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Standardization of an efficient protocol for *in vitro* clonal propagation of *Bacopa monnieri* L. - an important medicinal plant

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The present study was undertaken to evaluate the most suitable concentration of growth regulators for callus induction and subsequent organogenesis in *Bacopa monnieri* L. (commonly known as Brahmi). The best callus induction (71±2.2%) was found in MS medium supplemented with 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA from leaf explant. Combination of 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA and 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} IAA gave the most effective for shoot regeneration from callus. The elongated shoots rooted in ½ strength MS medium supplemented with different concentrations of auxins (NAA, IAA and IBA). IAA (6.5±0.57) was more suitable for root induction when compared to NAA and IBA (5.1±0.32 and 4.7±0.44). The success of plant tissue culture for *in vitro* culture of *B. monnieri* was encouraged by acclimatization of the plantlets in the field conditions. About 86% plantlets survived under field conditions.

Key words: *Bacopa monnieri* L., callus induction, *in vitro*, leaf explants, micropropagation

Introduction

Plants are the traditional source for many chemicals used as pharmaceuticals, biochemicals, fragrances, food colours and flavours. Most valuable phytochemicals are products of secondary metabolism and possess sufficient chemical or structural complexity, so that artificial synthesis is difficult (Leung, 1980). Plant tissue culture techniques now play an important role in the clonal propagation and quantitative improvement of the medicinally important plant. Though the conventional breeding techniques have considerably increased the productivity of modern crops, the application of biotechnology could speed up further crop improvement. It overcomes the

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barriers in conventional vegetative propagation and fulfils the demand for large scale cultivation in a short period by rapid multiplication. To date we can speed up the production rate of the average plant by approximately 10,000 times and a large number of productive plants can be multiplied routinely through tissue culture (Rao *et al.,* 1996).

Bacopa monnieri L. belongs to family Scrophulariaceae and is widely distributed in the warmer parts of Asia, Australia and America. It is an important medicinal creeping herb with light purple flowers, and in India commonly called as Brahmi. The plants have been selected based on their use in traditional systems of medicine, and research has identified a number of natural compounds that could act as nootropic agents[3]. Pharmacologically, it is understood that Brahmi has an unusual combination of constituents that are beneficial in curing mental inefficiency and illnesses and useful in the treatment of convulsive disorders like epilepsy (Anonymous, 2004). The plant has also been used in India and Pakistan as a cardiac tonic, digestive aid, and to improve respiratory function in cases of bronchoconstriction (Mukherjee and Dey, 1966). In Ayurveda, the plant has been used in the treatment of insanity, hysteria, sedative, vasoconstrictor and anti-inflammatory (Chopra *et al.,* 1956). The plant is reported to contain tetracyclic triterpenoid saponins, bacosides A, B, C and D, hersaponin, alkaloids viz. Herpestine and brahmine and flavonoids (Chatterjee *et al.,* 1965; Basu *et al.,* 1967; Rastogi *et al.,* 1994).

Earlier *in vitro* propagation of *Bacopa monnieri* was attempted through organogenesis from different explants such as shoot tips, axillary nodes, leaves and callus (Binita *et al.,* 2005; Debnath, 2008; Escandon *et al.,* 2006). The present study was aimed at developing a simple, rapid and an efficient protocol of plant regeneration from leaf explants of *Bacopa monnieri.*

Materials and methods

Plant material and explants

Young and healthy plants collected from Government Nursery, Ghatikia, Bhubaneswar, Orissa and raised in pots containing soil and farmyard manure (1:1) in the Botanical and research garden of Utkal University, Orissa, India. The young healthy leaves of *B. monnieri* were selected as explants for inoculation. Leaf explants were surface sterilized by cleaning thoroughly under running tap water for 15 min followed by immersing in 10% (v/v) detergent solution labolene (Qualigens fine Chemicals, Mumbai, India) for 5-7 min. They were washed thrice with double distilled water and kept in a laminar air flow chamber. The cleaned explants were finally treated with 0.1% (w/v) $HgCl_2$ for 5 minutes under aseptic conditions (inside laminar air flow) and washed five

times with sterile double distilled water to remove traces of $HgCl_2$ before inoculation.

Medium and culture condition

Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) containing 3% sucrose was used in all the experiments. All the plant growth regulators were filtered before use through 0.2 µm filter membrane (Minisart, Sartorius). The pH of the medium adjusted to 5.8±0.2, and were solidified with 0.8% (w/v) agar (Hi media, India). Molten medium was dispensed in 150 ml Erlenmeyer flasks (Borosil, Bangalore, India) and were plugged with non-absorbent cotton wrapped in one layer of cheesecloth. The medium was autoclaved at 1.16 kg/cm^2 pressure and 121^{0}C temperature for 15 min. the cultures were incubated under 16 h photoperiod in cool white fluorescent light (55 µmol m^{-2}s^{-1}) (Phillips, India) and maintained a constant temperature of 25±2^{0}C.

Callus induction

The leaf explants were placed on semisolid MS basal medium supplemented with different concentrations (1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75 and 3.0 mg l^{-1}) of benzylaminopurine (BAP) or Kinetin (1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75 and 3.0 mg l^{-1}) alone for callus induction. Next, different concentrations (0.25, 0.5 and1.0 mg l^{-1}) of naphthalene acetic acid (NAA) or indole 3-acetic acid (IAA) combining with BAP (2.0 mg l^{-1}) were tried for callus proliferation. The percentage of callus formation was calculated after 6 weeks of inoculation and howmany days was taken for callus induction was observed in every two days interval after 1 week of inoculation .

In vitro shoot regeneration

Calli were subdivided into five pieces (5-6mm) and placed in culture flask (150 ml Erlenmeyer flasks) containing 30ml of MS medium along with different concentration and combinations of NAA (0.25, 0.5, 1.0, 1.5 and 2.0 mg l^{-1}), IAA(0.25, 0.5, 1.0, 1.5 and 2.0 mg l^{-1}) and 2.0 mg l^{-1} BAP to induce adventitious shoot regeneration. The mean number of shoots and the % of shoot production from callus tissue were evaluated after 5 weeks of inoculation.

Rooting

For rooting, the *in vitro* raised shoots (3-5cm long) were excised and transferred to ½ strength MS basal medium containing 2% (w/v) sucrose and 0.8% (w/v) agar. The medium was further supplemented with individual

concentrations of 0.5-3.0 mgl^{-1} (NAA), (IAA) and indole 3-butyric acid (IBA). All the cultures were incubated at 25±2^{0}C under 16 h photoperiod with cool and white fluorescent lamps, as above. After 3 weeks of culture, the number of roots per shoot and their lengths were recorded.

Hardening and acclimatization

Selected rooted plants were harvested from rooting medium and washed in running tap water to remove agar. The plants were then transferred to pots containing a mixture of sterilized sand: soil: dry powdered cow dung (1:1:1). The potted plantlets were kept under transparent polythene to maintain humidity. Hardening was continued for 2 weeks with mild irrigation at 2-day interval and supplied with ¼ strength MS inorganic solution twice a week during the initial period until they were successfully acclimatized. After one month, the surviving plants were transferred to garden soil.

Statistical analysis

Experiments were setup in a Randomized Complete Block Design (RCBD) and each experiment was repeated two times. Data were recorded on the percentage of response, number of shoots per explants, number of roots and root length per shoot. Means and Standard errors were carried out for each treatment.

Results and discussion

Callus induction

Leaf explants were cultured on MS medium containing different concentrations of BAP and Kin alone or in various combinations with NAA or IAA for callus induction. Data were analyzed in every two days up to callus initiation and then finally after five weeks of culture for analysis the percentage of explants induced to develop callus. Morphogenic potentialities of the explant were found to differ depending on different growth supplementations (Table 1). Among the different concentrations of cytokinins (BAP/Kin) tried, BAP was found highly effective for initiation of callus (Fig. 1A). It was mostly soft, faster growing, light creamy-green and friable (Fig. 1B). The frequency of callus induction (63±4.5%) was recorded at the end of 6 weeks on medium containing 2.0 mg l^{-1} BAP. This result was more or less similar to others (Aloufa, 2002; Nagmani and Venketeswaran, 1987). The highest 58±0.98% callusing rate was observed in *Bacopa monnieri* on 1.5 mg l^{-1} Kin. Where a single concentration of BAP (2.0 mg l^{-1}) along with different concentrations

of NAA or IAA were applied, the maximum callusing rate of 71±2.2% was observed on 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA which was more than the 2.0 mg l^{-1} BAP. The role of auxin alone or in combination with cytoknin for callus proliferation is well documented (Hammerschlag *et al.,* 1985; Jain *et al.,* 1988; Niedz *et al.,* 1989; Verhagen and Wann, 1989; Roy and De, 1990). Individual effect of NAA and IAA on callus induction was poor.

Table 1. Effect of different concentration and combination of growth regulators on MS medium for callus induction through leaf explants of *B. monnieri*. The data were recorded 6 weeks after culture.

Growth regulators (mg/l)				**% of callus formation from explants (mean ± SE)***	**Days to callus induction ***
BAP	**Kin**	**NAA**	**IAA**		
Growth regulator free medium				--	--
1.0	--	--	--	34±5.1	14
1.25	--	--	--	41±2.5	14
1.5	--	--	--	43±2.2	14
1.75	--	--	--	56±2.8	14
2.0	--	--	--	63±4.5	14
2.25	--	--	--	37±0.56	14
2.5	--	--	--	22±0.76	20
2.75	--	--	--	--	--
3.0	--	--	--	--	--
--	1.0	--	--	41±4.8	--
	1.25	--	--	55±3.5	--
--	1.5	--	--	58±0.98	12
--	1.75	--	--	32±2.1	12
--	2.0	--	--	27±4.3	12
--	2.25	--	--	11±3.3	20
--	2.5	--	--	11±2.9	20
--	2.75	--	--	--	--
--	3.0	--	--	--	--
2.0	--	0.25	--	67±2.5	12
2.0	--	0.5	--	71±2.2	12
2.0	--	1.0	--	60±3.0	12
2.0	--	--	0.25	43±5.5	12
2.0	--	--	0.5	52±3.4	12
2.0	--	--	1.0	52±2.9	12

*Mean value ±SE of 3 replicates. Mean followed by same letter are not significantly different by Duncan's Multiple range test at 5% level. -- = No treatment (without any concentration of growth regulator).

Organogenesis of the shoots from callus tissue

Calli produced under light conditions was transferred to MS medium supplemented with various concentrations of auxins (NAA, IAA) along with a constant combination of BAP (2.0 mg l^{-1}). During this investigation shoot formation was highly influenced by concentrations and type of the growth regulators used in the experiment (Fig. 1C). Among the different concentrations and combinations, the best performance for shoot multiplication was showed in MS medium supplemented with 2.0 mg l^{-1} BAP+ 0.5 mg l^{-1} NAA. On this combination the percentage of explants produced shoot was 61±1.1. The number of shoots per callus was 16.7±1.46 (Table 2). But when different concentrations of IAA was applied along with 2.0 mg l^{-1} BAP, 54±4.4% of explant respond for shoot formation and the number of shoots per explant was 54±4.4 14.2±0.73. Cytokinin formulations were earlier shown to be critical for shoot elongation of many other plant species, including medicinal plants (Jha and Jha, 1989; Sharma *et al.,* 1993; Chen *et al.,* 1995; Saxena *et al.,* 1998; Rout *et al.,* 2000). Inclusion of either NAA or IAA in the culture medium along with BAP favoured shoot multiplication. Many authors report that cytokinin is required in optimal quantity for shoot proliferation in many genotypes but inclusion of a low concentration of auxin along with cytokinin increases the rate of shoot multiplication (Tsay *et al.,* 1989; Shasany *et al.,* 1998; Sharma and Singh, 1997).

Rooting

For root induction, well developed *in vitro* shoots were excised and cultured on ½ strength MS medium supplemented with various concentrations of NAA, IAA and IBA for root induction. Among different concentrations of auxins, IAA was found to be comparatively better response than NAA and IBA for producing roots. Best rooting was observed with half strength of MS medium supplemented with 2.0 mg l^{-1} IAA. In this combination, it was observed that the number of roots was 6.5±0.57 and average root length of 7.6±0.62cm after three weeks of culture (Table 3, Fig. 1D). The findings are in agreement with those observed in other plant species such as *Caphaelis* ipecacuanha (Jha and Jha, 1989) and *Plantago ovata* (Wakhlu and Barna, 1989).

Hardening and acclimatization

More than 93% of plantlets survival was observed on hardening in sterile mixture of sterilized sand: soil: dry powdered cow dung (1:1:1) (v/v) for one week. However, the rate of survival decreased to 7% after two-three weeks of acclimatization. It was observed that gradual acclimatization of *in vitro*

grown plants to external environment is mostly essential for *B. monnieri.* About 86% of the plants transferred to pots were survived and showed normal growth (Fig. 1 E, F).

In conclusion, an efficient protocol for micropropagation of an important medicinal plant, *B. monnieri,* was developed in this study by testing various concentrations of growth regulators and culture conditions. This protocol provides a successful and rapid technique that can be used for mass propagation of elite species and could permit genetic transformation studies, which so far have been limited.

Fig. 1. Callus induction and plantlet regeneration from leaf explants of *Bacopa monnieri* L. **A**. Swelling of leaf explants on MS medium with 2.0 mg l^{-1} BAP+.0.5 mg l^{-1}NAA. **B**. Callus development after 6 weeks of culture on MS medium with 2.0 mg l^{-1} BAP+.0.5 mg l^{-1} NAA. **C**. Shoot regeneration in MS + 2.0 mg l^{-1} BAP+.0.5 mg l^{-1}NAA after 5 weeks of culture. **D**. Root induction on ½ MS medium with IAA 2.0 mg L^{-1}. **E**. *In vitro* plantlet hardened in the potting medium containing a mixture of sterilized sand: soil: dry powdered cow dung (1:1:1). **F**. hardened plantlets in garden soil after 3 month of transfer.

Table 2. Effect of different concentrations of cytokinin (BAP) and auxins (NAA, IAA) for shoot regeneration from the callus of *B. Monnieri* after 5 weeks of culture.

BAP (mg/l)	**NAA (mg/l)**	**IAA (mg/l)**	**% of callus tissue producing shoots**	**Mean No. of shoots/callus (mean ± SE)***
Without any growth regulator			11±3.0	2.2±0.33
2.0	0.25	--	32±5.2	14.7±0.66
2.0	0.5	--	61±1.1	16.7±1.46
2.0	1.0	--	39±2.7	12.99±0.83
2.0	1.5	--	34±0.9	6.8±0.22
2.0	2.0	--	15±4.8	4.5±1.30
2.0	--	0.25	37±5.2	13.7±0.23
2.0	--	0.5	54±4.4	14.2±0.73
2.0	--	1.0	31±3.2	11.9±0.78
2.0	--	1.5	22±1.8	9.2±2.11
2.0	--	2.0	20±1.4	8.1±0.62

*Mean value ±SE of 3 replicates.
Mean followed by same letter are not significantly different by Duncan's Multiple range test at 5% level.
-- = No treatment (without any concentration of growth regulator).

Table 3. Effect of auxins (NAA, IAA and IBA) on rooting of *in vitro* raised shoots of *B. Monnieri* on ½ strength MS medium (2% sucrose) after 3 weeks of culture.

NAA (mg/l)	IAA (mg/l)	IBA (mg/l)	Mean no. of roots/ shoot*	Mean length of roots (cm)
No growth regulators			1.2±0.52	2.1±0.31
0.5	--	--	1.4±0.10	2.9±0.39
1.0	--	--	4.2±0.69	3.3±0.22
1.5	--	--	4.5±0.50	5.1±0.32
2.0	--	--	5.1±0.32	6.3±0.65
2.5	--	--	3.2±0.54	3.0±0.27
3.0	--	--	3.0±0.23	3.0±0.56
--	0.5	--	2.2±0.65	3.4±0.44
--	1.0	--	3.7±0.35	4.2±0.23
--	1.5	--	6.8±0.72	7.0±0.55
--	2.0	--	6.5±0.57	7.6±0.62
--	2.5	--	3.8±0.23	5.5±0.16
--	3.0	--	3.8±0.19	3.2±0.33
--	--	0.5	1.6±0.22	2.2±0.47
--	--	1.0	2.2±0.45	3.9±0.41
--	--	1.5	3.6±0.50	4.3±0.54
--	--	2.0	4.7±0.44	4.2±0.38
--	--	2.5	2.1±0.20	2.0±0.28
--	--	3.0	2.0±0.33	1.8±0.16

*Mean value ±SE of 3 replicates.
Mean followed by same letter are not significantly different by Duncan's Multiple range test at 5% level.
-- = No treatment (without any concentration of growth regulator).

figure legends

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