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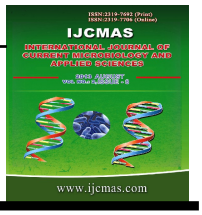

Original Research Article

In vitro propagation of *Bacopa monnieri* (L.) Wettst – A medicinally priced herb

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Keywords

Bacopa monneiri;
in vitro clonal propagation;
medicinal value;
field transfer.

A B S T R A C T

Tissue culture studies were performed on *Bacopa monnieri* (Brahmi). Nodal explants containing preformed axillary bud were used as the starting material. Surface sterilized explants were aseptically cultured on MS medium supplemented with different plant growth regulators. Best results of axillary bud induction were observed on medium containing 1.0 mg/l BAP wherein 100% bud break was achieved. Optimal results of further shoot multiplication were also obtained on same media combination and shoots were periodically subcultured alternately on media containing 0.5 mg/l BAP and basal MS medium without any PGR. Half strength MS media containing different concentrations of auxins were used for *in vitro* rooting of shoots. Medium supplemented with 1.0 mg/l IBA proved to be most efficient for development of healthy root system. Rooted plantlets were subsequently hardened, acclimatized and successfully established in field with 100% survival rate.

Introduction

Bacopa monnieri (L.) Wettst. (family Scrophulariaceae) is a creeping, glabrous, succulent herb, rooting at nodes whose habitat includes wetlands and muddy shores. Commonly known as Brahmi, the plant is 10-30 cm long with 1-2 mm thick, soft, glabrous stem having ascending branches. The leaves are 0.6-2.5 cm long and 3-8 mm broad. Flowers of brahmi are white or pale-bluish in colour, axillary, solitary, arranged on long slender pedicels. The fruits are ovoid, acute, 2-celled, 2-valved capsules and tipped with style base.

Brahmi has been extensively used in the traditional system of medicine for centuries including Ayurveda. It was earlier used as a brain tonic to enhance memory development, learning, and concentration and to provide relief to patients with anxiety or epileptic disorders. In India, brahmi has been used for treating dermatosis, anemia, diabetes and infertility since ancient times. With recent biotechnological advances, several therapeutic properties of brahmi have been clinically tested and established.

The plant is hence valued as a nerve and cardio-tonic, broncho-vasodilatory, digestive-aid, having anti-inflammatory, neuroprotective and hepatoprotective properties. Besides, the antioxidant properties of brahmi have also been studied which account for its anticancer activity (Sudharani *et al*., 011 ; Sundriyal *et al*., 2013).

The active chemical constituents accounting for the pharmacological effects of *B.monnieri* include alkaloids, saponins and sterols. Brahmin, an alkaloid was first reported as isolated compound of the plant. Subsequently, several other alkaloids like nicotine and herpestine have also been reported. *B.monnieri* contains major constituents such as des-saponin glycosides-triterpenoid and Saponins (Bacosides A & B). It also includes other minor constituents like, bacosides A1 & A3, hersaponin, betulic Acid, monnierin, alkaloids, herpestin and brahmine, flavonoids, luteolin-7-glucoside, glucoronyl-7-apigenin and gluucoronyl-7-luteonin, (Sundriyal *et al*., 2013).

Tissue culture technology has been known as an effective tool to propagate several valuable medicinal plants. The technique has been suitably applied in case of brahmi where natural regeneration through seeds or cuttings is severely impeded in areas with proper irrigation facilities. With the advent of herbal renaissance world over there is an increasing demand for this valuable medicinal herb in pharmaceutical markets. The present study was taken up with the aim to develop a clonal propagation protocol for brahmi with a tested healthy plant as the starting material, resulting in large scale production of disease free and superior quality plants.

Materials and Methods

Source of explants

Twigs of Brahmi were collected from healthy plants growing in medicinal garden of Non Wood Forest Products (NWFP) division, Forest Research Institute, Dehradun.

Surface sterilization

The nodal parts were carefully removed from the twigs and washed under running tap water for to remove superficial dust particle adhering to the surface. Nodal explants were then washed with liquid detergent (cetrimide: 1-2 drops/ 100 ml water) for 10 minutes followed by thorough rinsing with distilled water 4-5 times. This was followed by surfactant (Tween-20: 2-3 drops/100 ml water) treatment for 20 min which was again followed by thorough rinsing with distilled water. Shoot segments of approximately 2-3 cm with at least one node in each segment were excised. For surface sterilization, 1% fungicide (Bavistin) treatment was given for 15 minutes. followed by 0.1% $HgCl_2$ treatment for 1- 3 minutes under Laminar Air Flow. The explants so washed were carefully washed 4 -5 times with autoclaved distilled water.

In- vitro establishment of cultures

Surface disinfected nodal explants were inoculated onto full strength MS medium (Murashige and Skoog, 1962) having 3% sucrose and supplemented with different concentrations and combinations of PGRs viz. BAP (1.0-2.0 mg/l), Kn (1.0-2.0 mg/l), IAA or NAA (0.5 mg/l).

In- vitro shoot multiplication and maintenance

Shoots induced from nodal were excised and placed on fresh medium of same composition for establishment of initial stock of shoots. Single shoots were separated and further used to perform experiments to standardize best medium composition for optimal shoot multiplication. For the purpose, MS medium supplemented with cytokinin BAP (1.0- 2.0 mg/l) alone and in combination with IAA or NAA (0.5 mg/l) was tried.

In vitro generated healthy shoots were maintained by regular subculturing of propagules of 3 shoots each after every 3 weeks of culture. For subculturing, concentration of BAP in MS medium was reduced to 0.25 and 0.50 mg/l and compared with results on basal MS medium.

Medium and culture conditions

In all experiments, basal MS medium (without any PGR) was used as control. The pH of all media was adjusted to 5.8 (using 1.0N HCl or 0.1N NaOH) before adding 0.7% Agar as gelling or solidifying agent and sterilized by autoclaving at 15 psi (1.8Kg/cm²) pressure at 121ºC for 15 min. Cultures were incubated in culture room at 25±2ºC temperature and photoperiod of 16/8 hrs (light/dark) maintained by light supplied by cool-white fluorescent tubes at an intensity of 2500 lux.

In-vitro Rooting

In-vitro developed healthy shoots were transferred to root induction medium fortified with 2% sucrose and different concentrations of auxins IBA/NAA/IAA (0.5-1.0 mg/l).

Hardening and Acclimatization

In-vitro rooted plantlets were carefully washed under running tap water to remove all traces of agar. Thereafter the plantlets were transferred directly to pots containing a mixture of soil: sand: manure (1:1:1) and kept in Polyhouse for about 4 weeks where they were periodically watered. Well-developed plants with healthy shoots were hence successfully transferred to field conditions.

Experiments were repeated thrice and each experiment consisted of a minimum of ten replicates. Observations on mean number of shoots/ roots and mean shoot length/root length were recorded after 4 weeks of culture. Data collected is represented as mean ±standard error (SE).

Results

Standardization of surface sterilization and induction of axillary shoots

Treatment of nodal explants with 0.1% $HgCl_2$ for 2 minutes resulted in establishment of 100% contamination-free viable cultures. Induction of axillary shoots was observed in all the media combinations tried. Final observation after 4 weeks showed that MS media supplemented with 1.0 mg/l of BAP proved to be most efficient in shoot induction. On this medium an average of 5.00 ±0.31shoots with mean shoot length 4.29 ± 0.06 cm were obtained. On Kn supplemented medium, shoot induction reponse declined and similar observations were also made on medium containing cytokinin in combination with auxins (Table 1, Figure 1A).

Shoot Multiplication

Induced axillary shoots were excised from the explants and shifted to fresh medium containing 1.0 mg/l BAP to establish a stock of shoots used for *in vitro* multiplication (Figure 1B). Results in the present study showed the necessity of plant growth regulators for *in vitro* multiplication, as the shoots cultured on basal medium (i.e. devoid of any PGR) did not multiply and died after some time.

Shoot number and shoot length observed after 4 weeks varied in all the treatment tried. On BAP supplemented medium, results were superior to those recorded on media supplemented with other PGR. BAP at a concentration of 1.0 mg/l gave an average of 20.00 ± 0.24 shoots with mean shoot length 4.80 ± 0.51 cm after 4 weeks of culture (Figure 1C).

On increasing the concentration of BAP to 2.0 mg/l, a decline in shoot multiplication rate was observed. The shoots so developed were also shorter in length. Trials on effect of cytokinin:auxin ration on *in vitro* shoot multiplication of Brahmi showed that results were better than in medium with 2.0 mg/l BAP . However, comparative number, length and health of shoots on media fortified with BAP (1.0 mg/l) + IAA/NAA (0.5 mg/l) was not as good as in media containing 1.0 mg/l BAP (Table 2)

Sub culturing of shoots

For long term maintenance of shoot cultures MS media supplemented with concentrations of BAP were tried for routine subculturing. Propagules of 3shoots each were used for the purpose. MS medium supplemented with 0.5 mg/l BAP proved to be most optimal for routine subculturing. On this media an average of 19.00 ±0.24 shoots with mean shoot length 5.48 ±0.11 cm was obtained (Table 3). Basal MS medium (without any PGR) also gave good results (mean number of 16.00 ±0.58 shoots, mean shoot length 4.88 ±0.19 cm) and the shoots developed were healthy.

Periodic subculturing was hence done after every 3 weeks of culture on MS medium containing 0.5 mg/l BAP and basal MS medium in an alternate manner. The shoots so produced were long and healthy without any basal callusing. By this procedure over 70 subcultures have already been carried out without observing any declination in shoot multiplication rate. The shoots developed were healthy and without any basal callusing.

***In vitro* Rooting**

Response of rooting was different in different concentrations of auxins. Basal MS medium proved to be completely incompetent to induce *in-vitro* rooting while root induction was observed within 15-20 days in all the other combinations tried (Table 4). Maximum rooting was recorded in medium containing 1.0 mg/l IBA (Figure 1D). On this medium an average of 10.00±0.24 roots with average root length 11.32 ±0.31 cm was observed after 4 weeks. On medium supplemented with NAA or IAA, *in-vitro* rooting was hampered and the roots developed were small and less healthy.

Hardening and Acclimatization

In-vitro rooted shoots were maintained in the rooting medium for over 5 weeks. Thereafter they were shifted directly to pots containing soil: sand: manure (1:1:1) and maintained in Polyhouse for next one month (Figure.1E). In polyhouse, tissue culture raised plantlets were monitored for optimal growth and development and

Table.1 Effect of plant growth regulators on *in vitro* axillary shoot induction in *B. monnieri*

S.No	MS + PGR (mg/l)				Observations after 4 weeks	
	BAP	Kn	IAA	NAA	Mean shoot number	Mean shoot length (cm)
Control	0.0	0.0	0.0	0.0	0.00±0.00	0.00±0.00
1	1.0	0.0	0.0	0.0	5.00±0.31	4.29 ± 0.06
2	2.0	0.0	0.0	0.0	4.00±0.27	3.53 ± 0.09
3	0.0	1.0	0.0	0.0	3.00±0.22	3.58 ± 0.14
4	0.0	2.0	0.0	0.0	3.00±0.16	3.36 ± 0.12
5	1.0	0.0	0.5	0.0	4.00±0.37	3.31 ± 0.11
6	1.0	0.0	0.0	0.5	3.00±0.16	3.33 ± 0.08

Table.2 Effect of plant growth regulators on *in vitro* axillary shoot multiplication in *B.monnieri*

S.No	MS + PGR (mg/l)			Observations after 4 weeks	
	BAP	IAA	NAA	Mean shoot number	Mean shoot length (cm)
Control	0.0	0.0	0.0	0.00±0.00	0.00±0.00
1	1.0	0.0	0.0	20.00 ±0.24	4.80 ±0.51
2	2.0	0.0	0.0	15.00 ±0.58	3.46 ±0.24
3	1.0	0.5	0.0	17.00 ±0.37	3.78 ±0.26
4	1.0	0.0	0.5	16.00 ±0.40	4.12 ±0.09

Table.3 Effect of plant growth regulators on shoot sub-culturing in *B.monnieri*

S.N	MS + PGR (mg/l)	Observations after 4 weeks	
	6-Benzyl AminoPurine(BAP)	Mean shoot number	Mean shoot length (cm)
Control	0.00	16.00 ±0.58	4.88 ±0.19
1	0.25	14.00 ±0.73	4.18 ±0.09
2	0.50	19.0 0±0.24	5.48 ±0.11

Table.4 Effect of auxins on *in vitro* rooting in *B. monnieri*

S.No	MS (half strength)+PGR(mg/l)			Observations after 4 weeks	
	IBA	NAA	IAA	Mean root number	Mean root length
Control	0.0	0.0	0.0	0.00±0.00	0.00±0.00
1	0.5	0.0	0.0	7.00 ±0.24	9.22 ±0.26
2	1.0	0.0	0.0	10.00±0.24	11.32 ±0.31
3	0.0	0.5	0.0	7.00±0.54	9.45 ±0.41
4	0.0	1.0	0.0	8.00 ±0.37	9.32 ±0.34
5	0.0	0.0	0.5	7.00±0.37	9.36 ±0.47
6	0.0	0.0	1.0	7.00 ±0.44	10.0 ±0.33

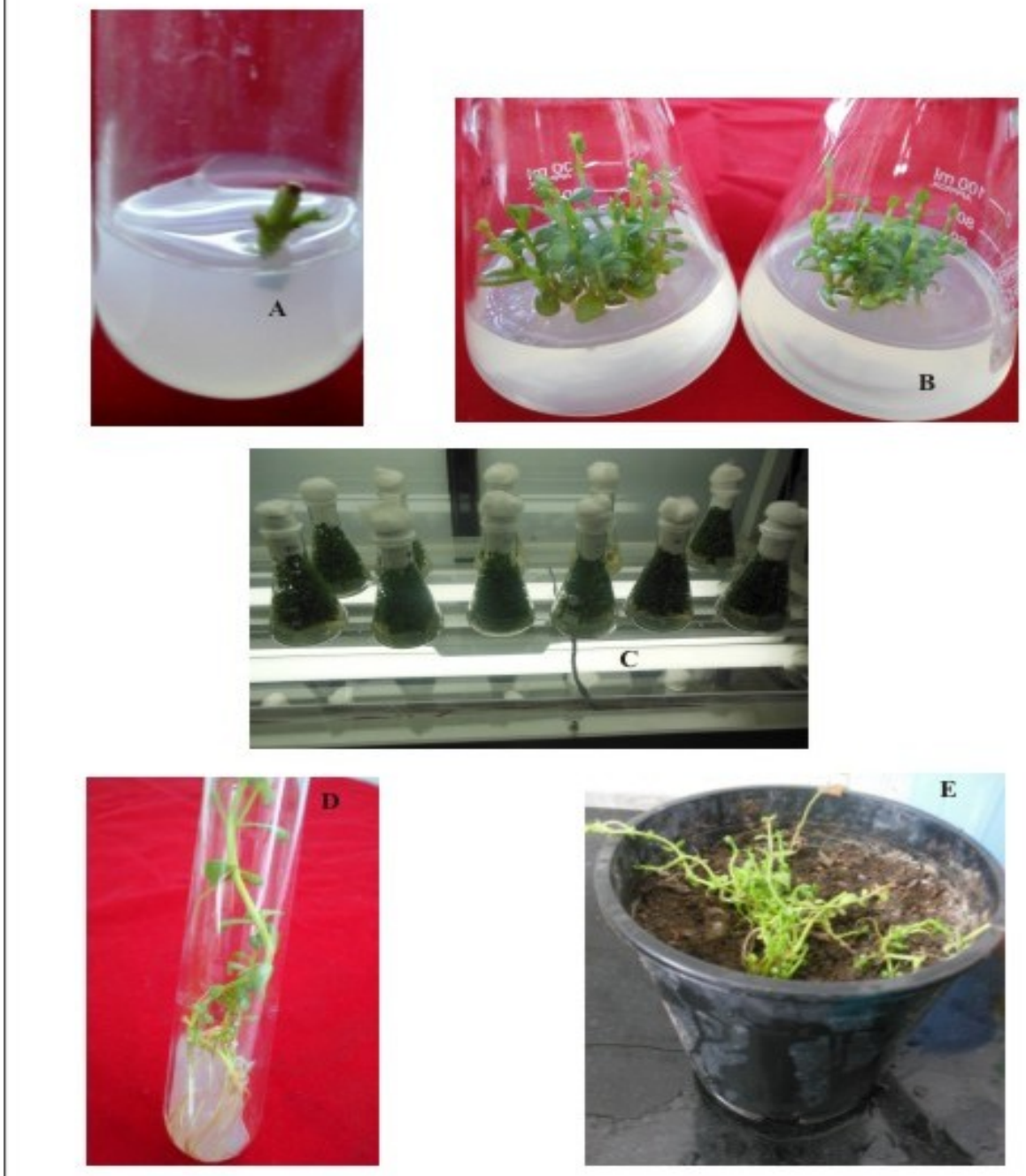

Figure 1 (A-E): *In vitro* propagation of *B. monnierri*
(A) Induction of axillary shoots from nodal explants (B) *In vitro* culture establishment (C) *In vitro* shoot multiplication (D) *In vitro* rooting *(*E) Tissue culture raised plantlets transferred to pots

watering was done as required. The plantlets eventually grew into long and healthy plants and subsequently transferred to the field conditions in open ground where 100% survival rate was observed.

In the present study, 0.1% mercuric chloride solution proved to be efficient in controlling contamination on nodal explants. Effectiveness of mercuric chloride in mitigating infection on brahmi explants has also been reported earlier (Shrivastava *et al*., 1999).

Surface disinfected nodal explants showed best results of axillary shoot induction on MS medium supplemented with 1.0 mg/l BAP. Several reports have verified superiority of BAP in enhancing bud induction at concentrations ranging from 1.0-5.0 mg/l (Shrivastava *et al.*, 1999; Chandra *et al.*, 2012). This is also in contradiction with earlier report by Sharma *et al*., 2010 wherein, a concentration of 0.1 mg/l has been shown to be the best for *in vitro* shoot induction. In our study, 1.0mg/l BAP also proved to be most efficient giving 10 fold shoot multiplication.. This observation is supported by previous studies on *B. monnierri* (Ceasar *et al.*, 2009; Vijyakumar *et al.*, 2010 ; Chaplot *et al.*, 2005; Tanveer *et al.*, 2010).

The observations showed that an increase in BAP concentration resulted in decline in shoot multiplication rate. This could be attributed to the fact that higher levels of cytokinin in the medium diminishes the multiplication potential.

In the present study periodic sub culturing of multiplied shoots was best observed on medium supplemented with 0.5mg/l BAP. However, the procedure of subculturing standardized was alternate subcultures on medium containing 0.5mg/l BAP and basal MS medium after every 3 weeks. This was done to minimize the negative effects of higher cytokinin concentration on shoot multiplication. An alternate cycle ensured that the shoots multiplied optimally and the cultures could be effectively maintained for long duration without any loss in regeneration potential.

Once the healthy shoots were generated, various combinations of auxins were tested for rooting. The role of Auxin is well established for enhancing rooting (Tiwari *et al*., 2000). In the present study IBA at 1.0 mg/l concentration proved to be most efficient for rooting, resulting in long and healthy roots. IBA has been found to enhance rooting in *Bacopa* (Ceasar *et al*., 2009) while it is contradictory to earlier reports in which IAA with other PGR has been used for enhancing roots (Vijyakumar *et al*., 2010; Gurnani *et al*., 2012 and Chaplot *et al*., 2005).

Tissue culture raised plants are delicate and need acclimatization before field transfer. For this purpose *in-vitro* regenerated plantlets were shifted to pots and kept in Polyhouse for about a month. The protocol resulted in development of healthy plants without any need of intermediary hardening treatment.

Conclusion

Bacopa monnieri has always been a topic of interest to a myriad of researchers. From tissue culture point of view several studies have been performed to propagate the plant *in vitro.* The objective of the present study was to develop a micropropagation protocol resulting not only in production of large number of healthy plantlets but is also suitable for

long term maintenance of short cultures of Brahmi. Use of low concentration of plant growth regulators and minimization of time required for field transfer of tissue culture raised plantlets are the highlights of the study. Besides, the multitude of disease - free plants produced open the scope for utilization of plant material for antimicrobial testing and suitable pharmaceutical preparations.

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