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An efficient *in vitro* propagation protocol of local germplasm of *Bacopa monnieri* (L.) found in Bihar: A plant with wide variety of medicinal properties

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Abstract
Bacopa monnieri L. Penn. is an important medicinal crop. The problems with natural propagation is death of seedlings at two leaved stage, short viability of seed, marshy areas requirements and slow growth of stem cutting. Therefore, in the present investigation, an effort has been made to develop a rapid and reproducible protocol for the *in vitro* mass multiplication through callus induction of *Bacopa monnieri*. Nodal and leaf segments were used as explants for regeneration. A friable, compact, globular, morphogenic, green and white colored callus was initiated in different combination of media. For callus induction from leaf segments, MS basal media supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA, supported the best result (75% callus induction) and MS salt supplemented with 4.0 mg/L BAP and 0.5 mg/L IAA supported best callus responses for nodal segment (85% callus formation). The MS salt supplemented with 1.0 mg/L BAP+ 0.5 mg/L NAA gave best results for shooting and multiple shooting induction from callus of leaf and nodal segment. Shoot induction percentage (85%), average number of shoots (15) and average shoot length (9.5 cm) was higher in case of callus developed from nodal segment as compared to leaf segments. Best performing rooting media (MS + 20g/l sucrose and 7g/l agar) induced rooting in 80% of shoots. Two weeks after hardening plants were transferred to soil in field/open conditions and 98% survival rate was observed, and the regenerated plantlets showed robust growth.

Keywords: medicinal plant**,** organogenesis, endangered species, ex-situ conservation, hormone, auxin, cytokinin

Introduction
Bacopa monnieri L. Penn. has originated in India, commonly known as "Brahmi" is a high value medicinal crop of the family Scrophulariaceae. It has been used as brain tonic in ayurvedic system of medicine to improve memory, concentration, and learning as well as to cure mental illness (Srivastava *et al*., 1999) [18], (Majumdar *et al*., 2011) [12], (Ahire *et al*., 2013) [1]. Report showed that bacosides A, B, C and D are the most important saponins are components of *B. monnieri* and also known as 'memory chemicals' due to their memory enhancement properties (Rastogi *et al*., 1994) [13], (Banerjee *et al*. 2008) [5]. In addition, the plant has anti-inflammatory, analgesic, antipyretic, anticancerous, anticonvulsive and antioxidant properties (Ahire *et al*., 2013) [1], (Elangovan *et al*., 1995) [8], (Kishore *et al*., 2005) [9]. This plant has been used in treatment of insomnia, asthama, hoarseness, rheumatism, leprosy, eczema, water retention, blood cleaning and insanity (Banerjee *et al*., 2008) [4], (Banerjee *et al*., 2010) [5]. *B. monnieri* also have phytoremadiation property and it phytoremediate toxic heavy metals (e.g. cadmium, chromium and mercury) from aquatic bodies (Ali *et al*., 2001) [3], (Shukla *et al*., 2007) [19]. Unluckily, the demand of this herb is fulfilled mainly by collection from wild sources/wastelands (Ved *et al*., 2007) [25]. Therefore, there is urgent need to standardization of propagation methods for ensuring the consistent accessibility of raw material and viable sources is imperative (Srivastava *et al*., 1999) [18]. The major hurdle in natural propagation with of *B. monnieri* is very short viability of seed and propagation through stem cutting is also very slow (Tiwari *et al*., 2001) [24],(Rathore *et al*., 2013) [14]. Besides, frequent seedling death at two leaves stage has also been observed. Thus, raising seedlings from seeds is a difficult task (Tiwari *et al*., 2001) [24], (Rathore *et al*., 2013) [14]. Vegetative propagation by stem is a slow process (Tiwari *et al*., 2001) [24], (Shah *et al*., 1995) [16]. In addition, it was also observed that the growth of *B. monnieri* is changes with seasons (Sharma *et al*., 2005) [17].Thus, normal propagation method is not sufficient to meet the demand of raw material of *B. monnieri*. The plant is already under threatened category due to untenable collection of raw material from wild/natural population (Tiwari *et al*., 1998) [22],

(Ceaser *et al*., 2010) [7]. Therefore, for constant and steady supply of raw materials and for reduction of load on natural/wild population, and development of an efficient and reliable *in vitro* plant regeneration protocol for this medicinal herb is an essential requirement. A number of reports on *in vitro* plant regeneration using different explants of *B. monnieri* are available. However, *in vitro* plant regeneration protocols of *B. monnieri* for local germplasm which are naturally found in Bihar is not available. Therefore, the objective of present study is to develop an rapid, effective, efficient and reproduciable *in vitro* plant regeneration protocol for local germplasm of *B. monnieri* found in Bihar.

Materials & methods

Source of explants

Shoots obtained from four months old plant of *Bacopa monnieri* (L.) Pennell (maintained in herbal garden nursery of the Hi –Tech horticulture research area of Dr. Rajendra Prasad Central Agricultural university) and nodal segment and young leaves were used as a explants.

Explants sterilization

The explants were thoroughly cleaned with running tap water for 15 min to remove all the dust particles adhere with and then treated with 0.2% surfactant Teepol (Himedia) for 10 minutes followed by repeated rinsing with double distilled water. Further sterilization was done with mixture solution of 0.1% streptomycin and 0.1% bavestin for 30 minutes and gently washed twice in sterile double distilled water. Finally sterilization was done under aseptic conditions (inside a laminar Airflow Hood), explants were surface sterilized with 50% ethanol (1 min) followed by 3 min treatment with 0.01% $HgCl_2$ solution and washing 3 times with sterile double distilled water Kumar *et al*. (2009) [10].

Media combination for callus induction

For callus induction experiment the sterilized explants were taken and were inoculated on five media combinations (MS basal as control, MS + 0.5 mg/l BAP + 0.5 mg/l NAA, MS + 1.0 mg/l BAP + 1.0 mg/l NAA, MS + 4.0 mg/l BAP + 0.5 mg/l NAA, and MS + 1.5 mg/l BAP + 1.0 mg/l IAA). The cultures were incubated at 25±2 ºC under 16 h photoperiod of 45 – 50 µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes (Philips, India) and with 55 – 60% relative humidity. Visual observations like callus induction, growth of callus, number of days taken for callus induction were recorded regularly.(BAP (6-benzylaminopurine), IAA (indole-3-acetic acid), NAA (α naphthalene acetic acid), 2,4D (2,4-Dichlorophenoxyacetic acid), MS (Murashige and Skoog))

Media combination for shoot regeneration and multiplication

For the regeneration of shoots from callus, the induced callus were sectioned into small pieces and transferred on seven media combinations with different concentration and combinations of auxin and cytokinin (MS basal as control, MS + 0.5 mg/l BAP + 0.5 mg/l NAA, MS + 1.0 mg/l BAP + 1.0 mg/l NAA, MS + 1.0 mg/l BAP + 1.0 mg/l IAA, MS + 1.5 mg/l BAP + 1.0 mg/l IAA, MS + 0.5 mg/l BAP + 1.0 mg/l 2,4-D, MS + 0.5 mg/l BAP + 2.0 mg/l 2,4-D). The cultures were incubated at 25±2 ºC under 16 h photoperiod of 45 – 50 µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes (Philips, India). Visual observations like number of days taken for shoot regeneration, number of shoots regenerated, and length of shoot per explants was recorded regularly. A mean of 10 replicates was taken per treatments.

Rooting and hardening of in vitro raised plantlets

Total six combinations (MS + sucrose (30 gm/l)+ Agar (7gm/l), MS + sucrose (30 gm/l) + Agar (8gm/l), MS + Sucrose (20 gm/l) + Agar (7gm/l), MS + Sucrose (20gm/l)+Agar (8gm/l), MS + Sucrose (10 gm/l) + Agar (7gm/l), MS + Sucrose (10 gm/l) + Agar (7gm/l)) of rooting media were used for rooting induction/regeneration from in vitro developed shoot. For hardening the complete plantlets were transferred in hardening unit for hardening. The roots of the plants are gently cleaned to remove the agar and other remaining of media sticking to them. The plantlets were transferred to plastic cupss containing sterilized hardening mixture of sand, soil, coco-pit and farm yard manure in1:1:1:1 ratio. Each pot was watered properly. Small holes were made in plastic pot for proper aeration of root. Finally after two weeks the plants were removed from plastic cups and finally plants were ready for field transfer.

Results and discussion

Callus induction

For callus induction, the balanced ratio of auxins or cytokinins is important. This ratio is also depends upon the type of explants used for callus induction. Experiments were performed to test the effect of different concentrations and combination of auxins and cytokinin for callus induction response from leaf and nodal segments. The callus induction was observed in all tested media combinations from both types of explants, while media combination SC1 (containing MS+0.5mg/l BAP + 0.5 mg/l NAA) supported maximum percent callus induction (75%) from leaf segments and SC4 (containing MS+4.0mg/l BAP + 0.5 mg/l NAA) supported maximum callus induction in case of nodal explants (Table1&2 and Fig.1A&B). In 12 to 18 days callus attained full growth. In contrast Sharma *et al*., (2005) [17] reported that media combination having IAA along with BAP gave best result. Similar results were also observed in Jojoba (Sanjeev *et al.,* 2013) [21] in Potato Cultivars (AL-Hussaini *et al.,* 2015) [2].

Shoot regeneration and multiplication

In order to get profuse and rapid shoot regeneration, multiplication medium supplemented with cytokinin and auxin combinations were used. Auxins, like 2,4-D, IAA, and NAA were added and tested along with varied concentration of BAP to study their effect and to maximize shoot regeneration, multiplication and elongation. Callus obtained from leaf and nodal segment were tested independently in all combination of media. All media combination supported the shoot regeneration from callus of leaf as well as nodal segments. The auxin NAA along with BAP combination media performed excellent for shoot induction and multiplication. The media combination SS1 (containing MS+ 1.0 mg/l BAP + 0.5 mg/l NAA) supported maximum shoot induction (85.1%), number of shoot (15.1) and shoot length (9.5cm) (Table. 3 & 4, Fig.1 (C -D). The callus of nodal segment gave fast shooting response than callus of leaf segment. The multiple shoot formation started after 12 to 16 days in same media. In contrast Behera & Naik, (2015) [6] reported that in case of Bacopa, 3.0 mg L-1 BA (shoot induction medium) offered the best result without any auxin addition and average of 6.5 shoots/explant with average shoot length of 3.69 cm was recorded.

Rooting of *in vitro* regenerated shoots

Application of different auxins are well established facts for induction of rooting in in vitro condition. However, in present investigation during subculturing it was observed that that rooting in *Bacopa in vitro* culture is quite impulsive. Hence the experiments were intended to study the rooting response with different treatment combination with two variations of agar (7 and 8 gm/l) and sugar (10, 20, 30 gm/l) without addition of plant growth regulators. Root formation induced in all media combination while media SR5 performed best among combinations in terms of percent root induction (80%), root length (3.1) and no of roots (6.3) were measured in rooted shoot (Table 5). Similar finding are also reported by Sharma *et al*., (2005) [17]. Tiwari *et al.;* (2000) [23], observed highest rate of rooting (90%) for Bacopa on full-strength MS medium containing 2.46 mM IBA. Kumari *et al*. (2014) [11] reported that media supplemented with 0.25mg/l IBA were induced the root formation after four weeks in *Bacopa*. In contrast in present my experiments rooting induced without application of any auxins in rooting media. Similar to my findings Srivastava *et al.*; (2017) [20] also reported rooting without growth regulators in *Bacopa*.

Table 1: Influence of different media combinations on callus induction of *Bacopa monnieri* (L.) Pennell from leaf explant

Media code	Medium details	% swelling of explant	% of callus induction	Appearance of callus
S	MS basal without hormone	-	-	-
SC1	MS + 0.5 mg/l BAP + 0.5 mg/l NAA	80.7	75.1	Cremish white
SC2	MS + 1.0 mg/l BAP + 1.0 mg/l NAA	70.3	65.9	Yellowish green
SC3	MS + 1.5 mg/l BAP + 1.0 mg/l IAA	70.0	60.3	Greenish white
SC4	MS + 4.0 mg/l BAP + 0.5 mg/l NAA	75.6	70.5	Light green

Table 2: Influence of different media combinations on callus induction of *Bacopa monnieri* (L.) Pennell from nodal segment

Media code	Media details	% swelling of explant	% of callus induction	Appearance of callus
S	MS basal without hormone	-	-	-
SC1	MS + 0.5 mg/l BAP + 0.5 mg/l NAA	65.3	60.4	Creamish white
SC2	MS + 1.0 mg/l BAP + 1.0 mg/l NAA	75.1	60.6	Greenish white
SC3	MS + 1.5 mg/l BAP + 1.0 mg/l IAA	80.6	70.8	Yellow green
SC4	MS + 4.0 mg/l BAP + 0.5 mg/l NAA	85.0	80.2	Greenish white

Table 3: Effect of different media combinations on shoot regeneration and multiple shooting from callus of leaf of *Bacopa monnieri* (L.) Pennell

Media code	Media details	% shoot regeneration	Mean Number of shoot ±SE	Mean shoot Length (cm) ±SE
S	MS basal without hormone	-	-	-
SS1	MS + 1.0 mg/l BAP + 0.5 mg/l NAA	82.0	12.1± 0.71	9.3±0.82
SS2	MS + 1.0 mg/l BAP + 1.0 mg/l NAA	80.0	10.6±0.56	8.1±0.60
SS3	MS + 1.0 mg/l BAP + 1.0 mg/l IAA	60.7	6.9±0.29	5.3±0.91
SS4	MS + 1.5 mg/l BAP + 1.0 mg/l IAA	65.2	6.3±0.37	5.5±0.81
SS5	MS + 0.5 mg/l BAP + 1.0 mg/l 2,4-D	72.9	8.2±0.42	7.3±0.68
SS6	MS + 0.5 mg/l BAP + 2.0 mg/l 2,4-D	70.3	8.7±0.71	7.5±0.41

Table 4: Effect of different media combinations on shoot regeneration and multiple shooting from callus of nodal segments of *Bacopa monnieri* (L.) Pennell

Media code	Media details	% shoot regeneration	Mean Number of shoot ±SE	Mean shoot Length (cm) ±SE
S	MS basal without hormone	-	-	-
SS1	MS + 1.0 mg/l BAP + 0.5 mg/l NAA	85.1	15.2±0.72	9.5±0.21
SS2	MS + 1.0 mg/l BAP + 1.0 mg/l NAA	85.1	15.0±0.43	7.3±0.85
SS3	MS + 1.0 mg/l BAP + 1.0 mg/l IAA	55.9	6.7±0.51	6.5±0.32
SS4	MS + 1.5 mg/l BAP + 1.0 mg/l IAA	55.6	6.9±0.21	6.0±0.83
SS5	MS + 0.5 mg/l BAP + 1.0 mg/l 2,4-D	75.2	8.1±0.10	7.5±0.70
SS6	MS + 0.5 mg/l BAP + 2.0 mg/l 2,4-D	70.1	8.0±0.71	7.2±0.11

Table 5: Effect of different media combinations on root induction of *Bacopa monnieri* (L.) Pennell

Media code	Media details	% root induction	Mean Number of root ±SE	Mean root Length (cm) ±SE
SR1	MS + sucrose (30 gm/l)+ Agar (8gm/l)	60.1	3.0±0.83	2.0±0.53
SR2	MS + sucrose (30 gm/l)+ Agar (7gm/l)	70.2	5.1±0.61	3.1±0.21
SR3	MS + Sucrose (20 gm/l) + Agar (7gm/l)	80.0	6.3±0.24	3.1±0.36
SR4	MS + Sucrose (20gm/l) + Agar (8gm/l)	75.9	6.0±0.70	3.0±0.13
SR5	MS + Sucrose (10gm/l) + Agar (8gm/l)	60.3	3.2±0.33	1.5±0.73
SR6	MS + Sucrose (10gm/l) + Agar (7gm/l)	52.2	3.0±0.70	2.2±0.67

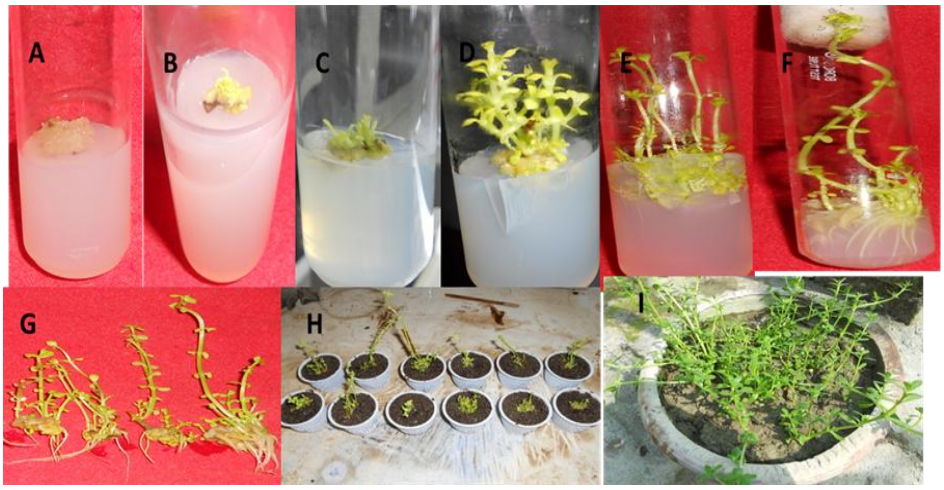

Fig 1: (A-I) callus inducation, organodgenesis and *ex vitro* acclimation. A. callus induction form leaf explants form leaf explants of Bocoma monnieri on MS containing 0.5mg/1 BAP and 0.5mg/1 NAA. B. callus induction from nodal explants of *Bacopa monnieri* on MS contanining 1.0 mg/a BAP and 0.5mg/1 NAA. D. Multiple shoot formation and elongation on MS containing BAP (0.5mg/1) and NAA (0.5mg/1). E. Elongation of multiple shoot on ptimal media. F. Root induction from regenerated shoot on optimal medium G. *In vitro* harvested *Bacopa* plantlets H. *In vito* raised raised *Bacopa* plantlets were transferred to plastic pots containing sterilized hardening mixture of sand, soil, cocnut powder and farm yard manure in 1:1:1:1 ratio for hardening and maintained in net-house. I. Acclimated plants growing outside in earthen pot.

Hardening of in vitro regenerated plants before field transfer

In vitro regenerated plants were hardened directly in the net house skipping the greenhouse stage. Initially high humidity was maintained by five sprays of water a day at 4 h interval. The plantlets hardened for two weeks in net-house. In vitro generated rooted plantlets were washed thoroughly with water to remove agar and any other remaining of the media. (Fig.1G). To protect from fungal attack during hardening plantlets were treated with 0.1% Bavistin. The rooted plantlets established well upon transfer to plastic cups containing containing sterilized hardening mixture of sand, soil, coco-peat and farm yard manure in 1:1:1:1 ratio. Contrast Sharma *et al*. (2005) [17] reported 4soil: 1agropeat ratio was best. However, Sherkar *et al*. (2014) [15] used 1:2:1 ratio of sand, soil farm yard manure to acclimatize in vitro generated tomato plant. and Small holes were made in plastic cups for proper aeration of root. The plantlets were acclimatized progressively by decreasing the moisture of whole system. Finally after two weeks the plants were removed from plastic cups and transferred to field conditions. 98% plantls survived in field conditions after two weeks hardening (Fig.1H). In vitro generated plants were morphologically similar to mother stock, there was no visible changes observed in respect to morphological features. All the transplanted plants (hardened plant) shown vigorous growth under field conditions.

Conclusion

In the this study, a highly efficient and reproducible *in vitro* callus induction, organogenesis, regeneration of new plantlets and *ex vitro* acclimation protocol was standardized for local germplasm of *B. monnieri* native to Bihar. The protocol developed could be used for mass micropropagation of *Bacopa monnieri*. Protocol can be used for a range of other biotechnological applications for further improvement of this species. The protocol discussed here will help in meeting the ever-increasing demands of the pharmaceutical industries and on the other hand save natural species from overexploitations.

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