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*Full Length Research Paper*

# *In vitro* rapid and mass multiplication of highly valuable medicinal plant *Bacopa monnieri* (L.) Wettst.

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**A protocol has been developed for micropropagation of *Bacopa monnieri* (L) Wettst, a medicinal plant of high commercial potential with legendary reputation as a memory vitalizer. Nodal segments containing axillary buds were surface sterilized with 0.1% solution of mercuric chloride for 5 min and were inoculated aseptically on culture medium, axillary bud break was achieved in 100% of cultures in semisolid MS medium supplemented with 0.2 mg/l BAP. These proliferated *in vitro* axillary shoots were excised and cut into groups of shoot clusters and subcultured on MS medium supplemented with 0.2 mg/l BAP for shoot multiplication. 100% *in vitro* rooting was obtained when shoot clusters were cultured on MS medium supplemented with 0.15 mg/l IBA. The rooted plantlets were hardened, acclimatized and successfully established in field.**

**Key words:** *Bacopa monnieri*, nodal segments, micropropagation, plant growth regulators.

## INTRODUCTION

The availability of plants used for medicine has reached a very critical phase due to rapid depletion of our forests. In their natural occurrence, the plants are scattered and it is difficult to collect and process them. Such herbal medicines are easily available at affordable prices for the common man, they are time tested and considered safer than modern synthetic drugs. Therefore, the necessity to cultivate them on large-scale has become very urgent.

*Bacopa monnieri* (L.) Wettst (Scorphulariaceae) commonly known as 'Brahmi' or Nirbrahmi has originated from India. It is a genus of spreading herbs, commonly growing in damp and marshy places throughout India, ascending up to an altitude of 1320 m, and it is a small creeping, glabrous, succulent, herb rooting at nodes. It is an ancient and renowned medicinal plant with legendary reputation as memory vitalizer (Anonymous, 1998). In the traditional system of Indian medicine (Ayurveda), 'Brahmi' is classified as medhya rasayana, that is, a drug that is supposed to counteract the effects of mental stress and improve intelligence and memory function. 'Brahmi' is found to be effective in cases of anxiety and neurosis. It possesses anti-inflammatory, analgesic and antipyretic activity (Vohra et al., 1997). It is also used to treat asthma, insanity, epilepsy, hoarseness, enlargement of spleen, snake bite, rheumatism, leprosy, eczema and ring worm, it is also used as a diuretic, appetitive and cardio tonic (Basu and Walia, 1994). In a recent study, *B. monnieri* was placed second in a priority list of most important Indian medicinal plants evaluated on the basis of medicinal importance, commercial value and potential for further research and development (Mohapatra and Rath, 2005; Sharma et al., 2007). It is also well known to contain steroidal saponins Bacoside A and steroidal saponins Bacoside B. Some other constituents present in Brahmi are alkaloids brahmine, herpestine etc. Compounds responsible for the pharmacological effects

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of bacopa include alkaloids, saponins and steroids. Many alkaloids like brahmine, herpestine, saponins d-mannitol, hersaponin, acid A and monnierin were isolated in India over 40 years ago. Other active constituents identified include betulic acid, stigmastarol, beta-sitosterol, as well as numerous bacosides and bacopa saponins. The constituents responsible for bacopa's cognitive effects are Bacosides A and B.

According to the National Medicinal Plants Board (NMPB), annual demand of *Bacopa* during the year 2004 - 2005 is 6621.8 tons with a annual growth rate of 7% annually. This requirement is rising rapidly in view of the popularity of the Bacopa based drugs. In view of wider market demand, there is need to conserve the wild stocks of *B. monnieri* (http://nmpb.nic.in/). 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. monnieri* that must meet the present requirement of 0.1 million quintal/year of the herb. The effects of *B. monnieri* have already been approved by clinical research trials.

There is thus an immediate need for assessing the natural populations, developing protocols for micro propagation, regeneration and agronomical practices. The present study deals with the rapid mass scale multiplication of *B. monnieri* using nodal explants containing axillary bud.

## MATERIALS AND METHODS

### Collection of explants

Nodal segments were collected from the juvenile shoots of growing at Non-wood Forest Products, Experimental Nursery, Forest Research Institute, Dehradun (altitude 610, latitude 30°N and longitude 78°E and annual rain fall 216 cm). Juvenile segments containing single axillary bud shoots tips were used as source material for micropropagation.

### Sterilization of explant

*B. monnieri* twigs with 3 - 4 nodes were collected, excised from plants maintained in the nursery, FRI, Dehradun were washed thoroughly in running tap water to remove the superficial dust, then explants were washed with dil. detergent (1 - 2% Cetavelon) solution for 10 min and then washed well in running tap water. Explants were surface sterilized with 5 min treatment with 0.1% (w/v) $HgCl_2$. *B. monnieri* was extremely sensitive to surface sterilizing agent, therefore, the surface sterilization procedure was optimized and this helped in preventing blackening of tissues and establishment of clean cultures. During surface sterilization treatment, it was found that treatment with 0.1% mercuric chloride as referred by Shrivastva and Rajani (1999) and Mathur and Kumar (1998), leads to blackening of the explants. Hence limited treatment of 0.1% mercuric chloride was given to plants 4 - 5 min, and it was proved to be the best sterilent as it produced the maximum number of aseptic explants. Finally, the explants were washed thoroughly (4 - 5 times) with sterilized distilled water. Throughout the experiments, MS medium with 3% (w/v) sucrose and gelled with 0.7% (w/v) agar (Qualigens, India) was used. The pH of all media was adjusted to 5.8 before autoclaving at 121°C (15 min). The cultures were incubated in a culture room at 25 ± 2°C under 16 h photoperiod.

## RESULTS AND DISCUSSION

### Axillary bud Initiation

Axillary bud break was achieved in all aseptic cultures on MS medium supplemented with (0.1 - 1.0 mg/l) BAP. The number of shoots proliferating ranged from 2 - 12 folds. The morphogenic differentiation of explants towards axillary bud multiplication was markedly influenced by the concentration of the growth regulator (BAP) in the medium. BAP treatment significantly increased the percentage of bud break and the number of shoots that proliferated. At 0.2 mg/l concentration of BAP in MS medium, distinct shoots proliferated in 13 - 15 folds in three to four weeks. Statistically, the optimum medium for axillary bud culture were MS medium supplemented with 0.2 mg/l BAP where shoots proliferated, and yielding maximum number of shoots cluster for shoot multiplication (Figure 1A). Axillary bud cultured on hormone free medium yielding less then 30% bud break response. Axillary shoots proliferation from cultured axillary buds were obtained when auxin (IBA) was added along with BAP.

Shoot proliferation was also obtained on MS medium supplemented with cytokinin alone. With the concentration of BAP (0.15 mg/l) maximum numbers of healthy and sizeable shoots were proliferated. With the use of Kn at concentration 0.2 mg/l, 12 to 14 folds were proliferated. These results are in line with those workers, indicating the efficiency of BAP for shoot culture initiation and multiplication in *B. monnieri,* reported by (Tiwari et al., 2000; Shrivastava, 1999; Tiwari et al., 2007).

MS media proved to be the best culture medium for the establishment of shoot culture in *B. monnieri*. In earlier reports on Bacopa, MS medium has been successfully used for shoot intiation and culture establishment.

### *In vitro* shoot multiplication

The proliferated *in vitro* axillary shoots were excised from mother explants and subcultured on semisolid MS medium supplemented with 0.1 mg/l BAP for further *in vitro* shoot multiplication. These multiplied *in vitro* shoots were subsequently dissected into propagules and were subcultured on fresh MS medium supplemented with BAP for further shoot multiplication. After every four weeks of subculture, it was noticed that the best *in vitro* shoot multiplication with sizable development were obtained on 0.2 mg/l BAP supplemented medium on hormone free medium, *in vitro* shoot multiplication rate drastically decreased and the shoot culture slowly died. Hence, incorporation of BAP was essential for the multiplication rate as well as for the development of *in vitro* shoots.

Optimal BAP response was obtained on 0.1 mg/l BAP in 4 weeks. Shoot length during the multiplication cycle showed a relationship with the concentration of BAP (Table 1 and Figure 1B). It increases with the increase

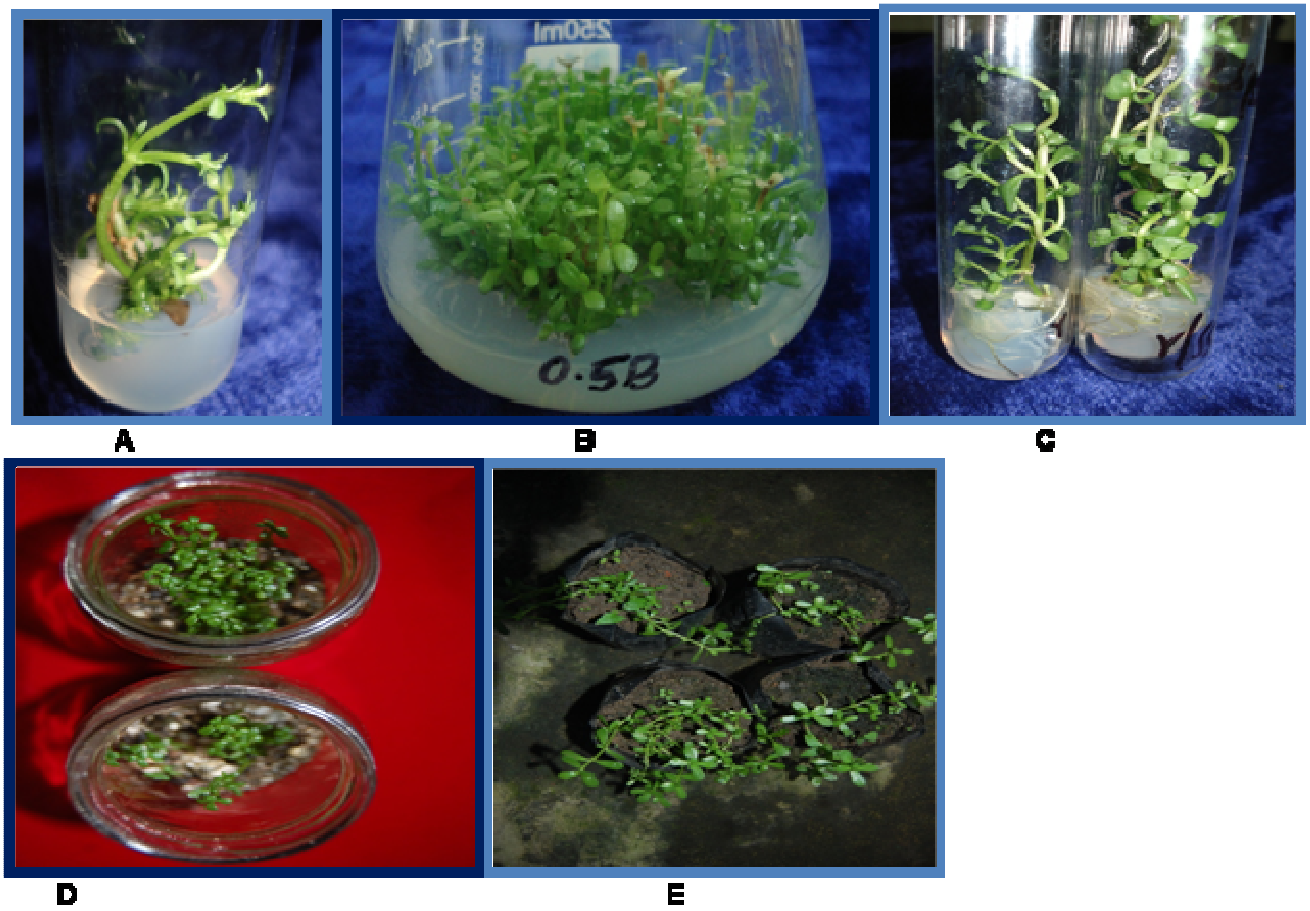


**Figure 1.** Different stages of Micropropagation of *B. monnieri* (L.) Wettst. **A,** Axillary bud Induction; **B,** *In vitro* shoot multiplication; **C,** *In vitro* rooted shoots; **D,** hardened and acclimatized plantlets; **E,** plants in polybags.

**Table 1.** Effect of cytokinin (BAP) and (Kn) in MS medium on *in vitro* shoot multiplication. Data were recorded after 4 weeks.

| Hormonal concentration (mg/l) | Average number of shoots developed | Multiplication rate | Average Shoot length (cm) |
|---|---|---|---|
| **Kn (mg/l)** | | | |
| 0 .00 | 13.0 ± 0.10 | 1.86 ± 0.14 | 0.70 ± 0.06 |
| 0.01 | 21.0 ± 0.19 | 2.98 ± 0.26 | 0.78 ± 0.08 |
| 0.10 | 23.0 ± 0.12 | 3.29 ± 0.17 | 0.76 ± 0.07 |
| 0.20 | 33.0 ± 0.16 | 4.74 ± 0.37 | 1.01 ± 0.08 |
| 0.30 | 29.0 ± 0.10 | 4.14 ± 0.14 | 0.44 ± 0.04 |
| **Significance** | *** | *** | *** |
| **CD** | 4.89 | 0.17 | 3.45 |
| **BAP (mg/l)** | | | |
| 0.00 | 11.4 ± 0.93 | 1.63 ± 0.13 | 1.34 ± 0.13 |
| 0.01 | 10.6 ± 0.93 | 1.51 ± 0. 13 | 2.02 ± 0.17 |
| 0.10 | 41.2 ± 2.54 | 5.89 ± 0.36 | 3.22 ± 0.20 |
| 0.20 | 35.2 ± 1.71 | 4.98 ± 0.24 | 2.27 ± 0.17 |
| 0.30 | 29.2 ± 2.32 | 4.36 ± 0.62 | 2.03 ± 0.07 |
| **Significance** | *** | *** | *** |
| **CD** | 7.23 | 1.06 | 0.20 |

***Significant at 0.1%; values are means ± standard deviation.

**Table 2.** Effect of auxin (IBA) on rooting of *in vitro* shoots in MS medium using nodal segments of *B. monnieri*. Data were recorded after 4 weeks.

| Growth regulators IBA (mg/l) | Average no. of roots developed | Average root length (cm) | Remarks |
|---|---|---|---|
| 0.10 | 10.5 ± 2.74 | 18.4 ± 7.40 | Less roots developed |
| 0.15 | 24.9 ± 4.19 | 27.5 ± 8.30 | Healthy roots |
| 0.20 | 24.0 ± 4.18 | 25.0 ± 12.94 | Roots with callus |
| 0.25 | 15.5 ± 2.09 | 15.0 ± 4.12 | Less roots developed |
| 0.30 | 15.1 ± 2.52 | 13.2 ± 4.35 | Roots not properly grown |
| **Significance** | *** | *** | |
| CD | 1.43 | 0.47 | |

***Significant at 0.1%; values are means ± standard deviation.

concentration of BAP in the MS medium. *In vitro* shoot multiplication rate steadily increased with each subculture cycle. Shoot multiplication was found to be the best when four to five shoots propagule units were used. Multiplication rate decreased when propagule of less than 4 shoots were used. The better growth of shoots multiplication in Brahmi on MS medium may be because of its higher concentration. These results are in agreement with the findings of other workers who have also noted the effectiveness of MS medium for optimum shoot multiplication in different Bacopa species (Tejavathi et al., 2001; Sharma, 2007; Banerjee and Shrivastava, 2008; George, 2004; Binita et al., 2005, Tiwari et al., 1998, 2000; Escandon et al., 2006). Medium strength also influences the multiplication rate of shoots as observed. When the explants were cultured on MS medium for multiplication, maximum shoot length was obtained on full strength medium in Bacopa species. This indicates the absolute requirement of higher concen-tration of salts and vitamins for multiplication.

## *In vitro* rooting

The ability of plant tissue to form roots depends on interaction of many endogenous and exogenous factors. The role of auxin in root development was established and reviewed by Torrey (1965, 1976). Thus, there is enough residual cytokinin present in shoots, therefore, little or no cytokinin is required in rooting medium by Hu and Wang (1983). In *B. monnieri*, rooting was observed in the absence of auxins also but the best rooting was observed by IBA when incorporated in MS at different concentrations (0.1 - 0.3 mg/l). For *in vitro* rooting, a propagule of 3 - 4 shoots of 2 - 3 cm was cultured on *in vitro* rooting medium. IBA supplemented MS medium produced maximum number of roots (25 to 28) at 0.15 mg/l IBA (Table 2).

## Hardening and acclimatization

For hardening, plantlets were washed to remove adhere agar and then transferred to autoclaved vermiculite.

These plantlets were nurtured with half strength MS medium (without organics) twice a week for two weeks and were kept in tissue culture room. After two weeks, these bottles were shifted to mist chamber having relative humidity of 80 - 90% with a temperature of 30 ± 2°C. The caps of bottles were removed and plantlets were allowed to remain in the bottle for 3 - 4 days (Figure 1D) before they were transferred to polybags containing a mixture of sand, farmyard manure and soil in a ratio of 1:1:1 (Figure 1E). In the mist chamber, the plants were kept for three weeks and were irrigated with half strength MS medium. Later, these polybags were shifted to high-density double deck agronet open shade house for acclimatization. After one month in shade house, the plants were transferred to bigger polybags/pots containing same soil composition and were irrigated with tap water. Plants were kept in shade house for two months.

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