High efficiency direct organogenesis of Bacopa monnieri - A highly valuable medicinal plant

Sumita Dasgupta[1], MN Reddy[2]
[1]*Assistant Professor, Bhagwan Mahavir College of Science and Technology, Surat - 395017, Gujarat India*
[2]*Professor, Department of Biosciences, Veer Narmad South Gujarat University, Surat - 395007, Gujarat, India*

Abstract **- *Bacopa monnieri*, popularly known as "Brahmi", a member of the Scrophulariaceae family, is a small, creeping herb grows in damp, marshy areas. It is a reputed medicinal herb in Indian systems of medicine. It is also under cultivation as medicinal crop. Traditionally, it was used as a brain tonic to enhance memory development, learning, and concentration, and to provide relief to patients with anxiety or epileptic disorders. The demand of this plant in the medicinal world is very high as compared to its supply. In the present work efficient protocol was developed for large scale mass propagation of this valuable medicinal plant. *In vitro* regeneration by direct organogenesis was achieved using nodes, internodes and young leaves. Maximum response of 99.82 $\pm$ 0.7 with a very high number of shoot bud (120 $\pm$ 0.3) was induced from leaf explants on Murashige and Skoog (MS) medium supplemented with 0.5 mg L^{-1} BA (6-benzyladenine) and 0.1 mg L^{-1} IAA (Indole-3-Acetic Acid). 100%of the regenerated shoots produced root on MS medium containing 0.1 mg L^{-1} IAA with maximum number of 14.5 $\pm$ 0.4 observed after 2 weeks of subculture. The rooted shoots were hardened and were successfully established in soil with a survival rate of 95%. HPTLC (High-performance thin-layer chromatography) profile of the methanolic extracts of the *in vitro* raised plants and mother plants revealed almost identical result with no significant differences.**

Index Terms **- Bacopa monnieri, direct organogenesis, leaf explants, phytochemical profile.**

INTRODUCTION

Bacopa monnieri Linn. Pennell or "Brahmi" a member of the Scrophulariaceae family has been used in the Ayurvedic system of medicine for centuries. This medicinal herb has drawn the interest of scientists all over the world who have studied the taxa and made interesting discoveries and revelations about this plant. Traditionally, it was used as a plant has also been used in India as a cardiac tonic, digestive aid, and to improve respiratory function in cases of bronchoconstriction [1]. It was used as a brain tonic to enhance memory development, learning, and concentration, and to provide relief to patients with anxiety or epileptic disorders [2]. Recent research has focused primarily on Bacopa's cognitive-enhancing effects, specifically memory, learning, and concentration and results support the traditional Ayurvedic claims. Research on anxiety and depression [3] [4] [5] [6] also support the Ayurvedic uses of Bacopa. Bacopa extracts also appear to have significant antioxidant activity in the brain, [7]. The leaves of Bacopa contain bacosides [8] [9] [10]. The authentication of the traditional claims of brahmi was initiated by Singh and Dhawan [11]. Their work demonstrated that triterpenoid saponins and their bacosides are responsible for Bacopa's ability to enhance nerve impulse transmission. In recent studies, B. monnieri has been placed second in a priority list of the major Indian medicinal plants assessed on the basis of medicinal significance, potential candidate, and commercial value for further research and development [12] [13].

The demand of this plant in the medicinal world is very high as compared to its supply. As a result it is the most adulterated species in Ayurvedic formulations [14]. Over exploitation has led the vulnerable natural plants on the verge of being extinct. Mass propagation of the elite clones as well as other alternative strategies for biotechnological production of its active principles, the bacopa saponins, have attracted the attention of researchers [15].

In vitro clonal propagation holds better scope and future than the conventional method of propagation for production of important plant-based phytopharmaceuticals. Independent of availability of plants, micropropagation offers a lucrative alternative approach to conventional methods in producing controlled amounts of biochemicals [16].

Plants developed from direct organogenesis are highly stable than those obtained from indirect oraganogenesis (callus) [17], with this concept the current study was undertaken to achieve direct organogenesis of B. monnieri using explant viz, nodes, internodes and leaves. The phytochemical profile of the in vitro raised plantlets was compared with the mother plants by HPTLC (High-performance thin-layer chromatography) techniques.

MATERIALS AND METHODS

Collection and explant preparation: Young and healthy branches were collected from one-year-old plant of Bacopa monnieri, grown and maintained by the Bappalal Vaidya Botanical Research Centre, Department of Biosciences of Veer Narmad South Gujarat University. The branches were washed with running water for 30 minutes and then were immersed in a beaker filled with 0.1% Tween-20 solution used as a wetting agent for 15 minutes. The explants were dissected after they were thoroughly washed with sterile distilled water. Healthy, green nodes, young leaves, and internodes were used as explants. The explants were first disinfected with 70% alcohol for 30-60 seconds, rinsed well with sterile distilled water followed by treatment with fungicide (1.0% carbendazim) and 0.03% streptomycin aqueous solution for 15 minutes afterwards they were washed with sterile distilled water. The explants were then transferred to laminar flow hood and surface sterilized with 0.1 %(w/v) Mercuric chloride (HgCl2) for 3 minutes, rinsed with sterile distilled water for 3-4 times to remove excess HgCl2.

Ten replicates were used per treatment in three repeated experiments. Data was recorded after 4 weeks.

Shoot induction and proliferation: The nodes (0.6-0.8 cm), internodes (1.0cm), leaf discs (0.8 cm2) were trimmed with a sterile scalpel and were transferred to a Murashige and Skoog (MS) media supplemented with 0.1-2.0mg L-1 BA (6-benzyladenine) or Kn (Kinetin) alone or in combination with 0.1-1.0mg L-1 IAA(Indole-3-Acetic Acid) /IBA (Indole-3-butyric acid) /NAA (1-Naphthalene Acetic Acid), 3% sucrose and 0.7% agar. The pH of the media was adjusted at 5.7.

Culture condition: All cultures were maintained at 16/8h light/dark photoperiod at a temperature of 25 ± 2°C.

Root induction: The regenerated healthy shoots were separated, washed well in sterile distilled water and were transferred to the rooting medium consisting of MS medium supplemented with 0.1-1.0 mgL-1 IAA / IBA/NAA.

Ten replicates were used per treatment in three repeated experiments.

Acclimatization: The rooted shoots were thoroughly washed to remove the adhering gel and were transferred to thermocol cups containing soil, sand and cow dung manure (1:1:1), kept under diffused light at 25 + 2 o C for 15 days. They were covered with polythene bags to check excessive transpiration and were nourished with MS salts on every alternate day. After 15 days the plantlets were transferred to green house in earthen pots containing garden soil and cow dung manure. They were covered with polythene bags and were watered regularly. 7 days later the bags were removed. After another 7days the plantlets were removed to garden soil for field establishment.

Phytochemicals

Extraction: The in vitro raised plantlets of Bacopa monnieri were dried at 40 o C and powdered. 1.0 gm of powder of dried plant materials was refluxed with 25 ml of methanol for 2 hrs, filtered. The filtrate was extracted further with another 25 ml of methanol for 1 hour and filtered. The combined filtrate was evaporated to dryness and the residue was dissolved in 10 ml of methanol [18].This was co-chromatographed with the methanolic extract of the mother plant similarly extracted.

Sample application -5.0 µl, 10.0 µl and 15.0 µl of both the extracts were co-chromatographed. The extracts were applied as bands on precoated silica gel 60 F 254 aluminium plate (MERCK) with the help of Linomat

5 applicator attached to CAMAG HPTLC system, which was programmed WIN CATS software.

Development of chromatogram-After the application of sample, the chromatogram was developed in Twin trough glass chamber 10 x 10 cm saturated with solvent system consisting of Toluene: Ethyl acetate: Methanol: Glacial acetic acid (3: 4:3:1) as the mobile phase, for 15 minutes.

Detection of spots- The air-dried plates were viewed in ultraviolet radiation and also under visual light after spraying with 20% methanolic H2 SO4 . The chromatograms were scanned at 254 nm at a speed of 20nm/s using CAMAG TLC scanner 3. The retention factor (Rf) values and fingerprint data were recorded by WIN CATS software.

RESULTS AND DISCUSSIONS

Nodes have been reported to be the better choice of explants for in vitro shoot multiplication in B. monnieri [15]. But in the present work very high number of 120 + 0.3 shoots were found to be produced from leaf explants on MS media supplemented with 0.5 mg L-1 BA and 0.1 mg L-1 IAA (Table1). Shoot buds were found to be emerging directly from the surface of the leaf explants after about 4days of inoculation. By the end of 2nd week the explant was completely covered with deep green tiny shoot buds (Figure 1). The number of shoots reached its maximum after 4weeks of inoculation (Figure 2). The rate of response for direct shoot induction was found to be 99.82 + 0.7 at this hormonal composition. Previous work [19] also reported superior morphogenetic potential of leaf explants with optimum adventitious shoot buds induction from leaf explants on media supplemented with 6.8 µM TDZ (Thidiazuron) with an average of 93 shoot buds produced after 7 weeks of incubation.

In the current study it was observed that MS media supplemented with combinations of cytokinins (BA or Kn) and NAA failed to initiate shoot bud from the leaf explants. Although the leaf segments were found to be the most responsive and produced maximum number of shoots but nodal explants were also found to be possessing promising regeneration capacity. The nodes when cultured on MS medium supplemented with lower concentration of cytokinin (0.1 mg L-1) showed shoot differentiation on the 4th or 5th day of inoculation but the rate of shoot proliferation was found to be very low particularly when supplemented with Kinetin. At a still higher concentration (0.5-1.0 mg L-1) shoot induction was noticed on the 6th or 7th day with but the shoots were thin and fragile with small leaves. Maximum number of shoots (49.66 + 2.3) was obtained at 0.5 mg L-1 BA and 0.1 mg L-1 IAA with 93.3% regeneration rate (Table 1; Figure 3). The regeneration capacity of the internodal explant was found to be the lowest as compared to the other explants (Table 1; Figure 4). In our work it was found that the optimum range of cytokinin for maximum shoot differentiation to be 0.5 mg L-1 -1.0 mg L-1. Further increase in the concentrations of cytokinins resulted in the decrease of the extent of shoot regeneration. This observation is similar to the findings of Shrivastava and Rajani [20].

In the present investigation it was observed that medium supplemented with only BA, produced week shoots. Same observation was also reported in micropropagation of Aristolochia indica [21]. Incorporation of IAA (0.01-0.5 mg L-1) along with BA was found to be stimulatory in improving the quality of the regenerating plantlets. The potentiality of IAA to enhance the rate of multiplication, was also observed in Plumbago zeylanica [22] and Costus speciosus [23], where it was observed that incorporation of IAA (0.01-0.1 mg L-1) into the culture medium improves the frequency of production of multiple shoots. BA was found to be more effective than Kn in evoking better morphogenic response from the explants. This observation is in accordance with the observation of Tiwari et al. [24]. The same observation was also reported by Binita et al. [14]. Roots were produced within 1 week of subculture to the rooting media. No problem was faced with rooting and hardening of this plant. Same observation is reported in the work of Escandon et al. [25]. Optimum root induction was obtained on MS media supplemented with 0.1 mg L-1 IAA in the present observation. The hardened plantlets were successfully established in the soil and growing well with a survival rate of 95%.

HPTLC chromatogram of methanolic extract of the tissue cultured raised plantlets and extracts of the mother plant were found be almost identical (Figure 7 and 8). The chromatogram scanned at 254 nm (Figure 9 and 10) showed 10 peaks in the extract of the tissue cultured raised plantlets whereas that of extracts of the mother plant were found to show 07 peaks. The

number of peaks indicates the presence of different phytoconstituents present in the sample. The Rf values (Figure 9 and 10) calculated for the phytoconstituents present in the tested sample would be helpful in the identification of the unknown compounds by comparing them with the reference standards, and from the values of peak area, the concentration of the compounds can be determined [26]. Three extra peaks obtained from the extract in-vitro raised plantlets might correspond to some new compound or some intermediates.

CONCLUSION

The protocol established in the present investigation is economical and will found to be helpful in mass multiplication of superior plantlets of Bacopa monnieri for medicinal purposes and also for ex vtiro conservation of the elite species. Further analysis of the three extra peaks detected in the TLC scan of cultured plants is required.

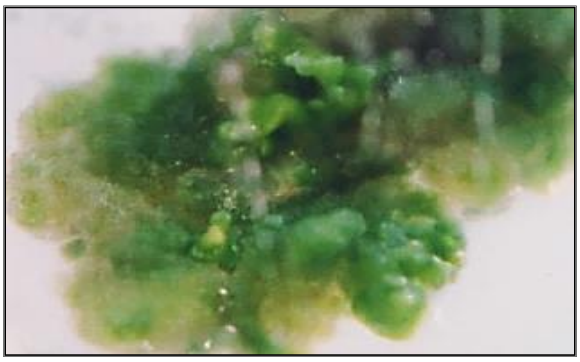


Fig-1

Fig-2

Fig-3

Fig-4

Fig-5

Fig-6

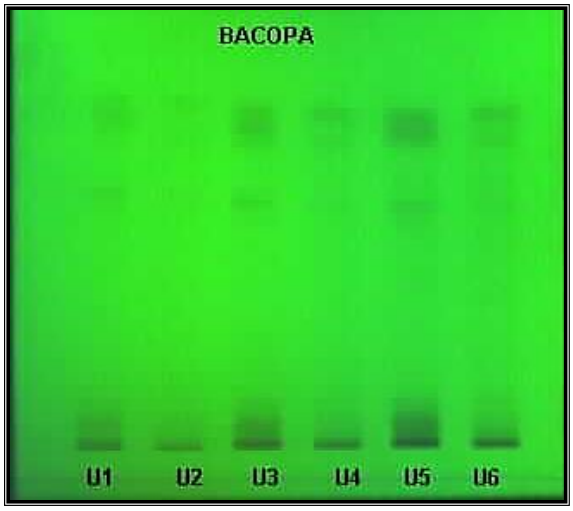


Fig-7

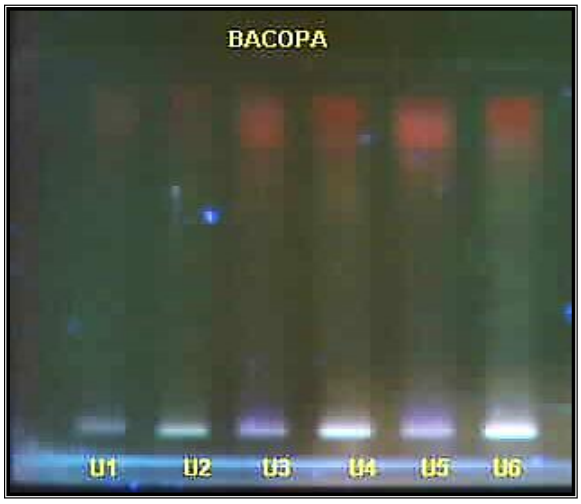
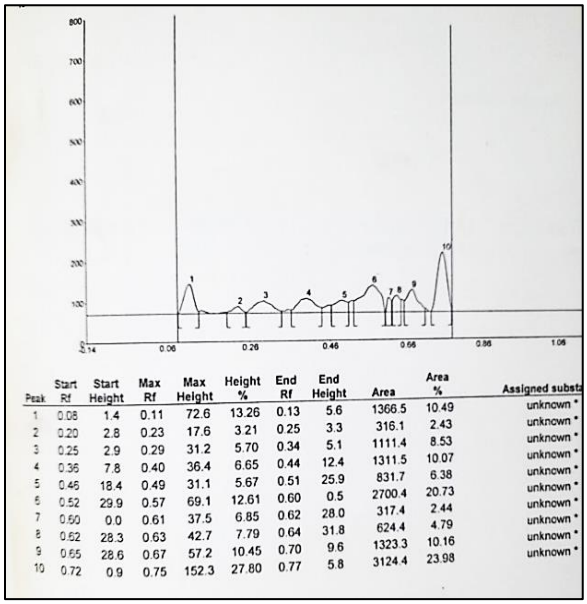
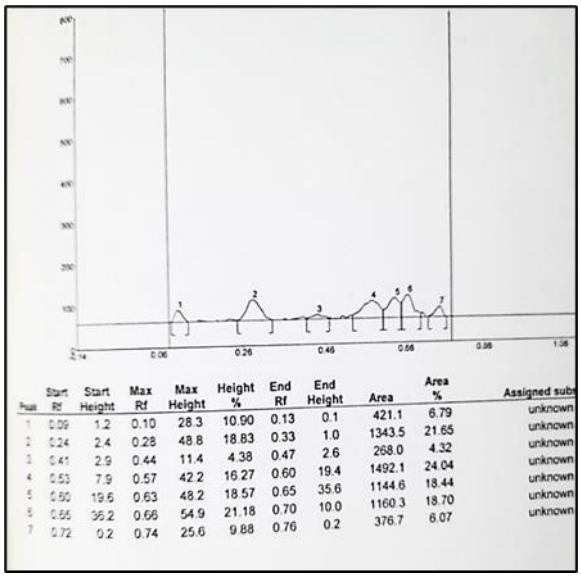


Fig-8



Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %	Assigned substs
1	0.08	1.4	0.11	72.6	13.26	0.13	5.6	1366.5	10.49	unknown *
2	0.20	2.8	0.23	17.6	3.21	0.25	3.3	316.1	2.43	unknown *
3	0.25	2.9	0.29	31.2	5.70	0.34	5.1	1111.4	8.53	unknown *
4	0.36	7.8	0.40	36.4	6.65	0.44	12.4	1311.5	10.07	unknown *
5	0.46	18.4	0.49	31.1	5.67	0.51	25.9	831.7	6.38	unknown *
6	0.52	29.9	0.57	69.1	12.61	0.60	0.5	2700.4	20.73	unknown *
7	0.60	0.0	0.61	37.5	6.85	0.62	28.0	317.4	2.44	unknown *
8	0.62	28.3	0.63	42.7	7.79	0.64	31.8	624.4	4.79	unknown *
9	0.65	28.6	0.67	57.2	10.45	0.70	9.6	1323.3	10.16	unknown *
10	0.72	0.9	0.75	152.3	27.80	0.77	5.8	3124.4	23.98	unknown *

Fig-9



Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %	Assigned subs
1	0.09	1.2	0.10	28.3	10.90	0.13	0.1	421.1	6.79	unknown
2	0.24	2.4	0.28	48.8	18.83	0.33	1.0	1343.5	21.65	unknown
3	0.41	2.9	0.44	11.4	4.38	0.47	2.6	268.0	4.32	unknown
4	0.53	7.9	0.57	42.2	16.27	0.60	19.4	1492.1	24.04	unknown
5	0.60	19.6	0.63	48.2	18.57	0.65	35.6	1144.6	18.44	unknown
6	0.65	36.2	0.66	54.9	21.18	0.70	10.0	1160.3	18.70	unknown
7	0.72	0.2	0.74	25.6	9.88	0.76	0.2	376.7	6.07	unknown

Fig-10

Figure 1-Shoot bud induction from the leaf explant at 0.5 mg L-1 BA and 0.1 mg L-1 IAA.

Figure 2- Multiple shoot induction from the leaf explant at 0.5 mg L-1 BA and 0.1 mg L-1 IAA.

Figure 3- Shoot bud induction from the node at 0.5 mg L-1 BA and 0.1 mg L-1 IAA.

Figure 4- Shoot bud induction from the internode at 0.5 mg L-1 BA and 0.1 mg L-1 IAA.

Figure 5- Root induction at 0.1 mg L-1 IAA.

Figure 6- Hardened plantlets.

Figure 7- Chromatograms at wavelength 254 nm; U1,U2,U3: 5µl,10µl and 15 µl of methanolic extracts of mother plant of B.monnieri ,U4,U5,U6 5µl,10µl and 15 µl Methanolic extracts of in-vitro raised plantlets of B.monnieri.

Figure-8- - Chromatograms at wavelength 366 nm; U1,U2,U3: 5µl,10µl and 15 µl of methanolic extracts of mother plant of B.monnieri ,U4,U5,U6 5µl,10µl and 15 µl Methanolic extracts of in-vitro raised plantlets of B.monnieri.

Figure 9- HPTLC profile of extract of in-vitro raised plantlets scanned at 254 nm.

Figure 10- HPTLC Chromatogram of extract of mother plant scanned at 254 nm.

Table 1-Effect of different combinations BA/ Kinetin and IAA on direct shoot induction from node, leaf and internodal explants of Bacopa monnieri (after 4 weeks of inoculation).

Growth regulators (mg L^{-1})	No. of shoots/explant + S.E			% Regeneration ± S.E.		
	Nodes	Leaf	Internodes	Nodes	Leaf	Internodes
0.1 BA	10.0 ± 0.5	14.48 ±0.8	5.52 ± 0.9	47.21 ± 5.5	41.66 ± 4.8	30.51 ± 5.5
0.5 BA	18.5 ± 0.3	26.67 +2.7	9.24± 2.6	58.39 ± 4.8	55.55 ±10.0	36.10 ± 2.7
1.0 BA	22.0 ±1.9	52.50± 2.7	12.21± 5.5	63.88 ± 7.3	69.44 ± 7.3	47.26 ± 7.2
1.5 BA	12.48 ± 1.2	21.22 ±2.4	7.90 ±0.7	55.52 ± 2.7	44.44 ± 10.0	33.30 ± 8.3
2.0 BA	9.50 ± 0.5	16.14± 0.9	5.46 ± 0.8	49.96 ± 4.6	36.10 + 5.5	30.52 ± 5.5
0.1 BA + 0.1 IAA	6.80 ± 0.4	8.83 ± 0.6	2.42 ± 0.4	36.10 ± 7.8	44.44 ± 2.7	27.78 ± 2.7
0.5 BA + 0.1 IAA	49.60 ± 2.3	120.0 ± 0.3	20.0 + 1.0	93.37 ± 0.8	99.82 ± 0.7	76.69± 8.8
1.0 BA + 0.1 IAA	43.0 ± 2.5	54.67 ± 5.5	18.64 ±4.8	73.33 ± 6.6	80.0 ± 5.7	70.0 ± 5.7
1.5 BA + 0.1 IAA	10.31 ± 0.9	19.34 ± 0.4	5.66 ± 1.2	47.27 ± 2.2	49.91 + 4.7	36.15 ± 7.3
2.0 BA + 0.1 IAA	3.32 ± 0.8	4.0 ± 0.5	2.50 ± 0.2	44.40 + 2.7	38.88 + 2.7	33.33 ± 8.3
1.0 BA + 0.5 IAA	6.67 ± 0.8	50.6 ± 2.7	12.0 ± 2.1	72.22 ± 2.7	69.44 ± 5.5	47.22 ± 2.7
1.5 BA + 0.5 IAA	3.67 ± 1.2	12.25 ± 1.5	4.84 ± 0.9	58.33 ± 8.3	61.11± 7.3	41.66 ± 4.8
2.0 BA + 0.5 IAA	3.0 ± 0.5	7.5 ± 0.5	3.66 ± 0.6	41.66 ± 4.8	44.44 ±2.7	36.11 ± 7.3
0.1 Kn	5.21 ± 0.3	3.2 ± 0.8	2.93 ± 0.2	49.99 ± 4.8	55.55 ± 5.5	41.66 ± 2.7
0.5 Kn	7.65 ± 0.5	11.25 ± 2.6	5.42 ± 0.8	55.55 ± 7.3	61.10 ± 5.5	52.77 ± 5.3
1.0 Kn	10.0 ± 0.5	17.5 ± 2.5	9.84 ± 0.8	61.11± 4.8	63.88 ± 2.7	58.33 ± 8.3
1.5 Kn	4.40 ± 0.2	5.65 ± 0.2	3.50 ± 0.5	52.77 ± 5.3	58.33 ± 4.8	24.99 ± 4.8
2.0 Kn	3.72 ± 0.3	3.33 ± 0.3	—	44.44 ±2.7	47.22 ± 2.2	19.44 ± 2.7
0.5 Kn + 0.1 IAA	33.6 ± 2.4	35.5 ± 0.8	10.80 ±1.1	66.66 ±4.8	72.22 ± 2.7	63.88 ± 5.5
1.0 Kn + 0.1 IAA	27.0 ± 2.7	27.33 ± 4.3	7.62 ± 0.8	61.11± 7.3	63.88 ± 2.7	55.55 ± 7.3
1.5 Kn + 0.1 IAA	11.20 ± 0.5	3.40 ± 0.5	—	47.22 ± 2.7	58.33 ± 4.8	41.66 ± 4.8
2.0 Kn +0.1 IAA	5.81 ± 1.4	5.6 ± 0.3	—	36.11 ± 7.3	55.55 ± 2.7	30.55 ± 2.7
1.0 Kn + 0.5 IAA	8.7 ± 1.2	14.33 ± 1.4	4.0 ± 1.1	61.10 ± 5.5	69.44 ± 5.5	50.0 ± 5.5
1.5 Kn + 0.5 IAA	4.6 ± 0.4	6.67 ± 0.8	—	38.88 ± 2.7	41.66 ± 4.8	24.99 ± 4.8
2.0 Kn +0.5 IAA	3.10 ± 0.3	3.60 ± 0.7	—	36.10 ± 5.5	33.33 ± 4.8	22.22 ± 2.7

Values represent means + S.E. (standard error) of 10 replicates per treatment in three repeated experiments.

Table 2- Effect of growth regulators on root induction of Bacopa monnieri (after 2weeks of subculture)

Growth regulators (mg L^{-1})	%Regeneration ±S.E	No. of roots ± S.E.	Length (cm)±S.E.
0.05 IAA	23.3 ± 56	1.67 ± 0.8	1.55 ± 0.1
0.1 IAA	100	14.5 ± 0.4	2.1 ± 0.2
0.5 IAA	86.62 ± 6.6	9.85 ± 0.3	2.65 ± 0.3
1.0 IAA	38.73 ± 3.3	3.21 ± 0.4	1.42 ± 0.3
0.05 IBA	6.67 ± 6.6	2.5 ± 0.5	1.87 ± 0.1
0.1 IBA	60.0 ± 0.9	7.0 + 0.2	2.2 + 0.2
0.5 IBA	74.08+ 5.7	11.2 ± 0.3	2.0 ± 0.1
1.0 IAA	13.67 ± 8.8	1.67 ± 0.8	0.75 ± 0.1

Values represent means + S.E. (standard error) of 10 replicates per treatment in three repeated experiments.

REFERENCES

[1] K.M.Nadkarni,.The Indian Materia Medica, Columbia, MO: South Asia Books.pp:624-625, 1988

[2] R.N.Chopra, 1958. Indigenous Drugs of India,2nd ed.Calcutta, India: U.N. Dhur and Sons. pp 341, 1958.

[3] Bhattacharya, S.K. and Ghosal, S..Anxiolytic activity of a standardized extract of Bacopa monniera in an experimental study,Phytomedicine, VOL.5, PP.77-82, 1998

[4] D.K.Ganguly, and C.L. Malhotra, Some behavioural effects of an active fraction from Herpestis monniera, Linn. (Brahmi), Ind J Med Res.,vol.,55.pp.473-482, 1967.

[5] S. Channa, A. Dar, M.Yaqoob,S. Anjum, Z. Sultani, Atta-ur-Rahman. 2003. Bronchovasodilatory activity of fractions and pure constituents isolated from Bacopa monnieri, J Ethnopharmacol.,vol. 86 pp.27-35, May 2003.

[6] K. Sairam, C.V. Rao, M.D. Babu, and R.K. Goel, Prophylactic and curative effects of Bacopa monniera in gastric ulcer models, Phytomedicine,vol. 8 pp. 423- 430. 2001.

[7] S.K.Bhattacharya, A. Bhattacharya, A.Kumar, A. Ghoshal, Antioxidant activity of Bacopa monniera in rat frontal cortex, striatum and hippocampus, Phytotherap. Res., vol.14,pp. 174-179,2000.

[8] N.Chatterji,R.P. Rastogi, M.L.Dhar,Chemical examination of Bacopa monniera Wetst. Part II: The constitution of bacoside A, Ind. J. Chem., vol.3,pp. 24-29,1965.

[9] U.P. Basu, and T.Dutta, 1967. Tetrahedron letter, vol. 31,pp. 2937,1967.

[10] P.Jain, and D.K. Kulshreshtha, Bacoside A1, A minor saponin from Bacopa monnieri, Phytochemistry,vol. 33,pp.449-451,1993.

[11] H.K. Singh, and B.N.Dhawan, Neuropsychopharmacological effects of the Ayurvedic nootropic Bacopa monniera Linn. (Brahmi), Indian J Pharmacol., vol. 29, pp. S359-S365,1997.

[12] H.P. Mohapatra, S.P. Rath, In vitro studies of Bacopa monnieri—An important medicinal plant with reference to its biochemical variations, Indian J. Exp. Biol., vol.43, pp.373–376,2005.

[13] N. Sharma, R. Satsangi, R. Pandey, S.Devi, S. Vimala, in vitro clonal propagation and medium-term conservation of Brahmi (Bacopa monnieri), J. Plant Biochem. Biotechnol.,vol.16, pp.139–142, 2007.

[14] B.C. Binita, M.A. Dave,and Y.T. Jasrai, 2005 Bacopa monnieri (L.) Pennell: A Rapid, Efficient and Cost Effective Micropropagation, Plant Tissue Cult. & Biotech., vol.15,pp.167-175,2005.

[15] P.S. Saha, S. Sarkar, R. Jeyasri, P. Muthuramalingam, M. Ramesh, S. Jha, In Vitro Propagation, Phytochemical and Neuropharmacological Profiles of Bacopa monnieri (L.) Wettst.: A Review. Plants (Basel)., vol.,9, no.4,411 doi: 10.3390/plants9040411, March 2020.

[16] S. Bhatia and K. Sharma, K. (2015). Micropropagation, Modern Applications of Plant Biotechnology in Pharmaceutical Sciences, Academic press 361–368. doi:10.1016/b978-0-12-802221-4.00011-x,2015.

[17] A. Kamenickà and M. Rypák, the regeneration of Actinidia chinesis Pl. cultured in vitro, Polnohospodarvo, vol. 35,pp. 811-818, 1989.

[18] Anonymous, Indian herbal pharmacopoeia. Indian drug manufacturer's association Worli, Mumbai, and Regional research laboratory Jammu-Tawi, India. 1: pp 33,1998.

[19] V. Tiwari, K.N. Tiwari and B.D. Singh,Comperative studies of cytokinins on in vitro propagation of Bacopa monnieri, Plant Cell Tissue and Organ Culture, vol. 66, pp. 9-16, 2001.

[20] N. Shrivastava and M.Rajani,Multiple shoot regeneration and tissue culture studies on Bacopa monnieri (L.) Pennell, Plant Cell Reports, vol.,18,pp. 919-923,1999.

[21] S. Manjula, A. Thomas, B. Daniel, and G.M. Nair, In vitro plant regenaration of Aristolochia indica through axillary shoot multiplication and Organogenesis, Plant Cell Tiss. and Org. Cult., vol.,51,pp.145-148,1997.

[22] Rout, G.R., Saxena, C., Samantaray, S. and Das, P. 1999. Rapid plant regeneration from callus cultures of Plumbago zeylanica. Plant Cell, Tissue and Organ Culture, 56: 47–51.

[23] H.C. Chaturvedi, P. Misra, P. and M.Jain,Proliferation of shoot-tips and clonal multiplication of Costus speciosus in long-term culture, Plant Sci Lett., vol. 35, pp.67–71,1984.

[24] V. Tiwari, B.D. Singh and K.N. Tiwari, Shoot regeneration and somatic embryogenesis from different explants of Brahmi. (Bacopa monniera), Plant Cell Reports., vol.17, pp.538-43,1998.

[25] A.S. Escandon, J.C. Hagiwara, and L.M. Alderete, A new variety of Bacopa monnieri obtained by in vitro polyploidization, Electronic Journal of Biotechnology, vol. 9 10.2225/vol9-issue3-fulltext-8,2006

[26] A.Bhargava, P.Shrivastava and A. Tilwari, HPTLC analysis of Fumaria parviflora (Lam.) methanolic extract of whole plant, Futur J Pharm Sci., vol.7,no. 1 https://doi.org/10.1186/s43094-020-00150-x,2021.