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An efficient micropropagation protocol of *Bacopa monnieri* (L.) Pennell through two-stage culture of nodal segments and *ex vitro* acclimatization

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ABSTRACT

An efficient plant regeneration protocol through two-stage culture of nodal segment has been reported here for a valuable medicinal plant, *Bacopa monnieri*. Multiple shoots with large number of shoot buds were induced from nodal explant on Murashige and Skoog's (1962) (MS) medium fortified with (1.0-5.0 mg L^{-1}) N^6- benzyladenine (BA). Of the different concentrations of BA tested, 3.0 mg L^{-1} BA offered the best result, where the nodes swelled and an average of 6.5 shoots with numerous buds/node were recorded. The swollen nodes, cultured on MS + 3.0 mg L^{-1} BA, were sub-cultured either on MS or MS supplemented with 1.0 mg L^{-1} GA_3 medium. The best result (114.2 shoots/ node) with an average shoot length of 6.4 cm was observed on MS media supplemented with 1.0 mg L^{-1} GA_3. Cent per cent acclimatization of healthy *in vitro* regenerated shoots was obtained by *ex vitro* rooting in plastic pots containing garden soil, which saves time and tissue culture cost by abolishing the *in vitro* rooting step.

1. INTRODUCTION

Bacopa monnieri (L.) Penell (Family: Scrophulariaceae), commonly known as Brahmi and Water Hyssop, is a valued herb since ancient times for its medicinal uses. The plant has been mentioned in different traditional systems of medicine including ayurveda, siddha, unani and homeopathy due to its therapeutic properties. It has been used as medhya-rasayana in ayurvedic system of medicine to enhance memory, concentration, and learning as well as to cure mental illness [1-3]. Studies showed that saponins such as bacosides (active triterpenoids) A, B, C and D are the most important components of *B. monnieri* and also known as 'memory chemicals' due to their memory enhancement properties [4-5]. Besides, the plant has anti-inflammatory, analgesic, antipyretic, anticancerous, anticonvulsive and antioxidant properties [3, 6-9]. It has been used in treatment of insomnia, asthama, hoarseness, snake bite, rheumatism, leprosy, eczema, water retention, blood cleaning and insanity [5, 10]. *B. monnieri* also has the ability to phytoremediate toxic heavy metals (e.g. cadmium, chromium and mercury) from aquatic bodies by absorbing and accumulating these metals in their shoots and roots [11-12].

On the basis of medicinal uses, commercial value and potential for further research and development, *B. monnieri* is designated as the second most important medicinal plant of India [1]. The annual demand of *B. monnieri* during the year 2004-2005 was 6621.8 tonnes with an annual growth rate of 7% [13]. The requirement of raw materials of *B. monnieri* is expected to increase further, which may be due to the popularity of using *Bacopa* based "memory enhancing" drugs. Most importantly, *B. monnieri* has been enlisted among 178 species of medicinal plants of India with high trade requirement (≥100 million tonnes/year). Unfortunately, the requirement of this plant material is fulfilled mainly by collection from wastelands [14]. Thus, development of methods for ensuring the availability of raw material of a consistent quality from regular and viable sources is imperative [1]. The seeds of *B. monnieri* have short viability of two months and thus, are poor propagules. Besides, two-leaved stage is the crucial period for this plant as frequent seedling death has been observed during this time. Thus, raising plants from seeds is a difficult task [15-16]. Vegetative propagation by stem is a slow process [15, 17]. Besides, it was observed that the growth of *B. monnieri* is dependent on seasonal changes [18]. Thus, conventional method of propagation is inadequate to meet the demand of raw material of *B. monnieri*. The unsustainable collection of raw material from natural population has already placed the plant under threatened category [19-20]. Thus, for constant supply of raw materials and reduction of pressure on natural/wild population, development of an efficient and reliable *in vitro* plant regeneration protocol for this wonder medicinal herb is essential. A number of reports on *in vitro* plant regeneration using different explants including nodal segments of *B. monnieri* are available. However, there is still scope for further

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improvement in *in vitro* plant regeneration protocols of *B. monnieri*. Therefore, the objective of this study was to develop an improved *in vitro* plant regeneration protocol for *B. monnieri* using nodal segments of field grown plants.

2. MATERIALS AND METHODS

2.1 Axillary shoot proliferation

The plants of *B. monnieri* maintained in the garden of Department of Botany, Ravenshaw University, Cuttack, Odisha, India were used as the explant source for axillary shoot proliferation experiment. The elite and healthy shoots were collected from these plants. After trimming the leaves and internode, the nodal segments (1.0-1.5 cm) were kept under running tap water for 25 mins, washed with 5% (v/v) liquid detergent (Teepol, Rickitt Benckiser Ltd., India) for 15 mins followed by rinsing with double distilled water for 3-5 times. Surface sterilization of explants was carried out by 0.1 % (w/v) aqueous solution of mercuric chloride ($HgCl_2$; Hi-Media, India) for 6 mins followed by washing with autoclaved double distilled water for 3-5 times inside the laminar air flow cabinet (Satyam, India). Surface sterilized nodal explants were cultured on either Murashige and Skoog's (1962) [21] (MS) medium alone or MS medium supplemented with different concentrations (1.0- 5.0 mg L^{-1}) of N^6- benzyladenine (BA) for shoot/shoot bud induction. After four weeks of culture, the swollen nodal explants with elongated shoots and large number of shoot buds cultured on optimum shoot induction medium, MS + 3.0 mg L^{-1}BA, were sub-cultured either by cutting them transversely or as such (without cutting) on either growth regulator free MS medium or MS supplemented with 1.0 mg L^{-1} GA_3 with the aim of further shoot proliferation and elongation.

All the media used for axillary shoot proliferation experiments were augmented with 3 % sucrose and 0.7% agar (Hi-Media, India). The pH of the media was adjusted to 5.8±0.1 before autoclaving at 121°C for 17 min. The cultures were kept inside the culture room at 25±1°C, with a 16 h photoperiod of 50 µ mol m^{-2} s^{-1} photon flux density provided with cool white fluorescent tubes (Phillips, India).

2.2 Rooting of *in vitro* regenerated shoots and acclimatization

The healthy *in vitro* regenerated shoots (3.5-5.0 cm) were excised and taken for both *in vitro* and *ex vitro* rooting. For *in vitro* rooting, the harvested shoots were inoculated on either half strength of MS or half strength of MS medium supplemented with different concentration (0.1-1.0 mg L^{-1}) of indole-3-butyric acid (IBA). *In vitro* rooted plantlets were taken out from the culture tube and agars were removed carefully from the roots under running tap water. The plantlets were then directly transferred to the clay pots (20 × 25 cm) containing garden soil. The pots were kept outdoor under shade for two weeks prior to transfer to outdoor. *Ex vitro* rooting was carried out by either planting the excised *in vitro* shoots (3.5-5.0 cm) (1) directly in the plastic pots (8.0 × 6.5 cm) containing garden soil or (2) after treating the shoots with different concentrations (1.0-5.0 mg L^{-1}) of IBA for 1 h. IBA was prepared in tap water. All the plastic pots (8.0 × 6.5cm) were kept in shade for two weeks. After two weeks of inoculation, total number of plants that survived, total number of roots per shoot and average root length were recorded.

2.3 Data recording and analysis

Experiments for shoot bud induction, shoot proliferation and rooting of *in vitro* regenerated shoots were repeated thrice. Five flasks were used having 2 explants in each flask for shoot bud induction. One explant per flask, totalling five flasks, was taken for shoot proliferation and elongation experiments. *In vitro* rooting experiments were carried out in culture tube having one explant per tube.

For *ex vitro* rooting 10 shoots were planted in each plastic pot. Both the rooting experiments had five replicas. The data including average number of shoots/ explant, average number of roots/shoot, average length of shoot and root were recorded every week. All data were subjected to Analysis of Variance (ANOVA) for a completely randomized design (CRD). The differences among the treatment means were tested by Duncan's New Multiple Range Test (DMRT) [22].

3. RESULTS

3.1 Influence of BA on shoot bud induction from nodal segments

It was observed that addition of a cytokinin e.g. BA to MS medium was not essential for bud break and shoot proliferation from nodal explants. MS medium devoid of any growth regulator exhibited 100 % bud break in 4-6 days and an average of 2.7 shoots per explant were regenerated in 4 weeks. Further, on MS medium supplemented with different concentration of BA, bud break was achieved in cent percent explants after 4 days of inoculation and it was found that only two shoots were elongated irrespective of medium tested. In this study, an interesting observation was made in all the BA supplemented media. It was found that gradually the base of the nodal region swells and numerous shoot buds were initiated (both above and below the medium), of which only few shoot buds (above the medium) were elongated in the same medium (Table 1; Fig. 1A). Amongst the different concentration of BA tested, 3.0 mg L^{-1} BA (shoot induction medium) offered the best result. An average of 6.5 shoots/explant with average shoot length of 3.69 cm was recorded in this optimal medium (Fig. 1B).

Table 1: Influence of BA on shoot bud induction from nodal explants of *B. monnieri* *

BA (mg L^{-1})	% response	Shoots/ explant	Shoot length (cm)
-	100	2.70^{f}	2.00^{d}
1.0	100	3.67^{d}	2.10^{c}
2.0	100	4.80^{b}	2.54^{b}
3.0	100	6.50^{a}	3.69^{a}
4.0	100	4.50^{c}	1.89^{e}
5.0	100	2.90^{e}	1.78^{f}

In a column, different letters in superscripts indicate statistically significant difference between the means (P≤0.05; Duncan's new multiple range test).
** Data collected at 4 weeks of culture.*

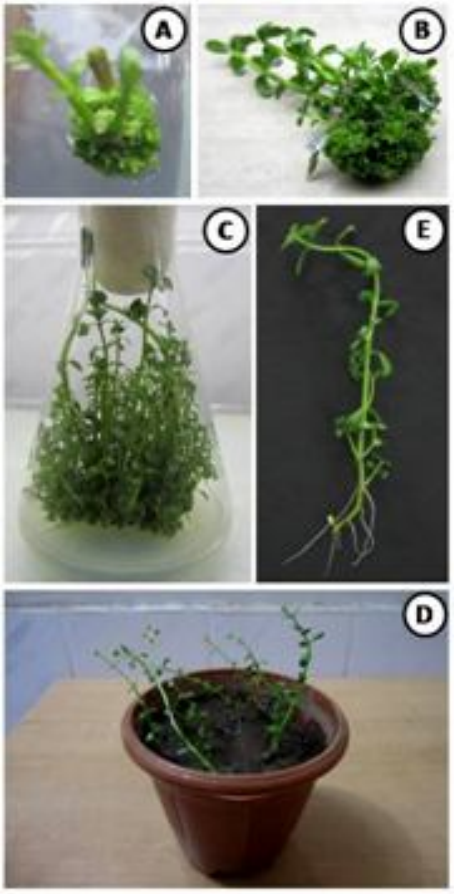

Fig. 1 (A) Initiation of shoots and shoot buts from nodal region in MS+3.0 mg L^{-1} BA after 14 days of culture, (B) Development of multiple shoots buds and shoots from nodal segment at 4 weeks of culture, (C) Proliferation of multiple shoots from intact nodal segment in MS+1.0 mg L^{-1} GA_3 following transfer from MS+3.0 mg L^{-1} BA at 3 weeks of culture, (D) Acclimatized plants of *B. monnieri* in pots through *ex vitro* rooting, (E) *Ex vitro* rooted shoot after 2 weeks.

Concentration of BA lower and higher than optimum level had inhibitory effect on shoot proliferation and the number of shoots per explant declined significantly. Interestingly, concentration of BA had no influence on frequency of shoot regeneration (Table 1). Development of a few roots from the base of the culture was a common phenomenon in all the media.

3.2 Evaluation of media for proliferation and elongation of shoot buds induced from nodal explants

A number of shoot buds were initiated on BA supplemented media; excepting a few, most of them failed to elongate in the same medium (Fig. 1B). The enlarged nodal region with induced shoot buds was transversely cut and sub-cultured either on growth regulator free MS medium or MS supplemented with 1.0 mg L^{-1} GA_3 for elongation of shoot buds. An average of 102 shoots /explant with an average length of 6.7 cm was recorded after 3 weeks of sub-culture (Table 2), when the transversely cut nodal segments were transferred from the shoot induction medium to MS medium augmented with 1.0 mg L^{-1} GA_3. However, the best response was observed when the uncut nodal segments were sub-cultured on MS medium augmented with 1.0 mg L^{-1} GA_3 (shoot proliferation medium), where 114.2 shoots/node with an average length of 6.4 cm were recorded (Table 2; Fig.1C).

Table 2: Evaluation of media for shoot proliferation and elongation from shoot bud induced nodal explants of *B. monnieri* *

Shoot induction media BA (mg L^{-1})	Shoot proliferation media	Shoots/ explant	Shoot length (cm)
1.0	MS	42.93^{i}	2.90^{ij}
	MS + GA_3**	68.06^{defg}	3.90^{fg}
2.0	MS	73.13^{def}	3.10^{i}
	MS + GA_3**	80.33^{cd}	5.20^{c}
3.0	MS	$78.20^{cde}(87.80)^{c}$	$4.15^{e}(3.80)^{fgh}$
	MS + GA_3**	$102.00^{ab}(114.20)^{a}$	$6.70^{a}(6.40)^{b}$
4.0	MS	33.06^{ij}	2.60^{k}
	MS + GA_3**	59.00^{gh}	4.50^{d}
5.0	MS	24.00^{jkl}	2.10^{l}
	MS + GA_3**	30.26^{jk}	4.00^{ef}

*In a column, different letters in superscripts indicate statistically significant difference between the means (P≤0.05; Duncan's new multiple range test). Data in the bracket are for intact nodes. * Data collected at 3 weeks of culture ** 1.0 mgL^{-1}*

3.3 Rooting of *in vitro* regenerated shoots and acclimatization of plantlets

The healthy *in vitro* regenerated shoots (3.5-5.0cm) were rooted on growth regulator free ½ MS or ½ MS medium supplemented with different concentration of IBA (0.1-1.0 mg L^{-1}). Cent per cent rooting was observed within 4 days of shoot inoculation in all the media tested. In half-MS medium 6.73 roots per shoot with an average root length of 4.60 cm was observed. On the other hand, maximum rooting was noted in ½ MS + 0.2 mg L^{-1} IBA, where an average of 10.2 roots with an average root length of 4.2 cm were recorded (Table 3). Callus formation at the basal end of the shoot followed by root initiation was observed in ½ MS augmented with higher concentration of IBA i.e.> 0.2 mg L^{-1}. The numbers of roots as well as length also significantly decreased in these media. Irrespective of the rooting media all the regenerated plantlets were successfully acclimatized. In the present study, cent per cent acclimatization through *ex vitro* rooting of shoots was easily achieved in *B. monnieri* without any auxin treatment (Fig. 1D, E). Further, no significant difference was found in the percentage of *ex vitro* rooting and acclimatization between the IBA untreated shoots and IBA (different concentrations) treated shoots. However, highest number (4.86) as well as longest of roots (3.5cm) were observed in shoots pre-treated with 2.0 mg L^{-1} IBA (Table 4).

Table 3: *In vitro* rooting of tissue culture regenerated shoots of *B. monnieri**

IBA (mg L^{-1})	Rooting (%)	Roots/shoot	Root length (cm)
-	100	6.73^{g}	4.60^{a}
0.1	100	9.80^{ab}	3.50^{c}
0.2	100	10.20^{a}	4.20^{b}
0.3	100	8.73^{c}	2.90^{d}
0.4	100	8.46^{cd}	2.50^{e}
0.5	100	8.26^{cde}	2.33^{ef}
1.0	100	8.00^{def}	2.20^{fg}

*In a column, different letters in superscripts indicate statistically significant difference between the means (P≤0.05; Duncan's new multiple range test). *Data recorded at 2 weeks.*

Table 4: *Ex vitro* rooting of *in vitro* regenerated shoots with or without IBA treatment*/**

IBA (mg L^{-1})	Rooting & Acclimatization (%)	Roots/shoot	Root length (cm)
0.0	100	2.26^{e}	2.20^{bcd}
1.0	100	2.46^{d}	2.30^{bc}
2.0	100	4.86^{a}	3.50^{a}
3.0	100	3.00^{b}	2.40^{b}
5.0	100	2.80^{c}	2.20^{bcd}

In a column, different letters in superscripts indicate statistically significant difference between the means (P≤0.05; Duncan's new multiple range test).
**Data recorded at 2 weeks.*
** IBA treatment was done for one hour.

4. DISCUSSION

Nodal segment is usually the preferred explant for *in vitro* clonal propagation as the cells of axillary meristem in the nodal segment undergo continuous and rapid mitosis so that the resulting shoots are less vulnerable to genetic changes. Thus, in this study we have selected nodal segment as the explant. Addition of plant growth regulators to basal medium is an essential requirement for axillary shoot proliferation from nodal segments of a number of plant species. In this study, growth regulator free MS medium alone was able to regenerate shoots by axillary shoot proliferation. Similar observation was also reported by [13, 23-26] for axillary shoot proliferation in *B. monnieri*. At the same time, Kaur et al. (2013) [27] reported the requirement of addition of plant growth regulator for bud break in *B. monnieri*. In this study only a single cytokinin i.e., BA was used for shoot regeneration. BA has also been found to be superior over other cytokinins for shoot regeneration in a number of earlier reports in *B. monnieri* [13, 26, 27, 33, 36]. The stability of ribosides and nucleotides occurring naturally in BAP compared to other cytokinins [37] possibly gives BAP an edge which may be the cause of enhanced shoot multiplication response in a number of plant species including *B. monnieri*. Treatment of explants in two different media, one for efficient shoot induction and another for elongation (preferably supplemented with no or low concentration of plant growth regulators) is known as two-stage culture procedure [20]. This versatile and efficient culture procedure yielded 135 shoots in *B. monnieri* by leaf mediated adventitious shoot organogenesis as reported by Ceasar et al. (2010) [20]. This type of two-stage culture system was also found to be beneficial for plant regeneration by axillary shoot proliferation of nodal explants in *Cassia angustifolia* [28] and *Manihot esculenta* [29]. The success of Ceasar et al. (2010) [20] and lack of report on the use of nodal explants for two-stage culture system in *B. monnieri* prompted us to undertake this experiment. We achieved 102 shoots when the transversely cut proliferated nodal segment (with numerous shoot buds) cultured on shoot induction medium, were transferred to the shoot proliferation medium. However, a further increase in shoot number, i.e., 114.2 shoots /node were observed when intact nodes were sub-cultured on shoot proliferation medium, which is the highest shoot number reported till date for axillary shoot proliferation through nodal segment in *B. monnieri*. This difference in the number of regenerated shoots between cut and intact node may be due to loss of a few shoot buds while cutting the nodes transversely. In previous reports of *B. monnieri*, the number of regenerated shoots per nodal segment was reported to be 56 [10], 41 [13], 43 [15], 8 [23], 18 [24], 19 [25], 20 [27],33 [30], 19 [31], 75-80 [32], 5 [33], 17 [34], 43 [35], 17 [36] and 19 [38], which are much less than the present study. Our result was in agreement with other reports on *Calamus nagbettai* [39] and *Cajanus cajan cv*. *Manak* (H77216) [40], where addition of GA_3 to MS media was found to be beneficial for shoot elongation particularly for undersized shoots resulted due to high levels of cytokinin. This may be due to the reason that GA_3 promotes cell division and cell elongation [41].

In many plant species including *B. monnieri* [13, 15, 20, 38] there was a requirement of auxin in the culture medium for rooting of shoots followed by step wise acclimatization process for soil establishment of *in vitro* regenerated plants. Now-a-days *ex vitro* rooting, i.e., simultaneous rooting and acclimatization is becoming popular due to significant reduction of time and cost of micropropagation, which is mainly due to elimination of a step (*in vitro* rooting) of micropropagation [42, 43].

In a number of plant species the treatment of shoots with auxins was required prior to *ex vitro* rooting [44]. However, in this study we could achieve *ex vitro* rooting mediated plant acclimatization of *B. monnieri* in garden soil without any auxin treatment in shoots. Although Ceasar et al. (2010) [20] obtained more number of shoots than our present report, their protocol has taken longer duration, i.e., fourteen weeks for complete plant regeneration in comparison to ours (nine weeks). This time difference was mainly due to our successful one step *ex vitro* rooting cum acclimatization process, which has taken only two weeks in contrast to seven weeks as reported by Ceasar et al. (2010) [20]. Thus, the results obtained here suggest that a single step rooting and acclimatization process reduces the cost and time for *in vitro* plant regeneration and is a better protocol than the one developed by Ceasar et al. (2010) [20], which has been supposed to be the best plant regeneration protocol in *B. monnieri* so far.

5. CONCLUSION

Here we have successfully developed an efficient plant regeneration protocol for *B. monnieri* using two-stage culture of nodal segment and *ex vitro* rooting. The regenerated plants will be useful for constant supply of uniform raw materials for commercial secondary metabolite extraction. This will reduce the pressure on natural population of this valuable medicinal plant species and thus be indirectly useful for conservation of this plant species.

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7. REFERENCES

1. Shrivastava N, Rajani M. Multiple shoot regeneration and tissue culture studies on *Bacopa monnieri* (L.) Pennell. Plant Cell Rep. 1999; 18(11):919-923.
2. Majumdar S, Garai S, Jha S. Genetic transformation of *Bacopa monnieri* by wild type strains of *Agrobacterium rhizogenes* stimulates production of *Bacopa* saponins in transformed calli and plants. Plant Cell Rep. 2011; 30:941-954.
3. Ahire ML, Walunj PR, Kishor PBK, Nikam TD. Effect of sodium chloride-induced stress on growth, proline, glycine betaine accumulation, antioxidative defence and bacoside A content in *in vitro* regenerated shoots of *Bacopa monnieri* (L.) Pennell. Acta Physiol Plant. 2013; 35:1943-1953.
4. Rastogi S, Pal R, Kulshreshtha DK. Bacoside A3 - a triterpenoid saponin from *Bacopa monnieri.* Phytochemistry. 1994; 36(1):133-137.
5. Banerjee M, Shrivastava S. An improved protocol for *in vitro* multiplication of *Bacopa monnieri* (L). World J Microbiol Biotechnol. 2008; 24(8):1355-1359.
6. Elangovan V, Govindasamy S, Ramamoorthy N, Balasubramanian K. *In vitro* studies on the anticancer activity of *Bacopa monnieri*. Fitoterapia. 1995; 66(3):211-215.
7. Tripathi YB, Chaurasia S, Tripathi E, Upadhyay A, Dubey GP. *Bacopa monnieri* L. as an antioxidant mechanism of action. Indian J Exp Biol. 1996; 4(6):523-526.
8. Rahman LU, Verma PC, Singh D, Gupta MM, Banerjee S. Bacoside production by suspension cultures of *Bacopa monnieri* (L.) Pennell. Biotechnol Lett. 2002; 24: 1427-1429.
9. Kishore K, Singh M. Effect of bacosides, alcoholic extract of *Bacopa monnieri* (L.) (brahmi), on experimental amnesia in mice. Indian J Exp Biol. 2005; 43:640–645.
10. Banerjee M, Modi P. Micropropagation of *Bacopa monnieri* using cyanobacterial liquid medium. Plant Tiss Cult Biotechnol. 2010; 20(2):225-231.
11. Ali G, Shrivastav PS, Iabal M. Responses of *Bacopa monniera* cultures to cadmium toxicity. Bull Environ Contam Toxicol. 2001; 66:342-349.
12. Shukla OP, Dubey S, Rai UN. Preferential accumulation of cadmium and chromium: toxicity in *Bacopa monnieri* (L.) under mixed metal treatments. Bull Environ Contam Tox. 2007; 78:252-257.
13. Sharma S, Kamal B, Rathi N, Chauhan S, Jadon V, Vats N, Gehlot A, Arya S. *In vitro* rapid and mass multiplication of highly valuable medicinal plant *Bacopa monnieri* (L.) Wettst. Afr J Biotechnol. 2010; 9(49):8318-8322.
14. Ved DK, Goraya GS. Demand and Supply of Medicinal Plants in India. NMPB, New Delhi and FRLHT, Bangalore, India; 2007.
15. Tiwari V, Tiwari KN, Singh BD. Comparative studies of cytokinins on *in vitro* propagation of *Bacopa monniera*. Plant Cell Tiss Org Cult. 2001; 66:9–16.
16. Rathore S, Singh N. *In vitro* conservation of *Bacopa monnieri*- an endangered medicinal plant. Global J Bio-Sci Biotechnol. 2013; 2(2):187-192.
17. Shah JD. Studies in growth and ecology of *Bacopa monnieri* (L.) Pennell.-a medicinal herb. Ph.D. Thesis, Banaras Hindu University, Varanasi, India; 1965.
18. Sharma N. Micropropagation of *Bacopa monneiri* L. Penn.- an important medicinal plant. M.Phil. Thesis. Thapar Institute of Engineering and Technology Patiala, India; 2005.
19. Tiwari V, Singh BD, Tiwari KN. Shoot regeneration and somatic embryogenesis from different explants of Brahmi [*Bacopa monniera* (L.) Wettst]. Plant Cell Rep. 1998; 17:538-543.
20. Ceasar SA, Maxwell SL, Prasad KB, Karthigan M, Ignacimuthu S. Highly efficient shoot regeneration of *Bacopa monnieri* (L.) using a two-stage culture procedure and assessment of genetic integrity of micropropagated plants by RAPD. Acta Physiol Plant. 2010; 32:443-452.
21. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 1962; 15:473-497.
22. Gomez KA, Gomez AA. Statistical procedure for agricultural research, Wiley, New York, USA; 1984.
23. Binita BC, Ashok MD, Yogesh TJ. *Bacopa monnieri* (L.) Pennell: a rapid, efficient and cost effective micropropagation. Plant Tiss Cult Biotechnol. 2005; 15(2):167-175.
24. Ramesh M, Saravanakumar RM, Pandian SK. Benzyl amino purine and adenine sulphate induced multiple shoot and root induction from nodal explants of Brahmi, *Bacopa monnieri* (Linn.) Penn. Green Page. 2006; 5(1):44-51.
25. Mohanta YK, Sahoo S. *In vitro* culture of highly valuable medicinal plant *Bacopa monnieri* (L.) Penn. for rapid and mass multiplication. Int J Pharma Sci Invent. 2014; 3(1):41-45.
26. Jain A, Pandey K, Benjamin D, Meena AK, Singh RK. *In vitro* approach of medicinal herb: *Bacopa monnieri*. Int J Innov Res Sci Eng Technol. 2014; 3(5):12088-12093.
27. Kaur J, Nautiyal K, Pant M. *In vitro* propagation of *Bacopa monnieri* (L.) Wettst- A medicinally priced herb. Int J Curr Microbiol Appl Sci. 2013; 2(8):131-138.
28. Iram S, Anis M. *In vitro* shoot multiplication and plantlet regeneration from nodal explants of *Cassia angustifolia* Vahl.-a medicinal plant. Acta Physiol Plant. 2007; 29:233-238.
29. Konan NK, Sangwan RS, Sangwan BS. Somatic embryogenesis from cultured mature cotyledons of Cassava (*Manihot esculenta* Kranz). Identification of parameters influencing the frequency of embryogenesis. Plant Cell Tiss Org Cult. 1994; 37(2):91-102.
30. Kumari S, Starlin NM, Huxley AJ. *In vitro* propagation of *Bacopa monnieri* (L.) - a wetland medicinal plant. J Basic Appl Biol. 2010; 4(3):138-142.
31. Showkat P, Zaidi Y, Asghar S, Jamaluddin S. *In vitro* propagation and callus formation of *Bacopa monnieri* (L.) Penn. Plant Tiss Cult Biotechnol. 2010; 20(2):119-125.
32. Yusuf A, Rajesh KT, Nikhilesh S, Rao PS. Effects of antioxidants and gelling agents on regeneration, *in vitro* conservation and genetic stability of *Bacopa monnieri* (L.) Pennell. Int J Ayur Herb Med. 2011; 1(3):51-67.
33. Mehta J, Ansari R, Syedy M, Khan S, Sharma S, Gupta N, Rathore R, Vaishnav K. An effective method for high frequency multiple shoots regeneration and callus induction of *Bacopa monnieri* (L.) Pennell: an important medicinal plant. Asian J Plant Sci Res. 2012; 2(5):620-626.
34. Pandiyan P, Selvaraj T. *In vitro* multiplication of *Bacopa monnieri* (L.) Pennell from shoot tip and nodal explants. J Agric Technol. 2012; 8(3):1099-1108.
35. Jain R, Prasad B, Jain M. *In vitro* regeneration of *Bacopa monnieri* (L.): a highly valuable medicinal plant. Int J Curr Microbiol Appl Sci. 2013; 2(12):198-205.
36. Asha KI, Devi AI, Dwivedi NK, Nair RA. *In vitro* regeneration of Brahmi (*Bacopa monnieri* (Linn.) Pennell - an important medicinal herb through nodal segment culture. Res Plant Biol. 2013; 3(1):01-07.
37. Letham DS, Palni LMS. The biosynthesis and metabolism of cytokinins. Annu Rev Plant Physiol. 1983; 34:163–197.
38. Kumari R, Priyadarshni M, Anjali K, Shukla LN. *In vitro* mass multiplication of *Bacopa monnieri* (L.) an endangered and valuable medicinal herb. Indian J Sci Res. 2014; 7(1):1248-1253.
39. Kumar HNK, Chandana E, Chauhan JB. *In vitro* propagation of *Calamus nagbettai*: an endangered plant. J Microbiol Biotechnol Res. 2012; 2 (2):270-275.
40. Srivastava J, Raghav PK. Effect of different gibberellic acid concentration on pigeon pea (*Cajanus cajan* [L.] Millsp.) *cv*. Manak (H77216) via cotyledonary node explants. Int J Adv Biotechnol Res. 2014; 5(2):151-156.
41. Taiz L, Zeiger E. Plant physiology. 5th ed. Sinauer Associates Inc., Publishers, Sunderland, Massachusetts, USA; 2010.

42. Phulwaria M, Rai MK, Harish, Gupta AK, Ram K, Shekhawat NS. An improved micropropagation of *Terminalia bellirica* from nodal explants of mature tree. Acta Physiol Plant. 2012; 34:299–305.
43. Rathore JS, Rai MK, Phulwaria M, Shekhawat NS. A liquid culture system for improved micropropagation of mature *Acacia nilotica* (L.) Del. ssp. *indica* and *ex vitro* rooting. Proc Natl Acad Sci Sect B: Biol Sci. 2014; 84(1):193-200.
44. Sathyanarayana BN, Dalia BV. Plant tissue culture, Practices and new experimental protocols, IK International Publishing House, Pvt. Ltd. New Delhi, India; 2007.

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