile phase were used; a mixture of methanol and water in the proportion of 80 : 20 was found to be suitable for the separation of bacoside A in *Bacopa monnieri* plant extract. A matching profile of representative chromatograms of *in vitro* generated plants, market sample and field-grown plants of *Bacopa monnieri* was observed (Fig. 2).

We report high level of shoot bud regeneration from various explants with continuous proliferation and elongation of shoot buds and root induction on MS medium supplemented with 1.1 µM BA and 0.2 µM IAA. Thus, a commercially viable protocol has been established for mass micropropagation of Medhya Rasayana - *Bacopa monnieri*. The procedure described here will go a long way to meeting on one hand the ever-increasing demands of the pharmaceutical industries and on the other save this species from extinction.

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