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A rapid *In vitro* propagation protocol of local germplasm of *Bacopa monnieri* (L.) induced through direct organogenesis from nodal explants

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Abstract
The natural propagation of local germplasm of *Bacopa* is affected by poor seed viability, death of seedlings at two leaved stage, marshy areas requirements and slow growth of stem cutting. An efficient *in vitro* propagation protocol through direct organogenesis from nodal explants has been reported here for local germplasm of *Bacopa monnieri*. For axillary bud break and shoot proliferation from nodal segments, MS basal media supplemented with 1.0 mg/l BAP and 1.0 mg/l IAA, supported the best response (85% induction) and the same media combination also offered the best result for multiple shoot formation. The average number of shoots (10) and average shoot length (8.2 cm) was observed from nodal segment. Best performing rooting media (MS + 20g/l sucrose and 7g/l agar) induced rooting in 81% of shoots. The average number of roots (6) and average root length (3.1 cm) was observed from in vitro regenerated shoot. Two weeks after hardening plants were transferred to soil in field conditions and 90% survival rate was observed, and the growth of hardened plantlets was vigorous.

Keywords: direct organogenesis, threatened species, tissue culture, hormone, auxins, cytokinin

Introduction
Bacopa monnieri (L.) originated in India and commonly known as "Brahmi". 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]. Important saponins like bacosides A, B, C and D are the components of *B. monnieri* and also known as 'memory chemicals' due to their memory enhancement properties (Rastogi *et al*., 1994) [14], (Banerjee *et al*. 2008) [4]. In addition, the plant has anti-inflammatory, analgesic, antipyretic, anticancerous, anticonvulsive and antioxidant properties (Ahire *et al*., 2013) [1], (Elangovan *et al*., 1995) [7], (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) [3], (Banerjee *et al*., 2010) [5]. Recently the Phytoremadiation properties of *B. monnieri* has also been reported and it remove toxic heavy metals (e.g. cadmium, chromium and mercury) from aquatic water bodies (Ali *et al*., 2001) [2], (Shukla et al., 2007) [19]. Unfortunately, the demand of this medicinal herb is fulfilled mainly by collection from wild sources/wastelands (Ved *et al*., 2007) [24]. The major issues with propagation of *B. monnieri* is very short viability of seed and propagation through stem cutting is also very slow (Tiwari *et al*., 2001) [23], (Rathore *et al*., 2013) [15], (Shah *et al*., 1995) [16]. Besides, frequent seedling death at two leaves stage has also been observed. In addition, it was also observed that the growth of *B. monnieri* is changes with seasons (Sharma *et al*., 2005) [17]. Thus, the conventional propagation method is not sufficient to meet the demand of raw material of *B. monnieri* for pharmaceutical industry. The plant is already under threatened category due to unsustainable way of collection of raw material from wild/natural population (Tiwari *et al*., 1998) [21], (Ceaser *et al*., 2010) [6]. 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 literatures 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, in present study an efficient *in vitro* micropropagation protocol has been developed through direct organogenesis from nodal explants for faster multiplication of local germplasm of *B. monnieri* found in Bihar.

Materials & Methods

Explants collection

The local germplasm of *Bacopa monnieri* (L.) Pennell maintained in herbal garden nursery of the Hi –Tech horticulture research area of Dr. Rajendra Prasad Central Agricultural university, Bihar, India were used as the source of explants. The explant, nodal segments with axillary buds, collected from healthy shoots of *Bacopa* plant.

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 1% savlon detergent for 10 minutes followed by repeated rinsing with double distilled water. Further surface 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 5 times with sterile double distilled water Kumar *et al*. (2009) [10].

Media combination for shoot/shoot bud induction and multiplication

Surface sterilized nodal explants were cultured on either Murashige and Skoog's (1962) [13] (MS) medium alone or MS medium supplemented with different concentrations (0.5-2.0 mg/L) of BAP (6-benzylaminopurine), IAA (indole-3-acetic acid), NAA (α naphthalene acetic acid) and 2,4D (2,4-Dichlorophenoxyacetic acid) for shoot/shoot bud induction. Seven media combinations with different concentration and combinations of auxins and cytokinin were used to induce shoot/bud break in nodal segments (MS basal without growth regulators, 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 bud break/shoot induction, number of shoots, 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

Multiple shoot were separated with help of sterilized blade inside the laminar air flow hood and inoculated in different combination of rooting media. Total four 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)) of rooting media were used for root 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 cups containing sterilized hardening mixture of sand, soil, coco-peat 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

Effect of cytokinin (6-BAP) and different auxins (NAA, IAA & 2, 4-D) on bud break/shoot bud induction and multiple shooting

In present experiments total seven shooting media combinations were tested for shoot induction and multiple shoot formation. Media combinations SS3 was found most effective for shoot induction as well as multiple shoot formation form nodal segment in terms of percent shoot induction (85.3%), shoot length (8.2) and no of shoots (10.4) formation (Table 1; Fig. 1A-D), while no shoot induction was observed in media without plant growth regulators. Emergence of a few roots from the base of the culture was a common phenomenon in all the media. The Shoot induction was started after 8 days after explants inoculation and multiple shoot formation was started 22 days after sub culturing in same media (Fig. 1B-D). Amongst the different concentration of BAP tested, 1.0 mg/ L BAP offered the best result, while among the auxins IAA (concentration1.0mg/L IAA) offered the best result (Table 1). In contrast to my finding Behera *et al.* (2015) [5] reported, 3.0 mg/ L BA offered the best result even bud break was initiated in without hormone containing media from nodal segments of *Bacopa*, while Sharma *et al*. (2005) [17] observed best result in 0.5 mg/l BAP + 0.5mg/L NAA containing media from nodal segments of *Bacopa*. Kaur *et al*. (2013) [8] reported the requirement of addition of plant growth regulator for bud break in *B. monnieri*.

Root induction of *in vitro* regenerated shoots

Addition of auxins in media play an important role in induction of rooting of *in vitro* generated shoots. However, in present experiments emergence of spontaneous roots (few) was observed during subculturing of shoot of *Bacopa*. Root formation induced in all hormone free media, this may be due to the reason that large amount of auxin synthesized in apical buds which moved towards basal side and induced spontaneous root initiation in *Bacopa.* In present investigation total four hormone fee rooting media tested with only variations in agar (7 and 8 gm/L) and sugar (20, 30 gm/L). Root formation induced in all media combination while media SR3 performed best among combinations in terms of percent root induction (81.3%), root length (6.1cm) and no of roots (3.1) were measured in rooted shoot (Table 2). Similar finding was reported by Sharma *et al*. (2005) [17] in *Bacopa*. Similarly Srivastava *et al*. (2017) [20] also reported rooting without growth regulators in *Bacopa*. Tiwari *et al.* (2000) [22], observed highest rate of rooting (90%) for Bacopa on full-strength MS medium containing 2.46 mM IBA. In contrast to my finding Behera *et al.* (2015)[5] reported maximum rooting was in ½ MS + 0.2 mg/L IBA, and Kumari *et al*. (2014) [11] reported that media supplemented with 0.25mg/l IBA were induced the root formation in *Bacopa*.

Hardening of in vitro regenerated plants before field transfer

Rooted plantlets were taken out of the culture tubes (Fig. E) and washed thoroughly with water to remove agar and any other remaining of the medium. 0.1% Bavistin treatment was given to the plants in order to protect them from the fungal attack in during hardening. 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 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. Small holes were made in plastic cups for proper aeration of root. In contrast Sharma *et al*. (2005) [17] observed 4 soil: 1 agropeat ratio was best hardening mixture. Finally after two weeks the plants were removed from plastic cups and transferred to field conditions. 90% plants survived in field conditions after two weeks hardening (Fig.1G). In vitro generated plants shown vigorous growth.

Table 1: Media combinations influenced the shoot bud induction and multiple shoot production from nodal segments (with auxiliary bud) of *Bacopa monnieri* (L.).

Media code	Media details	Response (%)	Mean Number of shoot ±SE	Mean shoot Length (cm) ±SE
S	MS basal without growth regulators	-	-	-
SS1	MS + 0.5 mg/l BAP + 0.5 mg/l NAA	45.1	5.3±0.23	5.8±0.94
SS2	MS + 1.0 mg/l BAP + 1.0 mg/l NAA	45.0	5.1±0.68	5.3±0.11
SS3	MS + 1.0 mg/l BAP + 1.0 mg/l IAA	85.3	10.4±0.37	8.2±0.08
SS4	MS + 1.5 mg/l BAP + 1.0 mg/l IAA	80.2	10.3±0.31	8.0±0.27
SS5	MS + 0.5 mg/l BAP + 1.0 mg/l 2,4-D	50.1	4.1±0.91	5.1±0.25
SS6	MS + 0.5 mg/l BAP + 2.0 mg/l 2,4-D	50.6	5.7±0.71	5.5±0.39

Table 2: Media combinations influenced the root induction in shoots of *in vitro* regenerated *Bacopa monnieri* (L.)

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	2.0±0.71	3.0±0.51
SR2	MS + sucrose (30 gm/l)+ Agar (7gm/l)	73.7	5.3±0.59	3.1±0.37
SR3	MS + Sucrose (20 gm/l) + Agar (7gm/l)	81.3	6.1±0.15	3.1±0.21
SR4	MS + Sucrose (20gm/l) + Agar (8gm/l)	79.1	6.0±0.01	3.3±0.73

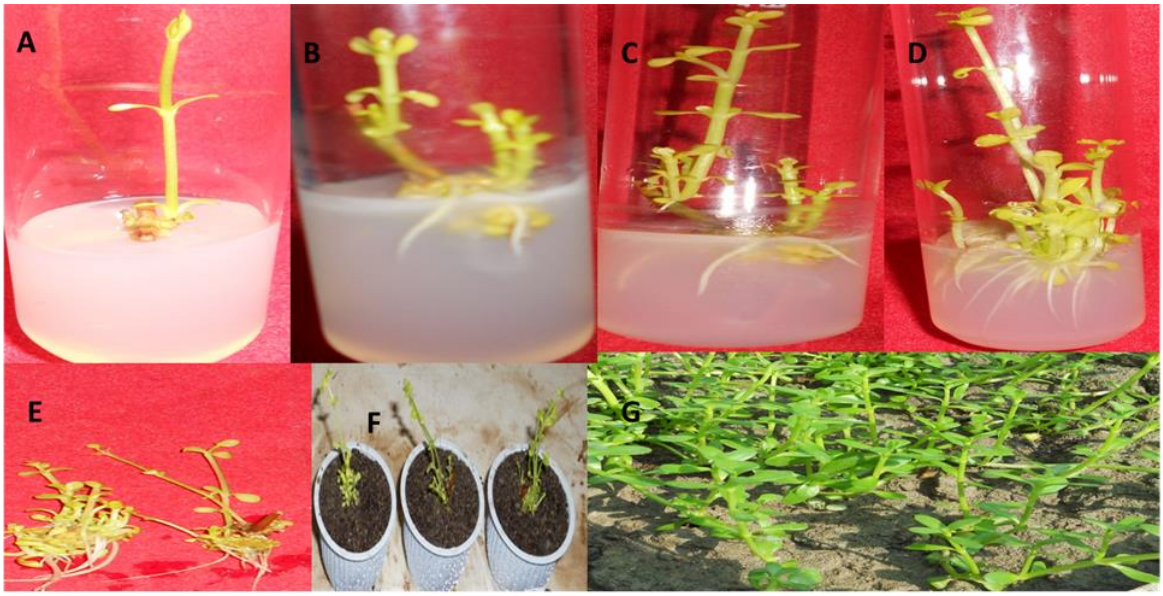

Fig. 1. (A-G) Direct organogenesis and *ex vitro* acclimation of *Bacopa monnieri* . A. Direct shoot induction from nodal explant of *Bacopa monnieri* on MS containing 1.0 mg/l BAP and 1.0 mg/l IAA. B. Multiple shoot induction from nodal explants of *Bacopa monnieri* on optimal media. C. Elongation of shoots from nodal explants of *Bacopa monnieri* on MS containing 1.0 mg/l BAP and 1.0 mg/l IAA. D. Profuse root induction from regenerated shoot on optimal medium. E. *In vitro* harvested *Bacopa* plantlets F. *In vitro* raised *Bacopa* plantlets were transferred to plastic cups containing sterilized hardening mixture of sand, soil, coco-peat and farm yard manure in1:1:1:1 ratio for hardening and maintained in net-house. I. Acclimated plants growing outside in soil.

Conclusion

In the present study, an efficient, rapid and reproducible protocol for *in vitro* micropropagation through direct organogenesis was standardized for local germplasm of *B. monnieri* found in Bihar. The protocol developed could be used for mass multiplication of *Bacopa monnieri*. Fast multiplication of *Bacopa* herbs can help in meeting the ever-increasing demands of pharmaceutical industries and it will also minimize the load on naturally grown plant or plants grow in wild.

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