ISSN 1996-0700

Asian Journal of Biotechnology

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Asian Journal of Biotechnology 3 (2): 135-152, 2011
ISSN 1996-0700 / DOI: 10.3923/ajbkr.2011.135.152

Micropropagation of *Bacopa monnieri* through Protoplast

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ABSTRACT

Bacopa monnieri is a vegetatively propagated medicinal plant, which is endangered so it is necessary to develop micropropagation protocols to preserve the germplasm and distribute it to new cultivars during cultivation in new areas. *Bacopa* is cultivated in marshy area such as rice fields; the plant is of commercial importance. *In vitro* culture of plants has gained importance during recent years because, besides other application, this technique can be used for the rapid multiplication of medicinal plants. The aim of the present work is to determine the culture conditions for micropropagation of this plant. In order to induce the callus formation from *Bacopa monnieri* and study the *in vitro* growth conditions of calli. Study the specific growth factor for root and shoot induction, transplantation and acclimatization of explants and isolation of the DNA from explants. Induction of callus from protoplast of *Bacopa monnieri* and regeneration of callus. Standardization of micropropagation protocol of *Bacopa monnieri*. In the case of *Bacopa monnieri*, the micropropagation technique from explants and protoplasts were established. We also raised plantlets from callus and also subjected the DNA for analysis and done RAPD to find the difference between the normal and *in vitro* plants, the result indicated that the plants are identical. Standardization of surface sterilization protocol, aseptic culture initiation, establishment and multiplication, suitable media for rooting and a suitable protocol for hardening in order to achieve quality transplant. Optimization of medium for callus induction, maintenance and regeneration.

Key words: Micropropagation, protoplasts, *Bacopa monnieri*, callus, regeneration

INTRODUCTION

In the folklore of Indian medicine, certain herbs have been used traditionally as brain or nerve tonics. One of the most popular of these used in neurotonics is *Bacopa monnieri,* a small, common, amphibious plant growing in marshy areas throughout the Indian subcontinent (Tiwari *et al.*, 2006). *Bacopa* is also called Brahmi, a name derived from Brahma, the creator god of the Hindu pantheon of deities. It is legendary for its diversity of usage. In the Ayurvedic *Materia Medica, Bacopa* has been recognized for its brain enhancement characteristics. *Bacopa monnieri,* a member of the Scrophulariaceae family, is a small, creeping herb with numerous branches, small oblong leaves and light purple flowers. In India and the tropics it grows naturally in wet soil, shallow water and marches. The herb can be found at elevations from sea level to altitudes of 4,400 feet and is easily cultivated if adequate water available. Flowers and fruits appear in summer and the entire plant is used medicinally. *Bacopa monnieri* has originated in India (Mohapatra and Rath, 2005).

According to scientists at the Central Drug Research Institute in Lucknow, India, certain memory chemicals in *Bacopa,* called *bacosides A* and *B,* help repair damaged neurons by enhancing proteins involved in the regeneration of neural-cell synapses (Rastogi *et al.*, 1994). These are the relay stations of the brain that facilitate the transmission of neural impulses. Thus *Bacopa* can be viewed as a neural nourisher, restoring depleted synaptic activity and leading to enhanced memory function. In scientific studies, it has been shown to exert a remarkable and unique effect on neurotransmitters (Shakoor *et al.*, 1994). *Bacopa* may even be able to revitalize intelligence. The pharmacological effects of *Bacopa monniera* are attributed to the presence of a number of biologically active compounds, including alkaloids, saponins (Ali *et al.*, 1999; Chatterji *et al.*, 1963, 1965) and sterols. The compounds responsible for the memory enhancing effects of *Bacopa monniera* are triterpenoid saponins called bacosides (Singh and Singh, 1980; Jain and Kulshreshtha, 1993).

Protoplasts are cells which have had their cell wall removed, usually by mechanical method (Tiwari *et al.*, 2000). Once the cell wall has been removed the resulting protoplast is spherical in shape. Digestion is usually carried out after incubation in an osmoticum (a solution of higher concentration than the cell contents which causes the cells to plasmolyse). This makes the cell walls easier to digest. Debris is filtered and/or centrifuged out of the suspension and the protoplasts are then centrifuged to form a pellet. On resuspension the protoplasts can be cultured on media which induce cell division and differentiation. A large number of plants can be regenerated from a single experiment-a gram of potato leaf tissue can produce more than a million protoplasts, for example. Protoplasts can be isolated from a range of plant tissues: leaves, stems, roots, flowers, anthers and even pollen. The isolation and culture media used vary with the species and with the tissue from which the protoplasts were isolated. Protoplasts are used in a number of ways for research and for plant improvement. They can be treated in a variety of ways (electroporation, incubation with bacteria, heat shock, high pH treatment) to induce them to take up DNA. The protoplasts can then be cultured and plants regenerated. In this way genetically engineered plants can be produced more easily than is possible using intact cells/plants (Zhou and Wu, 2006).

MATERIALS AND METHODS

Bacopa monnieri plants were collected from Aringar Anna Medicinal Farm Anna Nagar,Chennai, India in 2006. The explants were washed in running tap water for 30 min. In the next step, the explants were soaked in aqueous solution containing 5% Cetrimide for 10 min. This was followed by gentle wash in sterile double distilled water for 5 min. Then the explants were immersed in aqueous solutions of 10% Sodium hypochlorite for 10 min and were shaked regularly. After this treatment, the explants were sterilized with 0.1% Mercuric chloride aqueous solution for 5 min. The segments are then washed in 70% ethanol for 2 min. Then the explants were removed from the sterilizing solution and rinsed thoroughly for two times with sterile double distilled water.

Initiation of cultures: Sterilized explants were transferred aseptically to sterilized glass plate in the laminar flow hood. Then a cut was given on both basal as well as the top portion of the explants to remove undesirable/dead portions after surface sterilization. The forceps were earlier rinsed in the 70% ethanol and were flamed and cooled. Then the lid from one test tube was removed and test tube mouth was flamed to avoid any chance of contamination. Each nodal explant was then placed in an erect position in the test tube containing medium with the help of long forceps. The lid was finally closed carefully, flamed lightly and sealed with Klin film. The forceps were then again rinsed with 70% alcohol to avoid any chance of cross contamination. The same procedure was undertaken

for all the explants. These jars were finally kept in the growth room with temperature conditions 25±2°C, with a photoperiod of 16 h daylight and 8 h night break under the cool white fluorescent light of average 2500 lux (cool white fluorescent tube light 40 W GE).

Establishment of cultures: After approximately 9-10 days of inoculation, the axillary bud break was observed in some explants. When the explants attain bud proliferation, these cultures were then transferred to jars containing fresh medium. After 21-25 days incubation with a clean and sterilized forceps in the laminar flow hood, the initiated plants were taken out of the test tube, medium adhered to the plants were removed, undesirable/brownish leaves were removed from the plants and were taken to the culture bottles containing autoclaved semi-solid media having the same combinations as that of the culture initiation.

Then the bottles were placed in the culture room under the standard conditions of temperature (25±2°C) for 16/8 h of day/night break under the cool white fluorescent light of average 2500 lux (cool white fluorescent tube light 40 W GE).

Multiplication of shoots by repeated sub-culturing in multiplication media: The preparation and sterilization steps for the medium, instruments and chamber were repeated as before. Multiple shoots/cluster were transferred from the culture bottle to a sterile glass plate using flamed sterilized forceps, the brown leaves were removed from the primary shoots and sectioned into one node piece after removing the leaves. These nodal segments were transferred to the multiplication media. All this study was done with extreme care and inside the laminar flow hood to avoid any possible chance of contamination. These culture bottles were then incubated in the growth room. These steps were repeated every 25-30 days for the next sub-culturing.

Rooting of the shoots: Axillary shoots developed in cultures in the presence of cytokinin generally lack roots. In order to obtain complete plants the shoots must be transferred to a rooting medium, which is different from the shoot multiplication medium, especially in its hormonal composition. A low salt medium is found satisfactory for rooting of shoots in large number of plant species.

Rooting protocol: In the laminar flow, under sterile conditions the Klin film and cap was removed from the culture bottles in sterile condition (laminar flow hood) and with the help of sterile forceps, the multiplied shoots were removed from the medium and placed on the sterile glass plate.

With the help of sterile scalpel, elongated shoots up to 1-2 cm in length were cut and placed into the rooting medium. The culture bottles were then capped and placed in the growth room under the same condition. The time required for *in vitro* rooting of shoots may vary from 10-15 days.

Transplantation and acclimatization of the plantlets: The transfer of plants from the culture vessel to the soil require a careful, stepwise procedure. The roots of the plants were gently washed to remove the agar sticking to them. The most essential requirement for the successful transplantation is to maintain the plants under a very high humidity (90-100%). For the first 10-15 days by keeping them under mist or covering them with clear plastic. Some small holes may be poked in the plastic for air circulation. Inside the culture vessel the humidity was high and thus, the natural protective covering of cuticle was not fully developed. During this time plant attain ability to synthesize more food and develop cuticular covering. Plants are maintained under shade and are then ready to be used in open nursery.

Protocol for transfer of plantlets to soil: After 10-14 days of culture on rooting media, the rooted plantlets were transplanted to pots or trays for hardening prior to their final transfer to soil. Rooted plantlets were taken out of the culture bottles with the help of forceps and washed thoroughly with water to remove any remaining of the medium. 5% cetrimide treatment was given to the plants in order to protect them from the fungal attack in the near future. After this the plants were carefully planted in the plastic cups containing different soil mixtures in different ratios.

Protocol for callus induction: Three types of explants: leaf segments; internodal segments and nodal segments were used as a source of explant. Explants used for callus induction were taken from established cultures of *Bacopa*. The medium employed was MS Basal with different concentration and combinations of phytohormones such as NAA, Kinetin and 2,4-D. After inoculation of the culture, the bottles were properly capped and sealed. After labeling, these were transferred to the incubation room where they are incubated at 25±2°C in the rack covered with black paper.

Procedure for DNA isolation: *Bacopa* material (leaves) of 5 g was weighed and freezed quickly in liquid nitrogen and grinded to a fine powder using mortar and pestle. Seventy five millilter of Extraction buffer was added in a small volume and grinded thoroughly. The homogenate was transferred to 250 mL conical flask and to the homogenate, 5 mL of 20% SDS was added and mixed thoroughly using magnetic stirrer for 15-20 min. Then the contents were incubated at 65°C for 10 min. Fifty milliliter potassium acetate solution was added, mixed and incubated at room temperature for 30 min, in order to precipitate proteins and polysaccharides. The contents were centrifuged at 25,000 rpm for 15 min. The pellet was discarded and supernatant was collected. To the supernatant 1/6th volume of ice cold iso propanol was added and incubated at room temperature for 30 min. Then the DNA pellet were collected by centrifuging at 20,000 rpm for 15 min. The pellet were suspended in 3 mL of suspension buffer, 1.8 mL ice cold isopropanol and 180 μL of 3 M Sodium acetate and incubated at room temperature for 1 h. The DNA was repelleted by centrifugation. Then the pellet was washed with 80% ice cold ethanol and air dried. Finally pellet was suspended in TE buffer and stored at low temperature for further use. The DNA was subjected to Agarose Gel Electrophoresis.

RESULTS AND DISCUSSION

The surface sterilization procedure was optimized and this helped in preventing blackening of tissues and establishment of clean cultures. The sterilization procedure initially followed does not include any step with antibacterial treatment and large number of explants were found contaminated. Contamination was controlled after the addition of antibiotic, 5% Cetrimide and there was no adverse effect on bud sprouting and shoot multiplication.

Shoot initiation and establishment from *Bacopa monnieri* cultured on MS basal and MS Medium supplemented with various combinations of growth regulators i.e., BAP in combination with NAA and Kn is described in Table 1. Most of the research studies for other medicinal plant species have shown the use of cytokinin alone or in combination with other in different concentrations. For example for *Paederia foetida* and *Centella asiatica* multiple shoots were obtained in MS medium supplemented with BAP 1.0 mg L^{-1} (Singh *et al*., 1999) and in *Rauwolfia serpentina* on MS medium supplemented with benzyladenine and NAA (Sehrawat *et al*., 2001), whereas for *Bacopa* optimum shoot proliferation was achieved in different combination of hormones

Table 1: Observation for auxillary bud induction

Medium	Contamination	Bud breakage	Avg. shoot length (cm)	Cluster formation
BM	0.33±0.09	0.96±0.00	6.6±0.47	No cluster
BM2	0.25±0.01	0.54±0.00	2.6±0.044	0.68±.05
BM3	0.23±0.06	0.45±0.12	3.8±0.471	0.98±0.02
BM4	0.16±0.03	0.89±0.12	5.9±0.49	1.00±0.12
BM5	0.26±0.02	0.68±0.24	4.6±0.01	0.54+0.01

BM (0.1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA); BM2 (1 mg L^{-1} BAP + 0.2 mg L^{-1} NAA); BM3 (4 mg L^{-1} BAP, 0.4 mg L^{-1} NAA); BM4 (0.4 mg L^{-1} BAP + 0.5 mg L^{-1} kinetin); BM5 (1.0 mg L^{-1} BAP + 1.0 mg L^{-1} Kn)

in different concentrations. We have included coconut water and banana extract as growth regulators which showed good results. During initial week after inoculation, bud initiation was very low. However, bud initiation was found to be started in most of the cultures initiated from 9-10 days by showing a small newly sprouted bud, which proliferate into shoot buds with leaves during 21-25 days which were placed in the culture room under the standard conditions of temperature (25±2°C). All the experiments were performed thrice with 3 replicates per treatment.

Shrivastava and Rajani (1999), also reported that shoot bud initiation was observed visually on the ninth day of incubation in all replicates in the media having different concentrations of BAP, Kn, coconut water and banana extract. After 3-4 weeks thick mat of shoot buds spread over 90-100% of the surface of explant in the presence of 2 mM BAP, 4 mM Kn, coconut water and banana extract. Tiwari *et al.* (2001) reported use of range of cytokinins. Of the four cytokinins (6-benzyladenine, thidiazuron, kinetin and 2-isopentenyladenine) he reported that thidiazuron (6.8 μM) and 6-benzyladenine (8.9 μM) proved superior to other treatments. Optimum adventitious shoots buds induction occurred at 6.8 μM thidiazuron (TDZ). However, in our case, we have not used TDZ/2-ip, to ensure that the protocol standardized be cost effective.

It was observed from Table 1 that although bud break occurred in all the medium under study, following mediums were compared in terms of bud breakage percentage, average shoot length and percentage cluster formation. BM4 (0. 4 mg L^{-1} BAP + 0. 5 mg L^{-1} kinetin) was found to be the best initiation medium in terms of bud breakage and lower contamination percentage, however cluster formation was observed to be very low in this media in the initiation stage but was increased after establishment stage and had became highest during later stages. BM (0.1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA) was observed to be having highest shoot length with higher percentage of cluster formation which was not observed in case of BM4. In the other three mediums: BM2 (1 mg L^{-1} BAP + 0.2 mg L^{-1} NAA), BM5 (1.0 mg L^{-1} BAP + 1.0 mg L^{-1} Kn) and BM3 (4 mg L^{-1} BAP, 0.4 mg L^{-1} NAA), average shoot length and cluster formation was less as compared to above defined mediums.

Multiple shoot proliferation: After 25-30 days of first subculture, established cultures were transferred to culture jars having respective media combinations. Multiplication of shoot cultures were carried out by culturing nodal segments/clusters excised from *in vitro*-raised plants (Tejavathi and Shailaja, 1999), also found that maximum numbers of plants were obtained on medium containing Kin/2 i.p. (0.1 mg L^{-1}) and Kin (1 mg L^{-1}) in shoot tip and nodal cultures of *Bacopa*, respectively.

Shrivastava and Rajani (1999), reported that out of two cytokinins used BAP was found to be suitable than Kn as BAP resulted in quicker and better response then the latter while addition of NAA (0.2 mg L^{-1}) proved synergistic (Srivastava *et al.*, 2002). Tiwari *et al.* (2001) also reported that

addition of BAP resulted in the increase in number of shoots, mean shoot length and number of roots/explant. Srivastava *et al.* (2002) observed direct regeneration of shoots and roots occurred in nodal explants in *Bacopa* on MS medium containing NAA (0.1 mg L^{-1}) and BAP (0. 5 mg L^{-1}). Addition of higher levels (2.0 and 3.0 mg L^{-1}) of BAP in the media, helps in differentiation of shoots in 63 and 93% cultures from the nodal portion and shoots attained height of 5 cm in the ninth week in the above medium. Addition of 0.2 mg L^{-1} NAA to the above medium caused shoot regeneration in 95% of the cultures and shoots attained height of 5 cm in the 8th week. There is also report for use of high concentration of BAP (9-10 mM) for shoot regeneration (Yang and Read, 1997; Bhuyan *et al.*, 1997). Both these reports clearly supported our observations as well.

Observations were taken for evaluating the growth of explants by taking parameters such as average shoot length and number of nodes (15 shoots randomly selected per medium). The experiment was carried out in seven media having different concentrations of growth regulators each with 3 replicates, only results of best medium are given. The medium showing best results were BM4 and BM5 with highest average shoot length of 5.8 and 4.9, respectively with 100% cluster formation. Sub culturing was carried out after 25-30 days using the same medium combinations as for initiation and establishment stages. Shoot clusters obtained in each subculture was divided in approximately 1 cm^2 size with approximately 4-5 small shoots in each cluster and inoculated in each bottle.

Final observation was taken after 3rd subculture stage after initiation with 3 replication per treatment and summarized in the Table 2, cluster formation initiated from the basal node; progressively increases in size from each subculture and number of shoots initiated from the nodal portion vary from medium to medium. Shrivastava and Rajani (1999) also observed that each explant was transformed into a dense mass of profusely regenerating shoot buds (as cluster formation in our case).

It was observed that in case of BM4 highest number of shoots (>3 cm) originated from the basal node and average number of shoots formed in case of BM4 is 5. 80; 4. 9 in BM1; 3. 04 in BM7 and lowest no of shoot regenerated in case of BM3 is 2.70. In term of number of nodes/explant, same order is followed i.e., BM5 having 7.3, BM4 having 6.95, 4.89 in BM7 and in case of BM3 is 3.26. The texture of leaf was succulent and fleshy. It was also observed that number of clusters obtained by dividing each mother culture (cluster) is ranging from 1. 0 (BM3) to 4.1-4.4 (BM4 and BM5) and hence can be ascribed as suggested multiplication rate for each medium (Fig. 1, 2a, b).

It was observed that during each passage, the number of leaves/shoots has increased substantially along with the height of shoots. The leaf size was approximately 0. 4-0. 6 cm. It was observed that morphogenic responses exhibited in the form of shoots or roots are correlative to a specific auxin/cytokinin ratio and banana extract. In comparison to BM3 media, BM4 has shown a significant increase in no of shoots and average shoot length.

Table 2: Observation of multiple shoot proliferation

Medium	No. of shoots >3 cm	No. of nodes	Average shoot length
BM3	3.240±0.13	3.26±0.00	2.70±0.54
BM4	10.89±0.12	6.95±0.12	5.80±0.43
BM5	8.45±0.07	7.30±0.00	4.90±0.54
BM7	5.94±0.28	4.89±0.18	3.04±0.12

BM3 = (MS+4.0 mg L^{-1} BAP+0.4 mg L^{-1} NAA); BM4 = (MS+0.5 mg L^{-1} BAP+0.5 mg L^{-1} Kn); BM5 = (MS+1.0 mg L^{-1} BAP+0.5 mg L^{-1} Kn); BM7 = (MS+100 mL L^{-1} banana extract)

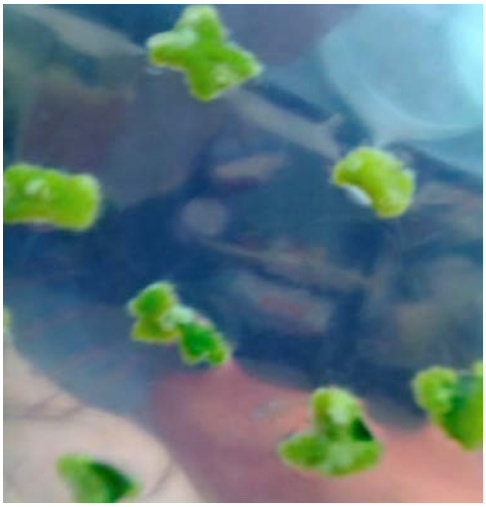

Fig. 1: *Bacopa monnieri* callus in leaf explants

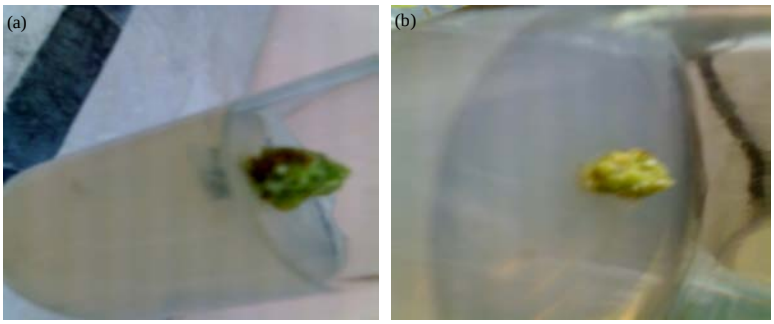

Fig. 2: (a, b) Callus induction and proliferation in BCM6 medium

Rooting: After two cycles of multiplication subculture, elongated shoots of 2-3 cm in length were excised and cultured on MS basal medium having different combinations of MS media with plant growth regulators (BAP,NAA,IAA, Kinetin, Banana extract and coconut water).

The experiments were conducted twice, with 3 replicates (with 3 shoots per bottle). Rooted shoots were taken after 2 weeks, shoot length, root length and no of roots per explant (total 9 explants per treatment each time), fresh weight and dry weight (keeping them in an oven with 50°C for 24 h) were measured.

Initiation of rooting took place after 10-15 days of inoculation. Single and multiple roots were formed from the base and the nodal portions and the length of the roots were 2-3 cm within 25-30 days.

It was observed from Table 3 that BM6 (MS + 100 mL L^{-1} coconut water) recorded highest S/R (2.97) and 2.63 for BM7 (MS+100 mL L^{-1} banana extract) with considerable higher no of roots (9.30 and 8.56, respectively). Similar response was also observed in case of BM4 and BM5 in terms of S/R of 1.56 and 1.33 and root number of 4.04 and 3.35, respectively. Present results indicate 100% root formation in 4 media (BM4, BM5, BM6, BM7). Tiwari *et al.* (2000) observed highest rate of rooting (90%) for *Bacopa* on full-strength MS medium containing 2.46 mM IBA.

Table 3: Observation for rooting

Medium	Root length (cm)	Shoot length (cm)	Shoot/ root	No. of roots	Fresh weight (g)	Dry weight (g)
BM4	2.68±0.01	6.00±0.24	1.56±0.04	4.04±0.04	1.07±0.07	2.38±0.07
BM5	1.80±0.19	6.70±0.09	1.33±0.70	3.35±0.04	1.21±0.08	2.70±0.06
BM6	3.85±0.07	8.45±0.24	2.97±0.04	9.30±0.14	1.06±0.07	1.90±0.02
BM7	2.69±0.24	4.69±0.09	2.63±0.21	8.56±0.04	1.05±0.02	2.09±0.02

BRM4 = MS+Sucrose(10 g L^{-1})+Agar (8 g L^{-1}); BRM5 = MS+Sucrose (20 g L^{-1})+Agar (7 g L^{-1}); BM6 = MS+Sucrose (20 g L^{-1})+Agar (8 g L^{-1}); BM7 = MS+Sucrose (30 g L^{-1})+Agar (7 g L^{-1})

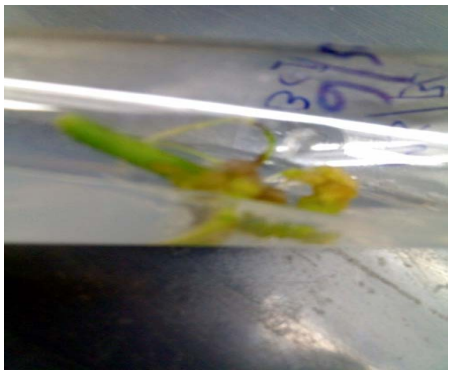

Fig. 3: *Bacopa monnieri* callus formation in shoot meristem

Fig. 4: *Bacopa monnieri* in shake flask

Ex-vitro rooting was also carried out using single shoots of 1-2 cm in height derived from MS medium and then transplanted in soil: vermicompost mixture in the ratio of 4:1 and soil: vermicompost mixture in the ratio of 2:1 which gave 100% survival rate.

Callus induction: Callus induction requires the presence of auxins or cytokinins or both or 2, 4, D in the nutrient media depending on the source of explant. Callus initiation was carried out by using leaf segment as source of explant (Fig. 3). Initiation was carried out using different growth regulators such as BAP, IAA and 2, 4-D containing media.

The explants enlarged within 12-14 days of inoculation; however callus formation started after 20-25 days at the ends of the explant. Appearance of callus was globular and was of pale green in colour. In the medium BCM6 (MS +100 mL L^{-1} coconut water), rapid callus growth was observed as pale yellow and of globular appearance (Fig. 4).

Table 4: Observation for the callus induction

Medium	Contamination	Regeneration	Appearance
BCM1	No Contamination	Regeneration	Globular, pale green
BCM2	No Contamination	No regeneration	Jelly type brown
BCM6	No Contamination	Regeneration	Globular, pale green
BCM7	Contamination	No regeneration	Jelly type green
BM1	Contamination	Regeneration	Jelly type pale green

BCM1 = MS + 0.5 mg L^{-1} BAP + 1 mg L^{-1} 2,4-D; BCM2 = MS + 0.5 mg L^{-1} 2,4-D; BM1 = MS+ 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA; BCM6 = MS+100 mL L^{-1} coconutwater; BCM7 = MS + 100 mL L^{-1} banana extract

Fig. 5: Embryo development from callus of *Bacopa monnieri*

Shrivastava and Rajani (1999) also reported that the addition of 1 mM BAP along with 2,4-D, induced a thin layer of granular callus after 4 weeks of culture. However, all the explants turned brown to black at the base after 6 weeks of culture. In case of BCM1 (MS +1 mg L^{-1} BAP + 1 mg L^{-1} IAA) initial small globular callus was formed, from where small shoot buds have been regenerated after 20-25 days. From an area of one cm^2 of callus, 3-4 shoots of height 1-1.5 cm were formed.

Shrivastava and Rajani (1999) also reported that IAA induced callus at the cut ends of both stem and leaf explants along with 1-5 shoots per explant, while in combination with BAP, it induced only shoots (1-10 per explant) within 2 weeks after incubation. These shoots attained height of average 4-5 cm after placing in light for 8-9 days when transferred into multiplication medium i.e., BM5 (MS +1 mg L^{-1} BAP + 1 mg L^{-1} kinetin). The response shown in Table 4 by regenerated shoots in multiplication medium was similar to the response shown by explants inoculated in multiplication medium in light. Loose jelly type callus formation was observed in BCM2 (MS + 0.5 mg L^{-1} 2,4-D), BCM7 (MS + 100 mL L^{-1} banana extract), BM6 (MS + 100 mL L^{-1} coconutwater) and BM1 (MS+ 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA) which however turned brown after few days.

Response of callus induction varies with the type of explant (Fig. 5-9). When the same was transferred to BM4 and BM5 then callus again started showing regeneration threads after 10-20 days in dark. Tiwari *et al.* (1998) has reported callus formation from nodal explants of *Bacopa*

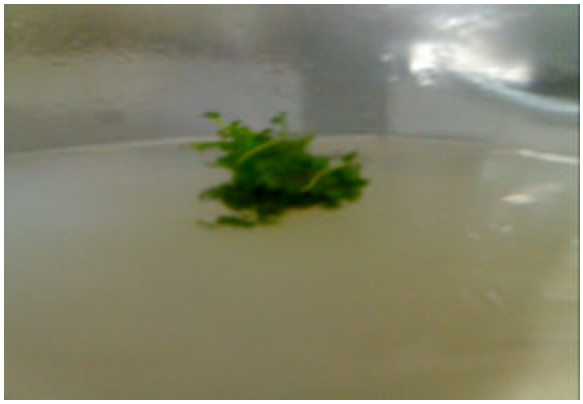

Fig. 6: *Bacopa monnieri* shoots from callus

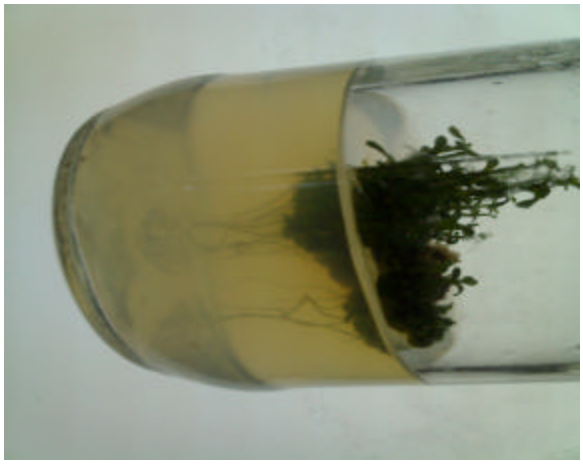

Fig. 7: *Bacopa monnieri* shoots and roots from callus

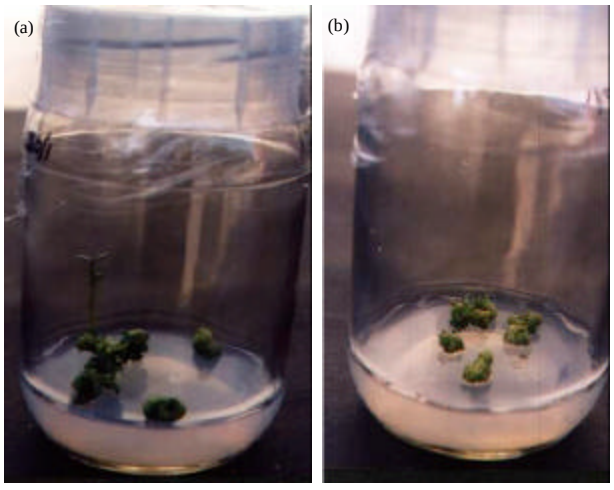

Fig. 8: Regenerated shoot buds in BM3 and MS medium

Fig. 9: Multiple shoot proliferation in BM3 medium

Fig. 10: Transplantation (25 days stage) open nursery l→r (HM1,HM6,HM8)

cultured on MS medium containing 0. 5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). Rani *et al.* (2003) reported that callus induction was observed from hypocotyl, root and cotyledonary leaf segments, grown on Murashige and Skoog (MS) medium supplemented with various concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (Kn),coconut water, Banana Extract.

Transplantation: Explants used for carrying out hardening experiment using different soil mixture were grown in MS basal medium as reported before. After sufficient rooting, *Bacopa* plantlets were transferred to the protrays and polybags having different soil mixtures in different concentrations for hardening (Fig. 10). Agar was removed from the rooted plantlets and then plantlets were washed with tap water. Potting mixtures of desired combinations were prepared and mixed properly and filled in the protrays and polybags. The polybags and protrays were sprinkled with water. The roots of each plant were dipped in 5% cetrimide solution to avoid future fungal

Table 5: Observation for the different soil mixtures

Treatment combination	Shoot length (cm)	Root length (cm)	Shoot/ root	No. of roots	Fresh weight (g)	Dry weight (g)
HM1	6.50±0.92	4.90±0.64	1.35±1.03	2±0.24	0.0032	0.0647
HM2	8.50±0.53	6.89±0.51	2.39±0.16	3±0.24	0.0038	0.0667
HM3	6.80±1.34	4.90±0.24	1.45±1.03	3±0.24	0.0049	0.0698

HM1 (soil:farmyard manure(4:1)); HM2 (soil:vermicompost(2:1)); HM3 (soil:vermincompost(4:1))

attack and then roots of explants were inserted carefully into the soil mixture. The plants were then again sprinkled with water and kept inside the polyhouse at tissue culture.

Then plants were transferred to the polyhouse with misting/pad fan cooling system where humidity maintained is approximately 80% and temperature was 28-30°C and kept there for 15 days. In the next step they were transferred to the shade house (75%) with overhead sprinkler system for irrigation for next 10 days. Finally they were transferred to the open area for 9-10 days before transferring them to the field. Success of hardening protocol was determined by calculating survival percentage. Other parameters included were Fresh Weight, Dry Weight (measured as discussed previously), Root Length, Shoot Length, Shoot/Root ratio and number of roots.

Survival percentage for all the mixtures used was >95. Tiwari *et al*. (2000) also reported that survival was 100% in sterilized soilrite. The observation shown by Table 5 was taken 30 days after transplantation. However in terms of root length (6.89 cm) and shoot length (8.50 cm), HM2 potting mixture (2 soil: 1 vermicompost) was found to be the best combination. Number of roots was found to be highest in case of HM2, soil and vermicompost mixture (2:1), however in terms of fresh and dry weight, soil+ vermicompost (4:1) (HM2) was found to be the better option with significantly higher number of roots and high shoot/root ratio.

It is observed that across five parameters two best hardening mixtures were HM2 and HM3. High shoot length is desired in case of *Bacopa* as once shoots are long, they fall down and new shoot buds form from each node as runner and further multiply once they touches soil and form roots. 1-2 side branches were observed in case of *Bacopa* during hardening process. Since the average survival of the *Bacopa* rooted plantlets were >95% and hence assumed that the different potting mixture did not have significant differences. However, for number of roots per plant, the treatment combinations did not show consistent results and hence it is observed that further experiments are necessary to ascertain the results.

For further experimentation, few plantlets (approximately 25) randomly selected from the hardening stage after 35 days. After 35 days the plants are surviving (100%) and started growing vigorously. Thus established the successful protocol of *Bacopa* micropropagation as undertaken under the present study.

Isolation of protoplast: Protoplasts were isolated from *Bacopa monnieri* using mechanical method (Fig. 11). Mannitol and sorbitol were used to maintain osmotic pressure. Protoplast isolated was checked for its viability using evan's blue staining procedure.

Culturing of protoplast: Isolated protoplasts were then inoculated in MS medium having coconut water as PGR (Plant Growth Regulators) and maintained as suspension culture for checking its growth rate. Growth rate was checked using spectrophotometer and readings were produced as graph by taking growth along Y axis and time period along X axis. Growth of protoplast was observed using spectrophotometer for a period of 35 days and graph was plotted. Protoplasts which

Fig. 11: Isolation of protoplast by mechanical method

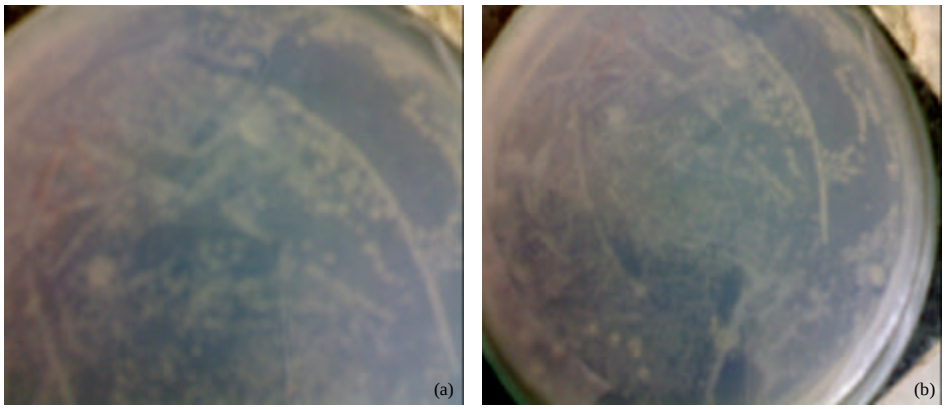

Fig. 12: (a, b) Protoplast culture by bergmann's plating technique

Fig. 13: Protoplast culture by hanging drop method

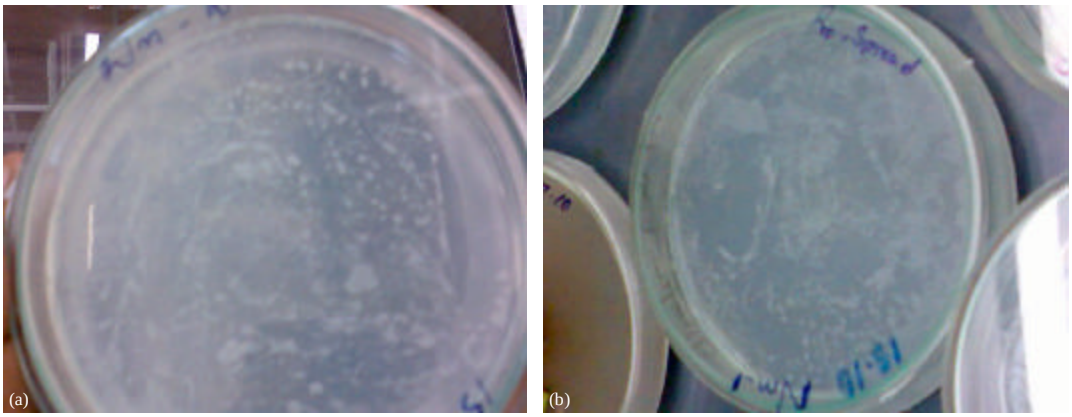

Fig. 14: (a, b) Protoplast of *Bacopa monnieri*

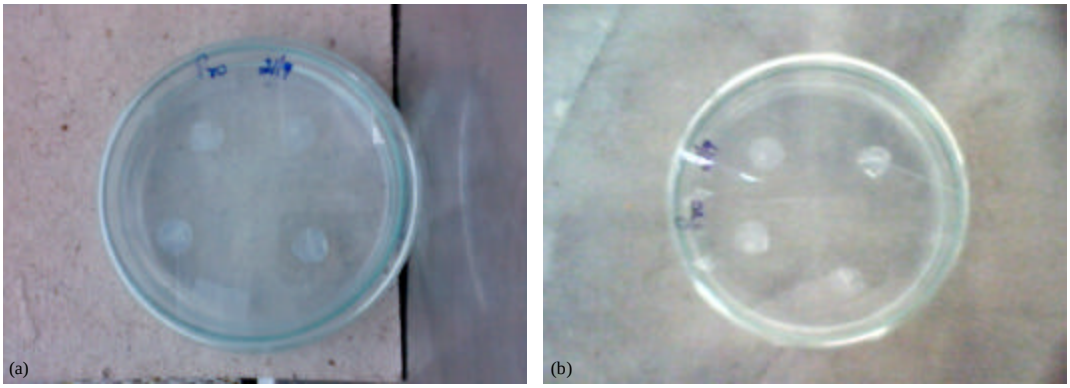

Fig. 15: (a, b) Induction of callus for regeneration

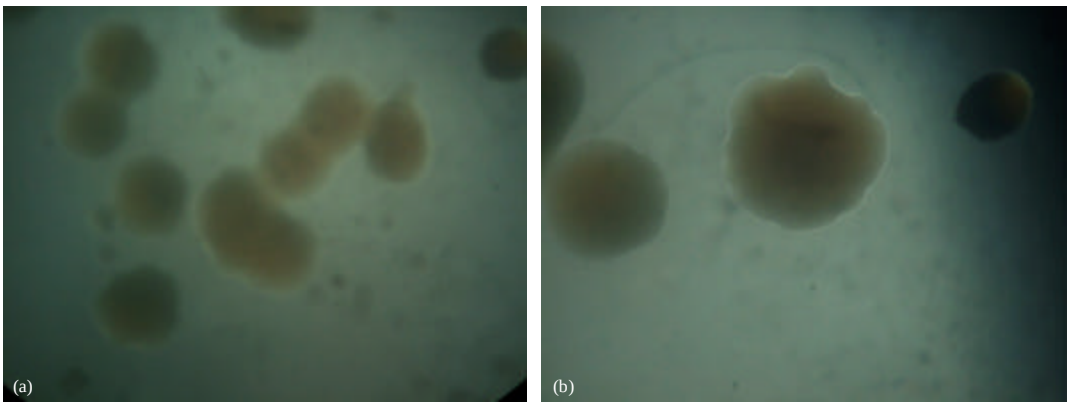

Fig. 16: (a, b) Microscopic colonies of protoplast (*Bacopa monnieri*)

were found viable were then cultured by bergmanns plating procedure and hanging drop method to form microscopic and macroscopic colonies (Fig. 12-16). Growth of protoplast was observed using spectrophotometer for a period of 35 days and graph was plotted.

Induction of calli and regeneration: Calli was not induced using protocol 1 and protocol 2 and so it was not successful. Calli was induced successfully and regenerated using protocol 3. Callus induction medium [Kao and Michayluk] was proved to be successful when compared to MS salts based culture medium which failed to induce callus. Multiple rootlets and shootlets were found to be produced when callus was transferred to medium supplemented with MS and 372.4 μg L^{-1} NAA. The establishment of reproducible protoplast to plant system for *Bacopa monnieri* will prevent it from becoming an endangered plant. This herbal plant if produced in large amount will result in bacoside production which has various medicinal uses. This technique of induction of callus was worthful when compared with *Braun's* experiment of plant regeneration.

Isolation of DNA: Fibe grams of *Bacopa monnieri* leaves were subjected to various mechanical (mortar and pestle, magnetic stirrer, heat treatment)treatment and chemical treatment (SDS, iso propanol, potassium acetate, sodium acetate, ice cold ethanol). Genomic DNA was isolated with yield of 250-300 mg mg^{-1} of *Bacopa monnieri* leaves.

The isolated genomic DNA from plant was observed as Orange red bands in agarose gel under the UV transilluminator and the molecular weight was determined as ≥3000 Kb.

Isolation of DNA: DNA was successfully isolated by mechanical and chemical treatment and molecular weight was determined as ≥3000 Kb.

Table 6: RAPD analysis plant population from the regenerated plant

Sequence	No. amplified bands	No. of polymorphic bands
AAATCGGAGC	0 (0)	0
GTCCTACTCG	7 (7)	0
GTCCTTAGCG	4 (4)	0
TGCGCGATCG	5 (5)	0
AACGTACGCG	5 (5)	0
GCACGCCGGA	6 (6)	0
CACCCTGCGC	6 (6)	0
CGGGATCCGC	6 (6)	0
GCGAATTCCG	7 (7)	0
CCCTGCAGGC	5 (5)	0
CCAAGCTTGC	3 (3)	0
GTGCAATGAG	3 (3)	0
AGGATACGTG	3 (3)	0
AAGATAGCGG	3 (3)	0
GGATCTGAAC	4 (4)	0
TTGTCTCAGG	6 (6)	0
CATCCCGAAC	6 (6)	0
GGACTCCACG	5 (5)	0
AGCCTGACGC	2 (2)	0
CTATCGCCGC	0 (0)	0

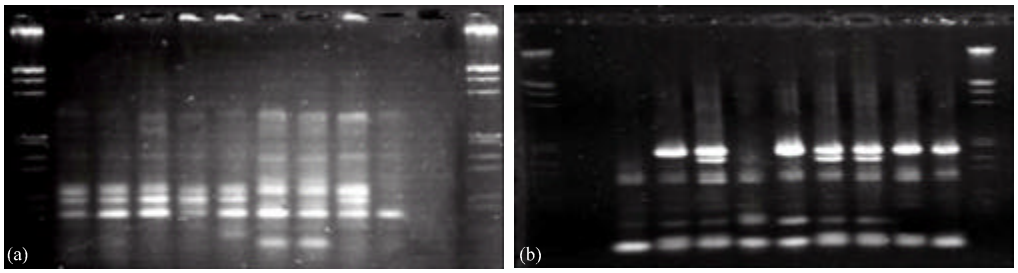

Fig. 17: (a, b) RAPD analysis of mother plant

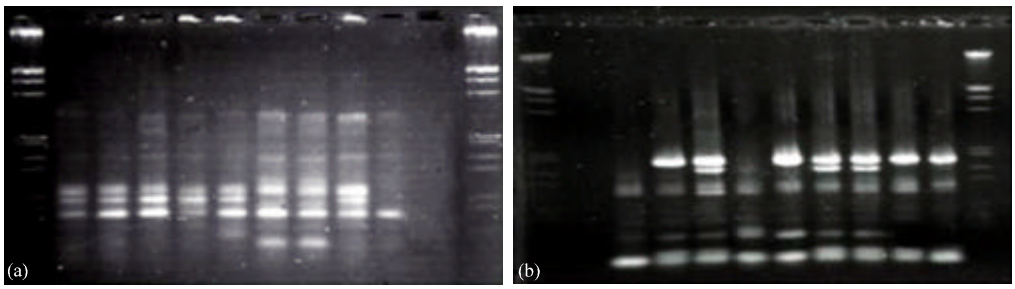

Fig. 18: (a, b) RAPD analysis of micropropagated plant

Restriction digestion of DNA: The various restricted patterns of DNA appear as clear bands under UV transilluminator.

RAPD analysis: Polymerase Chain Reactions (PCRs) were carried out in 25 J. ll volume. A reaction tube contained 25 ng of DNA, 0.2 unit of Taq DNA polymerase, 100 J. ll each of dNTPs, 1.5 mM $MgCl_2$ and 5 pmol of decanucleotide primers. The amplifications were carried out using a thermal cycler (MJ Research, USA) following the protocol of Darokar *et al.* (2001). The amplified products were loaded in 1.2% agarose gel containing 0. 51J. g mL^{-1} of ethidium bromide and photographed by Polaroid system. Custom-made decanucleotide primers were obtained from M/s Bangalore Genie, India. Twelve decamer primers having the sequences AAATCGGAGC, GTCCTACTCG, GTCCTTAGCG, TGCGCGATCG, AACGTACGCG, GCACGCCGGA, CACCCTGCGC, CTATCGCCGC, CGGGATCCGC, GCGAATCCG, CCCTGCAGGC, CCAAGCTTGC were used to analyse 100 *in vitro* regenerated plantlets from each cultivar (Table 6).

RAPD analysis: The RAPD analysis was performed to compare the mother and micro-propagated plant and 99. 9% homogeneity was observed (Fig. 17, 18).

CONCLUSION

Plants were the first medicines and even as modern humans have developed sophisticated pharmaceutical chemicals to treat illness. Medicinal plants remain an important tool for treating illness in most cultures. Human beings have been utilizing plants to basic preventive and curative health care. A rich heritage of knowledge on preventive and curative medicines was even available in Atharva Veda, Charkha, Sushruta etc. In the folklore of Indian Medicines (George *et al.*, 2004) certain herbs have been used traditionally as brain or nerve tonics. One of the most popular of

these used in neuro tonics is *Bacopa monnieri,* is also called Brahmi, a small, common, amphibious plant growing in marshy areas throughout the Indian Subcontinent. According to scientists at the Central Drug Research Institute in Lucknow, India, certain memory chemicals in *Bacopa,* called *bacosides* A and B, help to repair damaged neurons by enhancing proteins involved in the regeneration of neural cell synapses (Rastogi *et al.*, 1994).

In many medicinal plants, planting material is becoming endangered so it is necessary to develop micropropagation protocols to preserve the germplasm and for distribution during cultivation in new areas. Canter *et al.* (2005) and Debnath *et al.* (2006). Optimization of micropropagation protocol of *Bacopa monnieri* was carried out.

In micropropagation of *Bacopa monnieri,* a protocol for the sterilization, initiation and multiplication were achieved. Induction of callus were carried out in success and the callus were multiplied using the bubble column type of bioreactor. The protoplast isolation (Zhou and Wu, 2006) were carried out successfully. Achieved shoots and plantlets from the callus of protoplasts. The plantlets were transplanted to the Green house and found 95% survival. The analysis of genomic DNA of *Bacopa monnieri,* (Titanji *et al.*, 2007) were done and found the molecular weight >3000. RAPD analysis (Chaturvedi *et al.*, 2007) were carried out to establish the homogeneity of the mother and regenerated plant, found that there is no differences in characteristics of the *in vivo, in vitro* raised plants of *Bacopa monnieri.*

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