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In vitro regeneration of Brahmi (*Bacopa monnieri* (Linn.) Pennell) - an important medicinal herb through nodal segment culture

Asha K.I.*, A. Indira Devi, N.K. Dwivedi and R. Asokan Nair

NBPGR Regional Station, Vellanikkara, Thrissur-680 656, Kerala, India
*Corresponding author: ashakarthy@gmail.com

An efficient and cost effective *in vitro* plant regeneration protocol through nodal segment culture was achieved in the medicinally important herb *Bacopa monnieri* (L.) Pennell, the Memory Plus plant through axillary shoot proliferation in Murashige and Skooge medium augmented with varying concentrations of 6-benzylaminopurine (BAP) 1 - 5 mg/l. BAP at 2 mg/l was the most effective in multiple shoot induction and mean number of leaves, which gave an average of 17 shoots and 31.11 leaves, compared to other concentrations of the hormone tried in 35 days of culture. Regarding mean shoot length and number of nodes, basal MS giving 2.66 cm long shoots with 7.44 nodes is the best. MS basal medium, even though not promoting shoot multiplication, gave higher shoot length with elongated internodes. Healthy rooting of the *in vitro* developed shoots was achieved in half and full strength MS basal solid medium without the addition of any hormones. The healthy and vigorous *in vitro* regenerated micro shoots were separated out and were hardened on transfer to plastic cups with sterile soil and sand and were successfully acclimatized *ex vitro* in pots with potting mixture under green house conditions for 3 weeks. The survival rate was 100% and the plants established well in green house resembled the mother plants in habitat without any morphological variations. The very simple and cost effective protocol developed can be used to produce elite stable clones for *en masse* propagation for the large-scale cultivation of this very important medicinal herb.

Keywords: *Bacopa monnieri* (L.) Pennnell, brahmi, 6-benzylaminopurine, nodal segment culture, shoot multiplication, regeneration, micro shoots, *ex vitro*

India is known for the rich repository of medicinal plants, which are largely collected as raw materials for manufacture of drugs and perfumery products. Since ancient times, drugs of herbal origin have been used in traditional systems of medicines such as *Ayurveda* and *Unani*. *Bacopa monnieri* (L.) Pennell, a perennial, creeping herb inhabitant of wetlands and muddy shores known by the name 'Brahmi' or 'Nir-brahmi' of the family Scrophulariaceae has been used in the Indian Ayurvedic system of medicine as brain tonic to enhance memory development, learning and concentration and to provide relief to patients with anxiety or epileptic disorders. The plant has also been used as a cardiac tonic, digestive aid and to improve respiratory function in cases of broncho-constriction. Brahmi extract is also known to have anti-cancer (Elangovan *et al*., 1995) and anti-oxidant properties (Mukherjee and Dey, 1996). The principal active factors that have been reported from this plant are two steroidal sapogenins, bacoside A and B,

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alkaloids herpestine and brahmine, flavanoides, glycosides, betulic acid and phytosterols (Jain and Kulshreshtha, 1993).

The drug forms an important ingredient in a number of ayurvedic preparations like 'Brahmighritam' and 'Brahmirasayanam'. A herbal preparation from *Bacopa monnieri* under the brand name 'Promind' is being marketed for the improvement of memory. Other products like Anxocare, Brahmi, Mentat/Mind care, Mentat syrup are also available in the market.

With the release of new drugs like Memory Plus in the market, there is going to be over exploitation of the natural populations of *B. monneri* for meeting the estimated present requirement of 0.1 million quintal/year of the herb (Ahmad, 1993). The demand of *Bacopa* is met generally from natural population, which has put heavy strain on existing natural population, resulting in slow depletion of this already threatened herb. From the wild sources the total annual production is about 3000 tonnes mainly from the states of Tamil Nadu and West Bengal. Estimated consumption of this drug in India is 1000 tonnes per year. Moreover, the characteristics of rapid vegetative growth, available morphological variation and short sexual life cycle raise the possibility of using *Bacopa monnieri* in the developmental studies related to bioprospection, morphogenesis and secondary metabolism (Mathur and Kumar, 1998). In view of the popularity of the *Bacopa* based drugs and of the wider market demand and consequent over exploitation by the alternative medical practitioners and pharmaceutical companies, there is an immediate need for assessing the natural populations, to conserve the wild stocks and to develop protocols for micro propagation and regeneration of *B. monnieri*. Protocols for *in vitro* clonal propagation and conservation have also been conducted in *B. monnieri* by several workers (Shrivastava and Rajani, 1999; Tiwari *et al*., 2000, 2001; Tejavathi *et al*., 2001; Binita *et al*., 2005; Sharma *et al*., 2007; Banerjee and Srivastava, 2008; George *et al*., 2009 and Joshi *et al*., 2010).

Materials and Methods

Regeneration of plants through *in vitro* techniques has the potential of reproducing very large number of homogenous plants within a short period of time relative to continuous asexual propagation methods. A suitable nutrient medium is needed for the success and fulfillment of all experimental systems of plant tissue culture. In the present study, *in vitro* shoot multiplication through nodal segment culture was attempted in *B. monnieri*, the medicinal herb in solid MS basal medium augmented with cytokinin BAP at 1,2,3 and 5 mg/l. Different concentrations of BAP (1-5 mg/l) were tried and the data were recorded at regular 2 weeks intervals. Four shoot characters such as number of shoots induced, shoot length, number of nodes/shoot and number of leaves per shoot were recorded.

Nodal segments, excised from the young, healthy and actively growing potted mother plants of the accession IC324777, collected from Kozhinjampara in Palakkad District of Kerala and maintained in pots under green house conditions at this Regional Station of NBPGR (Fig.1) served as the explant source for the present *in vitro* plant regeneration experiments. The explants were initially washed in running tap water for 30 minutes and then with aqueous surfactant Labolene 0.1% (w/v) for 15 minutes to remove the superficial dust particles as well as fungal and bacterial spores and again in tap water followed by repeated rinses in sterile distilled water 3 times to remove the adhering detergent and surface contaminants. Further treatments were carried out in a laminar airflow cabinet. The explants were then surface sterilized with 0.1% (w/v) $HgCl_2$ for 5 minutes followed by rinsing with sterile double distilled water 3-5 times thoroughly to remove traces of $HgCl_2$ inside the laminar

airflow chamber. The surface sterilized explants were trimmed to 0.8-1.2 cm by removing off cut ends and leaves and inoculated onto culture tubes containing 15 ml of MS (Murashige and Skooge, 1962) basal solid medium supplemented with myoinositol (100 mg/l) and 3% (w/v) sucrose and individual concentrations of cytokinin BAP (1.0 - 5.0 mg/l) for shoot multiplication.

The cultures were then kept in the culture room maintained at a temperature of 25±2^{o}C under 12 h photoperiod at a light intensity of 3000 lux provided by cool-white fluorescent lights and at 70-80% RH. Throughout the experiments MS media with 3% (w/v) sucrose was used. The pH of the media was adjusted to 5.8 and solidified with 0.8% (w/v) agar and dispensed into culture tubes. The cultures were sterilized by autoclaving at 121^{0}C and 108 Pa for 20 minutes. In all the experiments, 10 replicates were maintained and the experiment was repeated twice. The experiments were monitored for 2 weeks and the data for number of shoots/explants, shoot length, number of nodes/shoot and number of leaves/shoot was recorded.

The responding explants were transferred to MS basal media or fresh media either of the same composition or to lower concentrations for further proliferation and elongation. The shoot multiplication rate was 15-20 times higher as compared to the first step. Multiplication could be continued by transferring each of the separated shoot or shoot clumps to the same media or to a medium with lower concentration of BAP in jam bottles. The shoot induction and multiplication was recorded in ten replicates per treatment, each repeated two times. At the end of the experiments, percentage of shooting, shoot length and number of shoots/explants induced were recorded at regular two weeks interval. The proliferated shoots of 2.5 to 4.5 cm in length were excised from cultures and were transferred to half and full strength MS medium for *in vitro* rooting. Root number and length were recorded after 35 days of culture. The well developed and rooted plantlets were rinsed thoroughly in sterile distilled water to remove the residuals and were potted in plastic cups in sterile soil with periodical watering for better rooting and hardening. After 14-21 days, the fully hardened plantlets were transferred to pots with potting mixture, sand and soil (3:1 w/v), in green house conditions for acclimatization.

Results and Discussion

Of the various methods of *in vitro* propagation, multiple shoot culture is the most frequently used multiplication technique in plant micropropagation systems as the same leads to the production of true to type plants. The production of plants from axillary shoots has proved to be the most applicable and reliable method of *in vitro* propagation. Nodal segment culture results in the stimulation of axillary shoot growth by overcoming the dominance of shoot apical meristem by incorporation of growth regulators in to medium (George and Sherrington, 1984). The use of nodal segments as initial explants for the *in vitro* propagation has already been reported in *Bacopa monnieri* by Tiwari *et al.,* 2000, 2001; Mohapatra and Rath, 2005 and Escandon *et al.,* 2006. These reports support the selection of nodal segment as explant for shoot multiplication experiments in the present species.

Effectiveness of MS medium for optimum shoot multiplication in different *Bacopa* species was reported by Tiwari *et al*., 1998, 2000, 2001; Tejavathi *et al*., 2001; George *et al*., 2004; Binita *et al*., 2005; Escandon *et al.,* 2006; Sharma *et al*., 2007 and Banerjee and Shrivastava, 2008. Cytokinins are known to promote cell division and act as a vital force for bud production and growth. In the present study shoot multiplication was achieved in *B. monnieri* by culturing single nodal segments in MS medium supplemented

with different concentration of BAP. The nodal segments when used as explants showed positive responses in accordance with the hormonal concentrations tried. Compared to the nodal segments inoculated onto basal MS, the shoot multiplication rate was very high in medium supplemented with BAP at different levels. Tiwari *et al*. (2001) also affirmed the potentiality of BAP in multiple shoot induction. Of the different concentrations of BAP used, it was found that BAP at 2 mg/l is very much effective in shoot multiplication in *B. monnieri* within 35 days of inoculation. A similar result showing effectiveness of 2 mg/l of BAP in *B. monnieri* was also reported earlier by Tiwari *et al.* (1998).

When compared with the check (single nodal segment in basal MS medium), shoot multiplication rate was high in medium supplemented with BAP. In basal MS single shoot alone was formed with eight culturable nodes and elongated internodes (Fig.2). But in medium supplemented with BAP at different levels, multiplication at different ranges was noticed in 35 days of culture.

When BAP (1 mg/l) was used, number of shoots ranged from 2 - 40 in 35 days (Fig.3) with an average 10.2 shoots (Table-1). Shoot length ranged from 1.5-2.8 cm. Number of nodes and leaves produced ranged from 1-10 and 6-37 respectively. With BAP (2 mg/l), very high rate of shoot multiplication in a range of 2-50 was obtained in 35 days of culture (Fig.4). This result is in agreement with the report of Gurnani *et al*. (2012) in the species where best shoot initiation was seen in MS media with 3 mg/l BAP concentration. Average shoot number was 17 and shoot length ranged from 1.5-2.5 cm (Table-1). Number of nodes ranged from 1-11 while, number of leaves ranged from 14-50. A maximum of 42 shoots were obtained ranging from 2-42, with an average of 13.7, in 35 days of culture in medium augmented with 3 mg/l of BAP (Fig.5; Table-1). Shoot length ranges from 0.2-2.5 cm with an average of 0.9. Number of nodes ranged from 1-8, and number of leaves from 11-50. When BAP at 5 mg/l was used, number of shoots produced ranged from 2-30 in 35 days of culture with root initiation (Fig.6). The average number of shoots produced in 35 days was 10 (Table-1). Shoot length ranged from 0.4-2 cm and the number of nodes and leaves obtained were 1-3 and 8-40 respectively. High frequency of adventitious shoot formation in presence of BAP from leaf explants in *B. monnieri* was reported by Joshi *et al*. (2010).

Table-1: Effect of different concentrations of BAP in MS basal solid medium on shoot multiplication from nodal explants and rooting of the induced shoot buds in *Bacopa monnieri* in 35 days of culture

Treatments*	Mean Number of shoots	Mean Shoot length (cm)	Mean Number of nodes	Mean Number of leaves	Rooting Percentage in 2 weeks
BA-1	10.22	2.42	4.67	22.00	95
BA-2	17.00	1.92	3.44	31.11	92
BA-3	13.67	1.08	3.11	22.78	85
BA-5	10.00	0.89	1.11	15.89	78
Basal MS	1.33	2.66	7.44	16.22	80
CD	1.70	0.07	0.07	1.28	1.32

*Mean of 10 replications per treatment each repeated 2 times

Plate-01

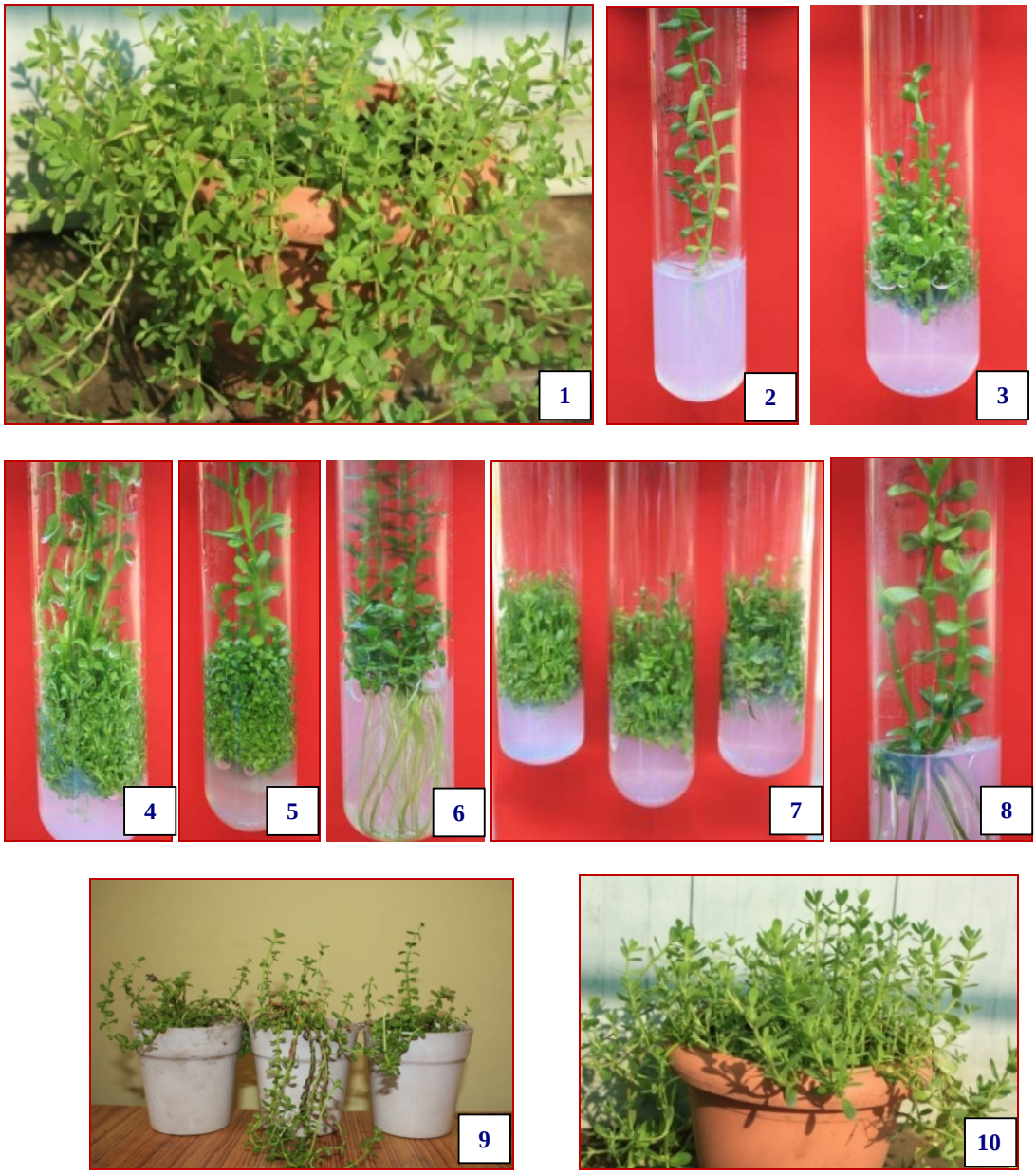

Plate-01. 1. IC324777, the accession of *Bacopa monnieri* collected from Kozhinjampara in Palakkad District of Kerala and maintained in the herbal garden of NBPGR Regional Station, Thrissur, Kerala, the source plant for nodal segment explants 2. Induction of single shoot in basal MS medium with elongated internodes 3. Multiple shoot induction 2-40 with an average of 10.2 in MS medium with 1 mg/l BAP 4. Best result of shoot multiplication in MS medium with BAP (2 mg/l), in a range of 2-50 shoots with an average of 17 in 35 days of culture 5. Initiation of 2-45 shoots, with an average of 13.7 in MS medium augmented with 3 mg/l of BAP 6. Reduction in the number of shoots induced to 2-30 with an average of 10 in MS medium with 5 mg/l of BAP 7. Multiplication of shoots on transfer to fresh media with lower concentration of BAP 8. Spontaneous rooting of shoots in shoot induction medium itself 9. Shoots transferred to plastic cups with sterile sand for hardening 10. Hardened regenerated plantlets in the greenhouse.

Multiplication could be continued by transferring each of the separated shoots/shoot clumps either to the same media or to a medium with lower concentrations of BAP (Fig.7). Shoot multiplication in BAP in MS semi-solid medium was reported by Sharma *et al*., 2010. Individual shoots on subculture to the same shoot initiation medium in 2 weeks initiated roots spontaneously (Fig.8). Similar result supporting spontaneous rooting of shoots in the same concentration of shoot induction media with cytokinin BAP was reported in *Alpinia purpurata* (Kochuthressia *et al*., 2010).

The rooted plants thus obtained were washed well in running tap water to make it free from agar gel and were separated and transferred to plastic cups with sterile sand for hardening (Fig.9). After 3 weeks, these plants were transferred to pots with potting mixture in greenhouse and were then transferred to the field and the survival rate was recorded (Fig.10). Almost 95-100% of the regenerated plants survived and showed vigorous growth without showing any morphological variations. Successful establishment of nodal regenerated plantlets was also reported in the species by Joshi *et al*., 2010; Sharma *et al*., 2010; Vijayakumar *et al*., 2010 and Gurnani *et al*., 2012. The commercially viable protocol presently developed can be put to use for mass micropropagation of this medicinally important herb that will go a long way to meet the ever increasing demands of the pharmaceutical industries, as well as to save this species from extinction due to over exploitation of natural population.

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