Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com
J P P
Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(2): 1614-1620
Received: 01-01-2020
Accepted: 03-02-2020

Ruchi Chauhan
Department of Biotechnology
Dr YS Parmar University of Horticulture & Forestry, Nauni, Solan, Himachal Pradesh, India

Poonam Shirkot
Department of Biotechnology
Dr YS Parmar University of Horticulture & Forestry, Nauni, Solan, Himachal Pradesh, India

Corresponding Author:
Ruchi Chauhan
Department of Biotechnology
Dr YS Parmar University of Horticulture & Forestry, Nauni, Solan, Himachal Pradesh, India

Micropropagation of endangered medicinal plant *Bacopa monnieri* (L.) Pennell

Ruchi Chauhan and Poonam Shirkot

Abstract
Bacopa monnieri (L.) Pennell, commonly known as “Brahmi” is an important medicinal herb of the family Scrophulariaceae. It has main importance as tonic for nervous disorders and mental diseases. It helps in improving intelligence, memory and learning ability. In the present work, a protocol for micropropagation of selected genotype of *Bacopa monnieri* (L.) Pennell has been standardized. Axillary buds were used as explants for *in vitro* plant regeneration studies in *Bacopa monnieri* (L.) Pennell. Axillary shoot buds showed best *in vitro* establishment (87.50%) on MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l Kn. Best *in vitro* shoot multiplication was obtained on MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l Kn. For *in vitro* rooting, MS medium supplemented with 0.4 mg/l IAA was found to be the best medium (93.33%). Regenerated plantlets were successfully acclimatized by using cocopeat followed by a mixture of soil: sand: FYM in ratio 1:1:1.

Keywords: Brahmi, *in vitro*, BAP, Kn, 2, 4-D

Introduction
The *Bacopa* genus consists of 20 species living in warm parts of the world, three of which are represented in India (Sharma, 2003) [19]. *Bacopa monnieri* (Linn.) Pennell is one of the most important medicinal plants which belong to Scrophulariaceae family. This herb is commonly known by various names such as Brahmi, Brahmi-Sak, Kiru-Brahmi, Neerbrahmi, Samrani and Safed Chamani. Its English name is thyme-left gratiola and water-hyssop (Anonymous, 1996) [2].

It grows sporadically in moist, wet and marshy areas across India. It is found naturally in West Bengal, Punjab, Haryana and Himachal Pradesh. Himachal Pradesh is situated between $30^{0}12^{0}40$” to $33^{0}12^{0}40$” north latitude and $75^{0}47^{0}55$ "to $79^{0}4^{0}20$" east longitude (Chauhan, 1999) [5] and here this plant is mainly found in moist places of Una, Hamirpur, Kangra, Bilaspur, Solan, Sirmaur etc. It grows naturally up to a height of 1000 m in marshy areas in the subtropical region (Sharma, 2003) [19]. The bloom of this plant occurs in the month of July and continues till December.

The entire herb is used in Ayurvedic, Unani and Siddha systems of indigenous medicine. It is astringent, bitter, cold and full of vitamin-C. The activity of the plant, *Bacopa monnieri*, has been attributed to a complex mixture of triterpenoid saponins, sterols and alkaloids (Rajani, 2008) [23]. Of these saponins, Bacoside-A and Bacoside-B have been found to be the most important. Other saponins include bacopasaponins-A, B, C, D, F, G and bacopaside I and II. Other chemical components of the plant are D-mannitol, herpaponin, betulic acid, alkaloids brahmine and herpestine, flavonoids, and phytosterols (Prabhuji *et al*., 2005) [15].

In India the plant is used for various types of skin problems - eczema, psoriasis, boils and ulcers. It is said to stimulate the growth of skin, hair and nails. Indian pennywort is also often used as an ointment for chronic arthritis. In Pakistan, the herbal medicine, Brahmi-Buti, is used for the treatment of skin diseases, leprosy, epilepsy, eczema, asthma, hoarseness of voice, and nervous system diseases (Shakur *et al*., 1994) [21].

With the release of new memory drugs in the market, the natural population of *Bacopa monnieri* is going to be exploited, which must meet the current requirement of 0.1 million quintals/year of the herb (Sharma *et al*., 2010) [20]. Thus there is an urgent need to assess natural populations, to develop protocols for micropropagation, regeneration and agronomic practices.

In view of the numerous medicinal applications of *Bacopa monnieri* and its endangered status, it becomes necessary to develop micropropagation protocols to preserve germplasm and distribute it for cultivation in new areas. The present work aims to determine the cultivation conditions for *in vitro* propagation of this plant. So this work was done with the objective of standardization of protocol for *in vitro* mass multiplication of *Bacopa monnieri* (L.) Pennell.

Materials and Methods

Plant Material

The present investigation was carried out in the Department of Biotechnology of Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan. In the present investigation the plant material used was selected from the previous study in which *Bacopa monnieri* (L.) Pennell genotype of Khaltoo village, Nauni, Solan was found to be elite genotype on the basis of the leaf length. The plant material was collected from the selected site and maintained in the glasshouse of Department of Biotechnology of Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, for further studies.

Methodology

Preparation of culture media

The explants were cultured on Murashige and Skoog's (1962) [14] medium supplemented with different concentrations of growth hormones. To reduce the time for weighing individual ingredients each time, concentrated stock solution of analytical grade chemicals (macro and micro elements, vitamins and growth regulators) were prepared and stored at 4 ^{0}C. Plant growth regulators were prepared fresh each time; auxins were titrated into solution with ethanol, whereas cytokinins were dissolved in dilute NaOH and then the final volume is adjusted with double distilled water. In preparing medium, each stock is added one by one in specified quantity in double distilled water, after bringing them to room temperature. After adding meso-inositol (100.0 mg/l), sucrose (30.0 g/l) and standard amount of growth regulators, the pH of the medium was adjusted to 5.6-5.8 with the help of 0.1 N NaOH and/or 0.1 N HCl. Agar- agar (8.0 g/l) was then added and dissolved by heating the medium. The medium was poured into flasks/tubes which were then plugged with non-absorbent cotton wool. Culture vessels containing the medium were sterilized in autoclave at pressure of 15 lbs per inch^2 at 121 ^{0}C for 15-20 minutes. After cooling, the media was stored in dark at 25 ^{0}C $\pm$ 2 ^{0}C for about a week to check the contamination before use.

Sizing and surface sterilization of explant

Young shoots were excised from the plant and leaves were removed carefully. The shoots were cut into small pieces of size approximately 1 cm with the help of sterilized scalpel blade so as at least one axillary bud per shoot is present. These axillary buds were used as explants. The explants i.e. shoot buds were thoroughly washed with running tap water to remove the superficial dust. Then they were initially treated with a mixture of aqueous surfactant and fungicide i.e. 0.2% (w/v) Teepol and 0.2% (w/v) bavistin respectively for 10-20 min, followed by washing 3-4 times with distilled water. Henceforth, the manipulations were carried out under aseptic conditions under laminar air flow hood. The explants were then surface sterilized using 0.1% $HgCl_2$ for 1-3 minutes; finally they were washed 4-5 times with autoclaved distilled water to remove any traces of sterilants.

In vitro establishment of axillary buds

For the establishment of axillary buds, the sterilized explants were inoculated on to MS medium supplemented with variable concentrations of BAP (0.25-1.0 mg/l) alone and in combination with constant concentrations of NAA (0.20 mg/l) and Kn (0.50 mg/l). The cultures were then placed in the culture room under the standard conditions of temperature (25 $\pm$ 2°C) for 16/8 hrs of day/night break under cool white fluorescent light of average 2500 lux (cool white fluorescent tubelight 40 W GE). The proliferation was indicated by unfurling of leaves, elongation of petiole and widening of leaf lamina. After four weeks the observations were recorded for percentage of buds proliferated.

In vitro multiplication of shoots

In vitro shoot multiplication was carried out on the MS medium supplemented with different concentrations of BAP (0.5-1.0 mg/l) alone and in combination with constant concentrations of 2, 4-D (0.2 mg/l) and Kn (0.5 mg/l). The cultures were kept in culture room and after four weeks observations were recorded for following parameters:

I. Average number of microshoots per shoot
II. Microshoot length (cm)

In vitro rooting of microshoots

Shoots of variable length ranging in height from 3-4 cm were excised at different stages of subculturing and transferred to MS medium supplemented with IAA (0.2-0.6 mg/l) and IBA (0.2-0.6 mg/l) for root induction. The cultures were transferred to the culture room and after three weeks observations were recorded for:

I. Per cent rooting based upon number of shoots forming roots
II. Numbers of roots per microshoot
III. Root length (cm)

Hardening and Acclimatization

The survival and establishment of plantlets was studied after transplanting the plantlets into different potting media.

I. Cocopeat
II. Sand
III. Soil
IV. Soil + vermicompost (1:1)
V. Soil + FYM (1:1)
VI. Sand + soil + FYM (1:1:1)

Per cent survival of the transferred plants was recorded after every 2 weeks.

Statistical analysis

The experiments were conducted in a completely randomized design (CRD). The data recorded for different parameters was subjected to analysis of variance (ANOVA) using completely randomized design [Gomez and Gomez, 1984 [8] (Appendix-1)]. Data transformation was carried out as needed to satisfy ANOVA requirements. Arcsine transformation was performed on percentage data (derived from count data) lying in the range of both zero to thirty per cent and seventy to hundred per cent while square-root transformation was performed on data consisting of small whole numbers i.e. data counting in rare events and for percentage data (derived from count data) lying within the range of 0 to 30 per cent and 70 to 100 per cent, but not both. The data that have been transformed were expressed in original units for presentation in the tables.

Results

Micropropagation of the selected genotype of *Bacopa monnieri* (L.) Pennell from Khaltoo village, Nauni, Solan may lead to mass production of the plants to overcome the increasing demand and unscientific exploitation which has given the plant species an endangered status. The information on various stages of micropropagation of *Bacopa monnieri*

(L.) Pennell and the results obtained during the course of this investigation are hereunder:

Establishment of shoot buds

For establishment of shoot buds, the sterilized explants were cultured on MS medium supplemented with variable concentrations BAP (0.25-1.0 mg/l) alone and in combination with constant concentrations of NAA (0.2 mg/l) and Kn (0.5 mg/l). A total of 13 treatments were given. Observations on sprouting of buds were recorded after four weeks and the results are presented in Table 1.

It has been observed that plant growth regulators had significant effect on per cent establishment of explants, while control medium (MS basal medium without growth regulators) did not show any response of establishment. Bud initiation was found to be started within 9-10 days by showing a small newly sprouted bud, which proliferated into shoot buds with leaves within 20-25 days. A maximum of 87.50% sprouting of buds was observed with treatment T_{11} (MS basal + 0.5 mg/l BAP + 0.5 mg/l Kn) followed by treatment T_9 (MS basal + 1.0 mg/l BAP + 0.2 mg/l NAA) where percentage of bud sprouting was found to be 83.33. With treatment T_{12} (MS basal + 0.75 mg/l BAP + 0.5 mg/l NAA), T_{10} (MS basal + 0.25 mg/l BAP + 0.5 mg/l Kn) and T_{13} (MS basal + 1.0 mg/l BAP + 0.5 mg/l Kn) sprouting percentage was found to be 79.16, 70.83 and 66.66 respectively. However, minimum per cent sprouting was observed with treatment T_2 (MS basal + 0.25 mg/l BAP) which was found to be 33.33 (Table 1).

Multiplication of *in vitro* raised shoots

For multiplication, the proliferated *in vitro* axillary shoots obtained on establishment medium were transferred to MS medium supplemented with variable concentrations of BAP (0.5-1.5 mg/l) alone and with constant concentrations of 2, 4-D (0.2 mg/l) and Kn (0.5 mg/l). A total of 10 treatments were given and observations for average number of shoots per explant and shoot length were recorded after 4 weeks and the results are presented in Table 2.

It has been observed that control medium have no effect on multiplication of microshoots however, maximum average number of microshoots per explant (7.0) and maximum average microshoot length (3.00 cm) was obtained with the treatment $T_{9,}$ supplemented with 1.0 mg/l BAP and 0.5 mg/l Kn. It was followed by treatment T_6 (MS basal + 1.0 mg/l BAP + 0.2 mg/l 2, 4-D) which resulted in 6.30 microshoots per explant and length of microshoot was found to be 3.80 cm. With the treatments T_{10} (MS basal + 1.5 mg/l BAP + 0.5 mg/l Kn), T_7 (MS basal + 1.5 mg/l + 0.2 mg/l 2, 4-D), T_5 (MS basal + 0.5 mg/l BAP + 0.2 mg/l 2, 4-D) and T_8 (MS basal + 0.5 mg/l + 0.5 mg/l Kn) average number of microshoots per explant were found to be 5.93, 5.86, 5.66 and 5.53 respectively and microshoot length obtained with these treatments were 2.84 cm, 2.78 cm, 2.60 cm and 2.24 cm respectively. However, average minimum number of 3.33 microshoots per explant were obtained with treatment T_2 (0.5 mg/l BAP) with microshoot length of 1.12 cm (Table 2). Therefore, treatment T_9 was found to be the best treatment for multiplication of microshoots but for elongation of microshoots, treatment T_6 yielded best results.

Rooting of microshoots

For *in vitro* root induction microshoots were transferred to MS basal medium supplemented with different concentrations of IAA and IBA. A total of five treatments were given and experiment was carried out to study the effect of different treatments on per cent *in vitro* rooting of microshoots, average root length and average number of roots per shoot (Table 3).

The rooting was initiated after 5 days of incubation and full rooting was obtained after a period of 30 days. Maximum rooting per cent of 93.33 was obtained in treatment T_6 comprising of 0.4 mg/l IAA. With this treatment maximum average number of roots per shoot was found to be 6.00 and their root length was 1.64 cm. This was followed by 86.66 per cent rooting, average root number of 5.66 and mean root length of 1.53 cm with treatment T_3 comprising of MS medium supplemented with 0.4 mg/l IBA. With the treatment T_7, T_5 and T_4 per cent rooting was found to be 80.00, 60.00 and 73.33 respectively and number of roots per shoot observed was 5.33, 5.00 and 5.13 respectively whereas mean root length with these treatments were 1.33 cm, 1.20 cm and 1.26 cm respectively. However, minimum rooting was observed in treatment T_2 where per cent rooting was found to be 66.66, number of roots per shoot were 4.66 and mean root length observed was 1.13 cm respectively.

Therefore, *in vitro* rooting in microshoots of *Bacopa monnieri* (L.) Pennell was observed on MS medium supplemented with 4.0 mg/l IAA. The fully developed plantlets were obtained after one month.

Hardening of *in vitro* raised plantlets of *Bacopa monnieri* (L.) Pennell

Fully developed *in vitro* raised plantlets obtained after the previous stage of *in vitro* rooting needed to be hardened and acclimatized. Hardening of the plantlets was carried out to acclimatize *in vitro* raised plantlets to *in vivo* conditions. After four weeks of incubation on rooting medium, plantlets of size about 3 cm with well developed roots were removed from the culture medium very carefully and the roots were washed gently under running tap water to remove the sticking medium. The plantlets were then dipped in 0.2% bavistin for 1-5 min followed by transferring to the plastic cups containing different hardening mixtures. Different hardening mixtures investigated consisted of cocopeat, sand and soil and their combinations. This experiment was carried out to study the suitability of these hardening mixtures on the per cent survival of the regenerated plantlets and results obtained have been presented in Table 4. Perusal of the data presented in the Table 4 shows a comparison of survival of *in vitro* raised plantlets in the sterilized substrates after six weeks of transfer from *in vitro* conditions to plastic cups kept in the culture room for 10-15 days.

It has been observed that per cent survival of the plantlets in the cocopeat is much higher as compared to the survival percentage of plantlets in other substrates. Total of 80 per cent plantlets survived after 6 weeks when hardened in cocopeat. Sand and soil resulted in 68 per cent and 42 per cent survival of the plantlets after 6 weeks of their transfer. When sand and soil were used as a hardened mixture in ratio 1:1, per cent survival observed was 45 whereas 48 per cent of plantlets survived in a mixture of cocopeat and sand in ratio 1:1, after 6 weeks of transfer to this mixture.

Therefore, cocopeat was found to be most suitable hardening mixture for the acclimatization of *in vitro* raised plantlets and was selected as the hardening mixture for the further acclimatization of plantlets. Hardened plantlets were transferred to small pots containing the sterilized mixture of soil: sand: FYM (1:1:1) and these were covered with glass jars and were transferred to glass house.

Acclimatization of *in vitro* raised hardened plantlets

The hardened plantlets which were transferred to small pots containing soil: sand: FYM in 1:1:1 ratio was kept covered with glass jars for 1 week and then the jars were removed and the pots were kept in the glass house. The growth rate and survival of the plantlets were observed for 2, 4, 6 and 8 weeks and the observations have been presented in Table 5.

Perusal of data presented in table 11 reveals that per cent survival of *in vitro* raised hardened plantlets after 2nd week of transplantation was found to be 91.67% with plant height of 2.96 cm and average number of leaves per plant were 19.50. After 4th week the survival per cent decreased and reached to 83.33% but plant height was increased to 4.22 cm and number of leaves per plant also increased to 25.50. After 6th week per cent survival remained to 75% but plant height increased considerably and reached to 5.18 cm. Number of leaves has also increased to 31.00. Per cent survival decreased with passage of time and remained only 66.67% after 8th week of transfer to pots, with mean plant height of 6.08 cm. However, there is a remarkable increase in average number of leaves per plant. After 8th week average numbers of leaves per plant observed were 39.00.

Discussion

The first step towards *in vitro* establishment of cultures is an appropriate choice of explant. The available literature has shown that in case of medicinal plants, nodal and internodal segments have been successfully used as explants (Razdan, 1993) [16]. It has been revealed that micropropagation of *Bacopa monnieri* could be carried out using axillary buds and internodal segments for producing true to type plants.

After excision of the explants, the most critical factor for successful establishment of cultures is the surface sterilization of the explants, since contaminants usually reduce the establishment rate of *in vitro* cultures. Excised shoot buds and leaves of *Bacopa monnieri* were surface sterilized using different sterilization treatments of a fungicide bavistin followed by $HgCl_2$. The maximum percent of uncontaminated cultures was achieved by using 0.2% bavistin for 15 min followed by 0.1% $HgCl_2$ for 2 min and finally washing with autoclaved distilled water 4-5 times. This method of sterilization has been found to be working satisfactorily provided enough care taken to remove residues of sterilants by proper washing with sterile water that otherwise have inhibitory effect on further growth of the explants whereas Sharma *et al*. (2010) [20] reported use of 1-2% cetavelon detergent solution for 10 min followed by 5 min treatment with 0.1% $HgCl_2$. Explants were surface sterilized with 50% ethanol followed by a 3 min treatment with 0.01% was reported by (Binita *et al*., 2005) [3]. Some authors have reported 0.1% (w/v) $HgCl_2$ along with other surface sterilants for sterilization of explants of various medicinal plant species such as *Gloriosa superb* (Hassan and Roy, 2005) [12], *Plumbago zeylanica* (Chinnamadasamy *et al*., 2010) [6], *Phyllanthus amarus* (Ghanti *et al*., 2004) [7], *Clitoria ternatea* (Rout, 2004) [17], *Nyctanthes arbortristis* (Rout *et al*., 2007) [18], *Ricinus communis* (Alam *et al*., 2010) [1], *Portulaca grandiflora* (Jain and Bashir, 2010) [13], *Solanum nigrum* (Sundari *et al*.,2010) [24] and *Boesenbergia rotunda* (Yusuf *et al*., 2011) [27]. Surface sterilization with $HgCl_2$ in case of *Bacopa monnieri* explants have also been reported by Sharma *et al*., 2010 [20].

In the present investigation, a protocol for establishment of axillary shoot buds and *in vitro* shoot multiplication has been developed using MS basal medium supplemented with different concentrations of growth regulators - BAP, Kn and 2, 4- D. Treatment T_{11} comprising of 0.50 mg/l BAP and Kn was found to be the best performing medium for establishment of axillary shoot buds giving a maximum value of 87.50 per cent of proliferated buds after seven days of culturing (Table 1). Earlier studies have shown that BAP alone could be used for *in vitro* shoot proliferation e.g. Sharma *et al*., 2010 [20] reported the use of BAP alone for the axillary bud break and shoot proliferation, they found out that 0.2 mg/l concentration of BAP results in 13-15 folds shoot proliferation. These results are in line with those workers, indicating the efficiency of BAP for shoot culture initiation and multiplication in *Bacopa monnieri*, reported by (Tiwari *et al*., 2000; Srivastava, 1999) [26, 23].

Development of rootlets leads to full plantlets and *in vitro* root induction is carried using growth regulators such as auxins which include IBA, IAA and NAA. In the present study 0.4 mg/l IAA produced maximum root formation of 93.33 per cent, whereas Sharma *et al*. (2010) [20] reported use of IBA in MS medium for best rooting. Different authors reported usage of IAA either alone or in combination with IBA for successful rooting in several medicinal plants such as *Plumbago zeylanica* (Chinnamadasamy *et al*., 2010) [6], *Nyctanthes arbortristis* (Rout *et al*., 2007) [18], *Bauhinia cheilantha* (Gutierrez *et al*., 2011) [10] and *Solanum nigrum* (Sundari *et al*., 2010) [24].

Most of the mortality of micropropagated plantlets occurs at the acclimatization stage. A substantial number of micropropagated plantlets do not survive the transfer from *in vitro* conditions to green house or field environment. Therefore, transferring of *in vitro* regenerated plantlets to soil remains a crucial step in micropropagation (Grout, 1975; Sutter and Langhans, 1982; Short *et al*., 1987) [9, 25, 22] due to reduced amounts of epicuticular wax and reduced vascular tissue developed. But such features pose no problem in *in vitro* conditions when microplants are surrounded by high humidity. In the present study the plantlets with well developed shoots and roots were able to survive when hardening was carried out in pots containing autoclaved mixture of soil: sand: FYM in the ratio of 1:1:1 with decrease in survival from 91.67% to 66.67% within a time period of 2-8 weeks. High humidity helped in enhancing the survival of plants by covering them with glass jars which were uncovered after about 10 days of transfer to soil and acclimatization of plantlets on the similar mixture has been reported by Hamirah *et al*. (2010) [11] in *Zingiber montanum.* However, in another report given by Chan *et al*. (2009) [4], the *in vitro* regenerated plantlets of *Gynura procumbens* were acclimatized in organic soil: sand mixture (1:1).

Thus in the present study a protocol has been developed successfully for micropropagation of *Bacopa monnieri* (L.) Pennell genotype of Khaltoo village Nauni, which can be further used for mass production of this genotype of this threatened plant species.

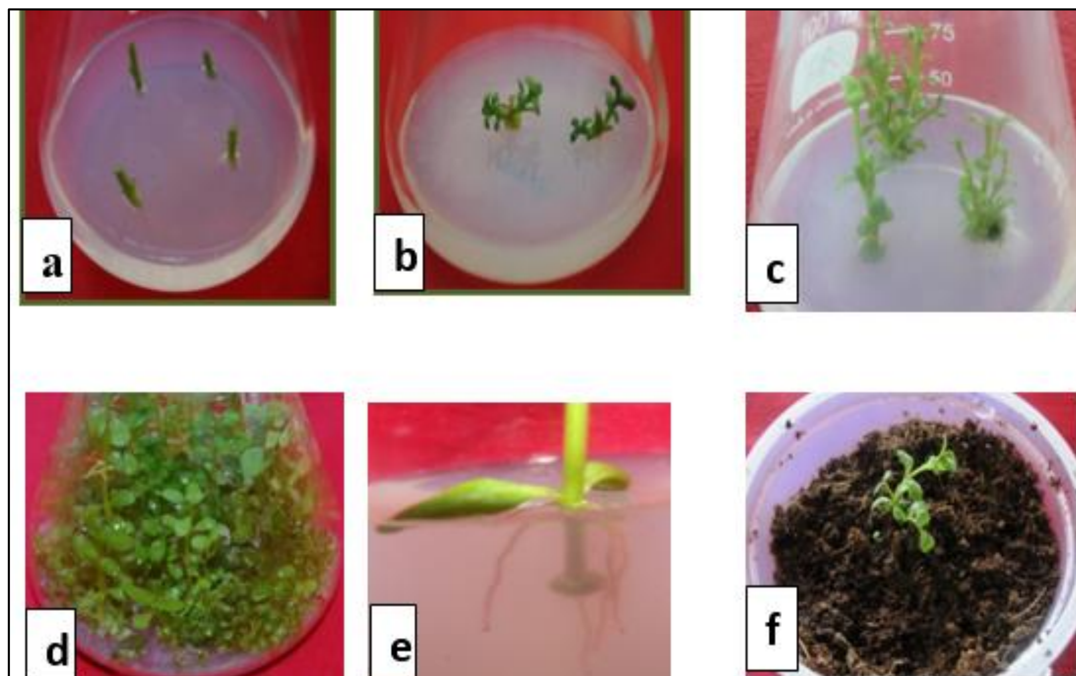

Fig 1: A&B. *In vitro* establishment of cultures from shoot buds of *Bacopa monnieri* (Linn.) Pennell. C&D. *In vitro* multiplied shoots of *Bacopa monnieri* (Linn.) Pennell E. Rooting of *in vitro* raised shoots F. Hardening of *Bacopa monnieri* plantlets in mixture of Soil: FYM: Sand

Table 1: Effect of various concentrations and combinations of growth regulators on the establishment of shoot buds of *Bacopa monnieri* (L.) Pennell after four weeks of culturing.

Treatment (T)	Plant growth regulators			Percentage of buds proliferated
	BAP (mg/l)	NAA (mg/l)	Kn (mg/l)	
T_1	-	-	-	0.000(0.000)*
T_2	0.25	-	-	33.33 (35.20)
T_3	0.50	-	-	41.66 (40.20)
T_4	0.75	-	-	45.83 (42.61)
T_5	1.00	-	-	54.16 (47.39)
T_6	0.25	0.20	-	50.00 (45.00)
T_7	0.50	0.20	-	58.33 (49.80)
T_8	0.75	0.20	-	62.50 (52.24)
T_9	1.00	0.20	-	83.33 (65.90)
T_{10}	0.25	-	0.50	70.83 (57.32)
T_{11}	0.50	-	0.50	87.50 (69.30)
T_{12}	0.75	-	0.50	79.16 (62.84)
T_{13}	1.00	-	0.50	66.66 (54.73)
	$CD_{0.05}$			0.79114
	SE±			0.38488

*Values expressed in parentheses are the arc sine transformation of percentage.

Table 2: Effect of various concentrations and combinations of growth regulators on the multiplication of microshoots of *Bacopa monnieri* (L.) Pennell after four weeks of culturing.

Treatment (T)	Plant growth regulators			Average no. of microshoots per explant	Microshoot length (cm)
	BAP (mg/l)	2,4-D (mg/l)	Kn (mg/l)		
1	-	-	-	0.00	0.00
2	0.50	-	-	3.33	1.06
3	1.00	-	-	4.13	1.11
4	1.50	-	-	4.66	1.12
5	0.50	0.20	-	5.66	2.60
6	1.00	0.20	-	6.30	3.80
7	1.50	0.20	-	5.86	2.78
8	0.50	-	0.50	5.53	2.24
9	1.00	-	0.50	7.00	3.00
10	1.50	-	0.50	5.93	2.84
	$CD_{0.05}$			0.16282	0.15867
	S.E±			0.078056	0.07552

Table 3: Effect of different concentrations of IBA and IAA on *in vitro* root induction in microshoots of *Bacopa monnieri* (L.) Pennell.

Treatment	Phytohormone		Percent rooting	No. of roots per shoot	Root length (cm)
	IBA	IAA			
1	-	-	0.00(0.00)*	0.000	0.000
2	0.2	-	66.66(54.74)	4.660	1.130
3	0.4	-	86.66(68.60)	5.660	1.530
4	0.6	-	73.33(58.91)	5.130	1.260
5	-	0.2	60.00(50.77)	5.000	1.200
6	-	0.4	93.33(75.07)	6.000	1.640
7	-	0.6	80.00(63.44)	5.330	1.330
$CD_{0.05}$			1.4654	0.62625	0.17171
SE±			0.68323	0.29199	0.080060

*Values expressed in parentheses are arc sine transformation of percentage

Table 4: Effect of hardening mixtures on acclimatization of *in vitro* raised plantlets of *Bacopa monnieri* (Linn.) Pennell after six weeks of incubation.

Substrate	Per cent survival
Cocopeat	80
Sand	68
Soil	42
Sand + Soil (1:1)	45
Cocopeat + sand (1:1)	48
$CD_{0.05}$	
SE ±	

*Values expressed in parentheses are arc sine transformation of percentage

Table 5: Effect of hardening at weekly interval on the growth and development of *Bacopa monnieri* (Linn.) Pennell

Treatments (weekly)	Per cent survival	Plant height (cm)	No. of leaves per plant
2th	91.67 (73.26)*	2.96	19.50
4th	83.33 (65.91)	4.22	25.50
6th	75.00 (60.00)	5.18	31.00
8th	66.67 (54.74)	6.08	39.00
$CD_{0.05}$	1.7186	1.8499	1.9789
SE ±	0.74526	0.80223	0.85816

*Values expressed in parentheses are arc sine transformation of percentage

Anova Tables

Table 1 A: Effect of various combinations of growth regulators on the establishment of shoot buds of *Bacopa monnieri* (Linn.) Pennell.

Source of variation	Degree of freedom	SS	MSS	F
		Per cent survival		
Treatment	12	11140.0	928.30	4177.71
Error	26	5.7773	0.2222	
Total	38	11145.0		

Table 2 B: Effect of various combinations of growth regulators on multiplication of microshoots of *Bacopa monnieri* (Linn.) Pennell.

Source of variation	Degree of freedom	SS	MSS	F
		Average number of shoots per explant		
Treatment	9	15.931	1.7701	193.69
Error	20	0.18278	0.0091391	
Total	29	16.114		

Table 3 C: Effect of various combinations of growth regulators on multiplication of microshoots of *Bacopa monnieri* (Linn.) Pennell.

Source of variation	Degree of freedom	SS	MSS	F
		Shoot length		
Treatment	9	37.174	4.1305	11.04
Error	20	7.4804	0.37402	
Total	29	44.655		

Table 4 D: Effect of different concentrations of IAA and IBA on *in vitro* root induction in microshoots of *Bacopa monnieri* (Linn.) Pennell.

Source of variation	Degree of freedom	SS	MSS	F
		Per cent rooting		
Treatment	6	11074	1845.7	2635.93
Error	14	9.8028	0.70020	
Total	20	11084		

Table 5 E: Effect of different concentrations of IAA and IBA on *in vitro* root induction in microshoots of *Bacopa monnieri* (Linn.) Pennell.

Source of variation	Degree of freedom	SS	MSS	F
		No. of roots per shoot		
Treatment	6	75.587	12.598	98.51
Error	14	1.7904	0.12789	
Total	20	77.378		

Table 6 F: Effect of hardening at weekly interval on growth and development of *Bacopa monnieri* (Linn.) Pennell.

Source of variation	Degree of freedom	SS	MSS	F
		Per cent survival		
Treatment	3	569.78	189.93	227.97
Error	8	6.6650	0.83312	
Total	11	576.44		

Table 7 G: Effect of hardening at weekly interval on growth and development of *Bacopa monnieri* (Linn.) Pennell.

Source of variation	Degree of freedom	SS	MSS	F
		Plant height (cm)		
Treatment	3	16.081	5.3604	5.55
Error	8	7.7228	0.9653	
Total	11	23.804		

Table 8 E: Effect of hardening at weekly interval on growth and development of *Bacopa monnieri* (Linn.) Pennell.

Source of variation	Degree of freedom	SS	MSS	F
		No. of leaves per plant		
Treatment	3	618.75	206.25	186.71
Error	8	8.8372	1.1046	
Total	11	627.59		

References

1. Alam Iftekhar, Sharmin Shamima Akhtar, Mondal Sanjoy Chandra, Alam Md. Jahangir, Khalekuzzaman Muhammad, Anisuzzaman M *et al*. *In vitro* micropropagation through cotyledonary node culture of castor bean (*Ricinus communis* L.). Australian Journal of Crop Science. 2010; 4:81-84
2. Anonymous. Wealth of India. Council of Scientific and Industrial Research, New Delhi. 1996, 143
3. Binita B Chaplot, Ashok M Dave, Yogesh T Jasrai. *Bacopa monnieri* (L.) Pennell: A Rapid, Efficient and Cost Effective Micropropagation. Plant Tissue Culture and Biotechnology. 2005; 15(2):167-175
4. Chan Lai Keng, Lim Su Yee. Micropropagation of *Gynura procumbens* (Lour.) Merr. an important medicinal plant. Journal of medicinal plant research. 2009; **3**(3):105-111
5. Chauhan NS. Medicinal and aromatic plants of Himachal Pradesh. Indus Publishing Company, New Delhi. 1999, 234-237
6. Chinnamadasamy Kalidass, Arjunan Daniel, Ramasamy Mohan Veerabahu. Rapid Micropropagation of *Plumbago zeylanica* L. – an important medicinal plant. Journal of American Science. 2010; 6(10):1027-1031
7. Ghanti Kiran S, Govindaraju B, Venugopal RB, Rao S Ramgopal, Kaviraj CP, Jabeen FTZ *et al*. High frequency shoot regeneration from *Phyllanthus amarus* Schum. & Thonn. Indian Journal of Biotechnology. 2004; 3:103-107
8. Gomez KA, Gomez AA. Statistical procedures for agricultural research (2 ed.). John wiley and sons, New York, 1984, 680.
9. Grout BW. Wax development on leaf surface of *Brassica oleracea* var. Curwang regenerated from meristem culture. Plant Science Letters. 1975; 5:401-405
10. Gutierrez IEM, Nepomuceno CF, Ledo CAS, Santana JRF. Micropropagation and acclimatization of *Bauhinia cheilantha* (an important medicinal plant). African Journal of Biotechnology. 2011; 10(8):1353-1358
11. Hamirah MN, Sani HB, Boyce PC, Sim SL. Micropropagation of red ginger (*Zingiber montanum* Koenig), a medicinal plant. AsPac Journal of Molecular Biology and Biotechnology. 2010; 18(1):127-130
12. Hassan AKM, Sayeed, Roy Shyamal K Roy. Micropropagation of *Gloriosa superb* L. through high frequency shoot proliferation. Plant Tissue Culture. 2005; **15**(1):67-74
13. Jain Ashok K, Bashir Mudasir. *In vitro* propagation of a medicinal plant *Portulaca grandiflora*. Hook. World Journal of Agricultural Sciences. 2010; 6(3):327-330
14. Murashige T, Skoog F. A revised medium for rapid growth of bioassay with tobacco tissue cultures. Physiologia Plantarum. 1962; 15:473-497
15. Prabhuji SK, Rao GP, Patil SK. Recent advances in medicinal plants research. Satish Serial Publication, New Delhi. 2005, 16-20

16. Razdan MK. An introduction to plant tissue culture. Andover, Hampshire: Intercept, 1993.
17. Rout Gyana Ranjan. Effect of cytokinins and auxins on micropropagation of *Clitoria ternatea* L. Biological Letters. 2004; 41(1):21-26
18. Rout GR, Mahato A, Senapati SK. *In vitro* clonal propagation of *Nyctanthes arbortristis* Linn. -a medicinal tree. Horticultural Science. 2007; 34(2):84-89
19. Sharma R. Medicinal plants of India. Daya Publishing House, New Delhi. 2003, 30-31
20. Sharma Sudhir, Kamal Barkha, Rathi Neelima, Chauhan Sudhir, Jadon Vikas, Vats Neha *et al*. *In vitro* rapid and mass multiplication of highly valuable medicinal plant *Bacopa monnieri* (L.) Wettst. African Journal of Biotechnology. 2010; 9(49):8318-8322
21. Shakoor A, Akram M, Asharaf CM, Siddiqui MR. Pharmagonistic study and chemical/pharmacological evaluation of Brahmi-buti. Hamdard Medicus. 1994; 37:92-109
22. Short KC, Warburton J, Roberts AV. *In vitro* hardening of cultured cauliflower and chrysanthemum plantlets to humidity. Acta Horticulturae. 1987; 212:309-344
23. Shrivastava N, Rajani M. Multiple shoot regeneration and tissue culture studies on *Bacopa monnieri* (L.) Pennell. Plant Cell Reports. 1999; 18:919-923
24. Sundari MS, Benniamin A, Manickam VS. Micropropagation and *in vitro* flowering in *Solanum nigrum* Linn. A medicinal plant. International Journal of Biological Technology. 2010; 1(1):29-32
25. Sutter E, Langhans RW. Formation of epicuticular wax and its effect on water loss in cabbage plants regenerated from shoot tip culture. Canadian Journal of Botany. 1982; 62:2896-2902
26. Tiwari V, Tiwari KN, Singh BD. Suitability of Liquid cultures for *in vitro* multiplication of *Bacopa monniera* Linn. Wettst. Phytomorphology 2000; 50(3, 4):337-34
27. Yusuf Nor Azma, Annuar MM Suffian, Khalid Norzulaani. Rapid micropropagation of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. (a valuable medicinal plant) from shoot bud explants. African Journal of Biotechnology. 2011; 10(7):1194-1199.