Shoot culture of *Bacopa monnieri*: standardization of explant, vessels and bioreactor for growth and antioxidant capacity

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Abstract Standardization of biomass production in different vessels and bioreactor using explants and media for growth, total phenolic content and antioxidant capacity of shoot culture of *Bacopa monnieri* is described. Maximum number of shoots per explant, higher explants response irrespective of the type of explants, and higher shoot length was obtained on MS medium containing BAP (2.5 mg l^{-1}) and IAA (0.01 mg l^{-1}) with 3 % sucrose. This medium was selected by varying BAP concentration and recorded optimal for shoot culture on gelled medium. The condition of 0.5 cm explant size and 20 explant/40 ml (1 explant/2 ml) was optimal for high explant response, number of shoots per explant regenerated and shoots length. Among the different vessels used, maximum growth index was achieved in Growtek bioreactor (10.0) followed by magenta box (9.16), industrial glass jar (7.7) and conical flask (7.2). The cultures grown in conical flask (100 ml) were used as control. The total phenolic content and antioxidant capacity of *in vitro* grown plants was higher to that recorded for *in vivo* material. Among *in vitro* regenerated plants, the activity was maximal in the tissues grown in 250 ml conical flask. The most critical function for vessels is to support the optimum profusion (growing area for maximum growth) of shoots and for *B. monnieri*, Growtek bioreactor supported 1980 shoots l^{-1} medium as compared to control (938 shoots l^{-1}). Growtek bioreactor was considered effective system to produce *B. monnieri* biomass in culture without loss of antioxidant properties.

Keywords Antioxidant activity · *Bacopa monnieri* · Growtek bioreactor · Shoot culture

Introduction

Bacopa monnieri (L.) Pennell (commonly known as Brahmi, Scrophulariaceae) is a creeping annual plant found throughout the Indian subcontinent in wet, damp and marshy areas. Besides, legendary reputation as memory vitalizer, it is also claimed to be useful in the treatment of cardiac, respiratory and neuropharmacological disorders like insomnia, insanity, depression, psychosis, epilepsy and stress (Howes and Houghton 2009; Rajani 2008). These pharmacological properties of the plant are mainly due to the saponins called bacosides, which are complex mixture of structurally closely related compounds, glycosides of either jujubogenin or pseudojujubogenin (Rajani 2008). Memory enhancing property of bacosides has increased its demand for its use in commercial preparations. The *in vitro* propagated medicinal plants can furnish uniform, sterile and compatible biomass for downstream processing (Banerjee and Shrivastava 2008).

Growing demand of *B. monnieri* plant material is met both from wild and cultivated plants. Many reports are offered for *in vitro* culture study of this vital medicinal plant (Praveen et al. 2009; Caesar et al. 2010). Therefore, development of quick and innovative methods is obligatory for large scale production of this medicinal plant for commercial uses. Recent studies suggest that cognition promoting functions of the plant may be partially attributed to the antioxidant effects of the bacosides (Russo and Borrelli 2005). The most important specific requirement for vessels is to provide adequate growing area for shoots to select maximum growth and biomass (Smith and Spomer 1995).

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In the present study, we report standardization of parameters for maximum biomass production and total phenolic content and antioxidant capacity of shoot culture of *B. monnieri*.

Materials and methods

Shoot culture The plants of *B. monnieri* were collected from the botanical garden of the institute. Plant material (leaf and stem) was first washed with liquid detergent, then it was surface sterilized in ethanol for 30 s. After it, they were immersed in 0.1 % of aqueous solution of HgCl_2 for 10 min, rinsed four times with sterilized distilled water. The plant material was then cut down to make suitable size explants (~1 cm) and inoculated on to the surface of solid MS (Murashige and Skoog 1962) medium. Subsequently, the cultures were grown in the liquid medium of same composition.

Culture conditions All cultures were incubated in the culture room at 26.0 ± 0.5 °C under white fluorescent light (Philips cool TL 12 W) with a total irradiance of $36 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 16 h photoperiod and 55–60 % relative humidity. The pH of the medium was adjusted to 5.8 and autoclaved at 121 °C for 15 min. For each treatment, at least six culture flasks containing 120 explants were used as replicates. For determining the growth index (GI), cultures were carefully removed from the flasks, fresh weight was determined, and then were dried in an oven at 60 °C to a constant weight and dry weight was determined. GI was calculated as—[final weight – initial weight/initial weight].

Optimization of shoots Different concentrations of 6-benzylamniopurine (BAP, 0.5–10 mg l^{-1}) with 3-indole

Table 1 Shoot multiplication in *B. monnieri* treated with different concentrations of BAP and IAA after 6 weeks of culture

BAP [mg/L]	IAA [mg/L]	Type of explant	Response [%]	Average shoots/explant	Shoot length [cm]
0.5	0.01	Node	50.25	1.88±0.08	2.92±0.04
		Internode	55.00	1.56±0.31	2.5±0.05
1.0	0.01	Node	62.50	1.93±0.17	3.16±0.32
		Internode	60.10	1.58±0.06	3.0±0.21
2.5	0.01	Node	70.20	2.20±0.70	3.82±0.17
		Internode	65.28	1.69±0.64	3.2±0.40
5.0	0.01	Node	40.30	1.45±0.54	2.23±0.09
		Internode	42.60	1.32±0.46	2.1±0.30
10.0	0.01	Node	Callusing	0	0
		Internode	Callusing	0	0
F value				11.3 ^b	117.0 ^b

^a Non-significant ($P > 0.05$)

^b Significant ($P < 0.01$)

Table 2 Optimization of size and number of explants (node) grown on MS medium with BAP 2.5 mg/l and IAA 0.01 mg/l

Size [cm]	No. of explants in 40 ml medium	Response [%]	Average shoots/explant	Shoot length [cm]
0.5	10	60.2	1.53 ± 0.06	3.20 ± 0.17
	20	70.5	3.00 ± 0.04	3.96 ± 0.07
	30	65.0	2.23 ± 0.09	3.0 ± 0.09
1.0	10	64.0	1.92 ± 0.06	3.05 ± 0.61
	20	65.2	1.96 ± 0.98	3.25 ± 0.44
	30	58.5	1.15 ± 0.04	2.50 ± 0.04
2.0	10	62.2	1.45 ± 0.36	2.12 ± 0.02
	20	64.7	1.68 ± 0.88	2.64 ± 0.10
	30	55.0	1.10 ± 0.29	2.20 ± 0.17
F value			4.18 ^b	14.0 ^b

^a Non-significant ($P > 0.05$)

^b Significant ($P < 0.01$)

acetic acid (IAA, 0.01 mg l^{-1}) were used to compare shoot induction, multiplication and shoot length from nodal and internodal explants.

After selecting the optimized medium, the size and number of nodal explant was varied to obtain optimal condition for shoot multiplication. Shoot cultures were maintained on MS medium containing BAP (2.5 mg l^{-1}) and IAA (0.01 mg l^{-1}), 3 % sucrose with agar (0.8 %) or without agar. Using these cultures, time course study of growth was determined up to 60 days. The cultures containing optimized nodal explants (size and number) were harvested at 10 days interval up to 60 days.

Culture vessels Four type of vessels; conical flasks (100 and 250 ml), magenta boxes (400 ml), commercial glass jars (500 ml) and Growtek bioreactor (1 L) were used for comparison. Growtek bioreactor (1 L, Tarson, India) was used with 10 % v/v aeration provided by inserting a needle through septum in the side tube. The details of Growtek

Fig. 1 Time course study of growth of shoots

Table 3 Effect of different culture vessels on the growth of nodal explants of *B. monnieri* grown on MS basal medium with BAP 2.5 mg l⁻¹ and IAA 0.01 mg l⁻¹

Culture Vessel	No. Of Shoots/L	Fresh Weight [g/L]	Dry Weight [g/L]	GI
Control- Conical flask (100 ml)	938.25±8.82	163.2±0.30	14.25±0.10	8.5
Conical flask (250 ml)	850.5**±4.41	146.0**±2.09	12.25*±0.04	7.16
Magenta box	1176.25**±6.61	187.7**±1.54	15.25±0.11	9.16
Glass jar	891.5**±5.82	126.5**±1.38	13.0±0.08	7.66
Growtek Bioreactor	1,979.16**±9.2	197.9**±7.2	16.5**±1.46	10.0*
F value	12,800 ^c	213 ^c	20.2 ^c	10.3 ^b

^a Non-significant ($P>0.05$)^b Significant ($P<0.05$)^c Significant ($P<0.001$)

* or ** Means are significantly different when compared with control at ($P<0.05$) or ($P<0.001$), according to Dunnett multiple comparison test. Mean data without * are non-significantly different from the control value

bioreactor's design have been described earlier (Sharma et al. 2011). The explants used for the experiment were single nodal pieces of 0.5 cm obtained from 6 weeks old tissue-cultured plants in all the experiments. In glass jars and magenta vessels, explants were placed on cotton pad support (Ruuhola and Julkunen-Tiitto 2000). Liquid cultures were agitated on a rotary shaker at 80 rpm. The cultures were harvested after 40 days and all the parameters such as number of shoots, fresh weight, dry weight, growth index (GI), total phenolic content and antioxidant potential were determined. In addition, antioxidant strength and total phenolic content of *in vivo* material was also recorded to compare *in vitro* regenerated plants and *in vivo* plants of *B. monnieri*.

Table 4 Effect of different culture vessels on the total phenolic content and antioxidant activity of *in vitro* shoots of *B. monnieri* and their comparison with *in vivo* material

Vessel type	Total polyphenols (mg GAE/gram)	DPPH% inhibition	SOD% inhibition
Control-100 ml conical flask	40±0.71	75.69±0.88	64.43±0.38
250 ml conical flask	55**±0.82	80.77**±0.76	81.42**±1.04
Glass jars	33**±0.41	74.24±0.55	66.43**±0.23
Magenta box	36**±0.99	70.82**±0.38	59.96**±0.48
Growtek bioreactor	34**±0.15	74.0*±0.69	68.19**±0.65
<i>In vivo</i> sample	5.30**±0.42	54.12**±0.81	35.83**±0.77
F value	1,860 ^c	515 ^c	1,610 ^c

^a Non-significant ($P>0.05$)^b Significant ($P<0.05$)^c Significant ($P<0.001$)

* or ** Means are significantly different when compared with control at ($P<0.05$) or ($P<0.001$), according to Dunnett multiple comparison test. Mean data without * are non-significantly different from the control value

Total phenolic content The dried, powered samples (100 mg) were extracted for 4 h at room temperature by shaking on a test tube rotator with 5 ml of 60 % methanol. The sample suspensions were centrifuged at 10,000 g for 15 min at 10 °C and supernatants were filtered through Whatman no.1 filter paper and the filtrate was stored at -20 °C till analysis. The samples were analyzed for total phenolic content (TPC) using spectrophotometer by the method of Farkas and Kiraly 1962. Gallic acid was used as the reference standard. All determinations were carried out in triplicate and the results are expressed as mg gallic acid equivalent per gram (mg GAE/g) of extract.

DPPH radical scavenging activity DPPH radical scavenging activity was determined according to method described previously (Sreeramulu et al. 2009). This method is based on the ability of the antioxidant to scavenge the DPPH

Fig. 2 Correlation between TPC and DPPH assays. Correlation coefficient $R=0.8763$

Fig. 3 Correlation between TPC and SOD assays. Correlation coefficient $R=0.8374$

cation radical. Percentage inhibition by the sample extract was calculated.

Superoxide radical scavenging activity Superoxide radical scavenging activity was determined according to method described by Jain et al. 2008. Superoxide anions were generated using PMS/NADH system and were subsequently made to reduce NBT which yields a chromogenic product, which is measured at 560 nm. Percent inhibition was calculated.

Statistical analysis All the data were recorded in triplicate ($n=3$). The data was analysed by analysis of variance followed by Dunnett multiple comparison test (comparing all versus control) using Prism statistical software. To correlate the results of antioxidant potential, obtained with different

Fig. 4 Correlation between SOD and DPPH assays. Correlation coefficient $R=0.9352$

Table 5 Correlation coefficient (R) between assays

	SOD	DPPH
SOD		0.9352
TPC	0.8374	0.8763

methods, a regression analysis was performed and correlation coefficients were calculated.

Result and discussion

Effect of BAP Induction of shoots occurred from stem and leaf explants on almost all the media tested. Irrespective of shoot formation, initially cultures grew slowly. The results obtained with varying concentrations of BAP incorporated in MS medium on shoot regeneration from stem explants are presented in Table 1. Maximum number of shoots per explant, higher explant response irrespective of the type of explants, and higher shoot length was obtained on MS medium containing BAP (2.5 mg l^{-1}) and IAA (0.01 mg l^{-1}) with 3 % sucrose (Table 1). Therefore, this medium and nodal explants were used in the next experiment. At 10 mg l^{-1} concentration of BAP in the medium, callusing occurred.

Explant number and size The explants of varying size and number were used in 40 ml MS medium containing BAP (2.5 mg l^{-1}) and IAA (0.01 mg l^{-1}) based on results shown in the Table 1. The results obtained are presented in the Table 2. It is evident from the results that the condition of 0.5 cm explant size and 20 explant/40 ml (1 explant/2 ml) was optimal for high explant response; number of shoots/explant regenerated and shoots length. Higher number of explants (30) was not observed favorable for growth as compared to 10 explants/40 ml medium. Increasing the size of explants did not enhance shoots number in the same proportion and use of large sized explants also consumed more primary material for the culture.

Time course of growth The explants transferred on MS medium containing BAP (2.5 mg l^{-1}) and IAA (0.01 mg l^{-1}) with 3 % sucrose showed rapid growth during 3 weeks period (Fig. 1). During this period, shoot number and dry mass production increased rapidly which slowed down during next 20 days. After 40 days, cultures showed decline in growth.

Vessels type The results obtained with number of shoots and biomass production using different culture vessels are presented in Table 3. Among the different vessels used, maximum GI (10.0) was recorded in Growtek bioreactor followed by magenta box (9.16), conical flask 100 ml (8.5), glass jars (7.66), 250 ml conical flask (7.16) (Fig. 5). Variable results

were recorded in relation to the number of shoots l^{-1} and vessel type. However, significantly higher number ($\sim 2,000$) of shoots l^{-1} was recorded in Growtek bioreactor followed by magenta box. It is evident from Fig. 5 that the cultures grew well directly in liquid medium, irrespective of vessel type and amount of liquid medium. However, Growtek bioreactor was provided with 10 % v/v aeration because of large volume of the liquid medium (Fig. 5d, e).

Plant tissue culture vessels with their caps or closures make the boundaries between the internal microenvironment and the external environment of outside air (Huang and Chen 2005). They have reported that the conical glass flask had the smallest air exchange rate and the rectangle box had the largest value. But the uniformity of internal transmittance for round vessels was better than that of square or rectangular box. The type of vessel and lid affects the gaseous composition inside the vessel as well as light penetration. Therefore, the growth of tissues in culture (shoot elongation, proliferation, fresh weight increase, and possibly the hyperhydric degradation processes) is also affected (Islam et al. 2005). In the present study, Growtek bioreactor with 10 % aeration showed maximum shoots and growth than other culture vessels.

Though, there are reports about regeneration of shoots (Praveen et al. 2009; Ceasar et al. 2010) and bacoside content (Parale et al. 2010) in organized or unorganized cultures of *B. monnieri*, no attempt has been made to optimize vessel or correlate the growth with antioxidant potential of the regenerated biomass. It is evident from the available publication that requirement for plant growth regulators and type of explants varied in different reports (Praveen et al. 2009; Ceasar et al. 2010). It may be inferred that genetic variation in the plant may be responsible for these variation. No proper selection of cultivars has been done and the plant material is collected from wild. The plant propagated vegetatively, hence ex vitro rooting of shoots directly in soil can be applied. This technology can be used for uniform biomass generation and about 2,000 shoots l^{-1} with 16.5 g l^{-1} dry weight was achieved in the present work.

In the present work stem explants were used though leaf explants were shown as good choice of explants in previous works (Praveen et al. 2009; Ceasar et al. 2010). Firstly, leaf explants require two stage culture systems; stage I involve growth on static medium and stage II is transfer from static to liquid medium. Secondly, shoot buds produced from leaf explants were too small, making impossible to count (Praveen

Fig. 5 Shoot culture of *B. monnieri* in different culture vessels: **a** 250ml Conical flask, **b** Magenta box, **c** Glass jar, **d-f** Growtek bioreactor with aeration, filter, light arrangement and culture taken out with raft

et al. 2009) and take long time to become shoots. In present work, dry biomass production was higher or comparable to earlier works (Table 3).

Total phenolic content and antioxidant capacity The total phenolic content as well as antioxidant capacity of *in vitro* regenerated plants was observed maximum as compared to *in vivo* plants. The total phenolic content of *in vitro* regenerated plants grown in 250 ml conical flask was 55 mg GAE/g as compared to 5.3 mg GAE/g recorded for *in vivo* plant, showing a ~10 fold higher activity in *in vitro* system (Table 4). *In vitro* system not only offers an ideal system for biomass production but also a potential system for the higher phenolic content/antioxidant activity. Therefore, this system can be used for large scale biomass production of this medicinal plant instead of field collection of heterogeneous material. The results of regression analysis are shown in Figs. 2, 3 and 4 and Table 5. Maximum correlation was found between SOD and DPPH ($R=0.9352$, Figs. 4 and 5) showing that the two assays are strongly correlative. The correlation between TPC and DPPH assay was also significant having correlation coefficient ($R=0.8763$, Fig. 2). The lowest correlation was found between TPC and SOD ($R=0.8374$, Fig. 3). A high positive correlation between free radical-scavenging activity and the total concentration of phenolic compounds in plant extracts has also been reported by other workers (Wangenstein et al. 2004; Zheng and Wang 2001). It may be concluded from the present work that the *in vitro* culture of *B. monnieri* in large vessel size can be used for biomass generation with potential antioxidant activity.

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