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Micropropagation and Assessment of Antibiotic Selection *in Vitro* of *Bacopa monnieri* (L.) Pennell

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ABSTRACT

Bacopa monnieri (L.) Pennell (Brahmi), Scrophulariaceae, is one of the sources of *medhya rasayan* drugs (that counteract stress and improves intelligence and memory) of Ayurveda. The aim of the present study was to assess tissue culture conditions and antibiotic selection which could be useful for genetic transformation of this important medicinal plant. An efficient, simple and reproducible system for plant regeneration through leaf and nodal explants was devised. Sensitivity of the nodal explants against cefotaxime (Cef), kanamycin (Kan) and hygromycin (Hyg) was established. Leaf and nodal explants produced about 90 shoots on Murashige and Skoog (MS) medium fortified with 1.0 mg/L 6-benzyladenine (BA) after 28 days of incubation. Subculture to fresh MS medium resulted in shoot elongation; the shoots obtained grew well and were healthy. The transfer of shoots to liquid MS medium supplemented with 0.2 mg/L α-naphthaleneacetic acid (NAA) resulted in 100% rooting. The rooted shoots, on transfer to plastic containers containing a mixture of garden soil + sand (1:1), could acclimatize within 14 days under glasshouse conditions. The acclimatized *in vitro*-grown plants showed 100% survival after transfer to soil in earthen pots. A threshold limit of survival of nodal explants was observed at 500 mg/L Cef, 100 mg/L Kan and 15 mg/L Hyg.

Keywords: cefotaxime, hygromycin, kanamycin, nerve tonic, plant regeneration
Abbreviations: BA, 6-benzyladenine; **Cef**, cefotaxime; **Hyg**, hygromycin; **IAA**, indole-3-acetic acid; **Kan**, kanamycin; **Kin**, kinetin; **MS**, Murashige and Skoog; **NAA**, α-naphthaleneacetic acid; **SRM**, shoot regeneration medium; **TDZ**, thidiazuron

INTRODUCTION

In Indian medicine, certain herbs are used traditionally as brain or nerve tonics. One of the most popular of these is *Bacopa monnieri* (L.) Pennell, which belongs to the Scrophulariaceae family. It is a small, amphibious plant growing in marshy areas. It is one of the sources of the *medhya rasayan* drug of Ayurveda prescribed for a variety of therapeutic indications including epilepsy, insanity and memory enhancement (Satyavati *et al*. 1976). The plant is also used for nervous breakdown, dermatitis, antiulcerogenic and adaptogenic activities (Russo and Borelli 2005). It is also reported to possess antiinflamatory, analgesic, antipyretic, sedative (Kishore and Singh 2005; Ganjewala and Srivastava 2011), free radical scavenging and lipid peroxidative activities (Anbarasi *et al*. 2005).

Phytochemical studies have shown that *Bacopa* contains many active constituents including alkaloids, brahmine, herpestine and saponins. The saponins include bacoside A, A3 and B, bacopasaponin A to F, betulic acid, β-sitosteron and stigmasterols (Chatterji *et al*. 1963; Jain and Kulshreshtha 1993). Bacoside A yields bacogenins A1, A2, A3 and A4 upon hydrolysis (Chatterji *et al*. 1965). The pharmacological properties of Brahmi are mainly due to the presence of major bioactive saponins called bacosides and bacoside A is a major chemical entity shown to be responsible for memory-facilitating action of Brahmi (Singh and Dhawan 1997).

Today medicinal plants are important to the global economy (Nalawade and Tsay 2004; Bapat *et al*. 2008), as approximately 85% of traditional medicine preparations involve the use of plants or plant extracts (Vieira and Skorupa 1993). This leads to a sudden rise in demand for herbal medicines (Srivastava and Shrivastava 2008). *B. monnieri* was placed second in a priority list of the most important Indian medicinal plants evaluated on the basis of their medicinal importance, commercial value and potential for research and development (Anonymous 1997).

Metabolic engineering is one of the important approaches to improve and modify secondary metabolite contents of medicinal and aromatic plants. *Agrobacterium*-mediated genetic transformation is an effective and widely used method to transfer a desirable foreign gene into the plant genome (Qin *et al*. 2011). Establishment of efficient regeneration protocol and antibiotic conditions for elimination of *Agrobacterium* in explants after infection are the key factors for selection and recovery of genetically modified plants (Opabode 2006; Karami *et al*. 2009; Qin *et al*. 2011).

Antibiotics are useful for control of bacterial growth and as selective agents in transformation studies, but they also have significant effects on regeneration capacity of explants and plant growth (Teixeira da Silva *et al*. 2003). Antibiotic sensitivity of plants is depends on plant species, type of explant, growth conditions and culture system (Teixeira da Silva and Fukai 2001; Grewal *et al*. 2006; Qin *et al*. 2011). Therefore, it is necessary to first screen the type and concentration of antibiotics with least phytotoxic effects on plants and that can effectively control the growth of *A. tumefaciens* in the explants after infection (Qin *et al*. 2011).

Reports are available on shoot regeneration but meager information is available on genetic transformation in *B. monnieri*. Therefore, in the present study, efforts were made to develop an efficient protocol of shoot regeneration and antibiotic selection in *B. monnieri*.

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Original Research Paper

MATERIALS AND METHODS

Plant material and shoot regeneration

Healthy plantlets of *B. monnieri* were obtained from the National Research Institute of Basic Ayurvedic Sciences, Pune. The plants were potted in earthen pots containing garden soil and maintained in the glasshouse of the Department of Botany, University of Pune, Pune. Leaf and nodal explants were excised from healthy plants and washed with running tap water then washed thoroughly with 0.01% (v/v) Tween-20 (HiMedia, Mumbai, India) solution followed by sterilized distilled water. Explants were then surface-sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) (Qualigens, Mumbai, India) solution for 5 min and washed 5–6 times with sterilized distilled water to remove the traces of $HgCl_2$. All the chemicals and reagents including antibiotics were purchased from HiMedia and plant growth regulators were procured from Sigma-Aldrich (Banglore, India).

The surface-sterilized leaf and nodal explants were cultured onto MS (Murashige and Skoog 1962) medium supplemented without and with different concentrations (0.1–5.0 mg/L) of cytokinins (6-benzyladenine, BA; Kinetin, Kin; thidiazuron, TDZ) and 3% (w/v) sucrose. The pH of medium was adjusted to 5.8 and solidified with 0.8% (w/v) agar–agar prior to autoclaving at 121°C for 15 min. The cultures were incubated under controlled conditions such as 25 ± 2°C, 60 ± 10% relative humidity, and an 8-h photoperiod (photon flux density (PFD) = 40 µmol m^{-2} s^{-1}) provided by white fluorescent tubes (Philips, Kolkata, India).

The shoots obtained were sub-cultured regularly at 28-day intervals on MS liquid medium supplemented with 1.0 mg/L BA in culture glass bottles (400 mL capacity). Shoot cultures were maintained over a period of 2 years.

Root initiation and acclimatization

Well developed shoots (4-6 cm in height) were separated and transferred for rooting onto MS liquid medium supplemented with auxins α-naphthaleneacetic acid (NAA) or indole-3-acetic acid (IAA) (0.1–0.5 mg/L) or lacking them. Observations recorded were: percentage rooted shoots, mean number of roots/shoot and average root length after 28 days of incubation.

Individual plantlets were transferred to plastic containers (7 cm diameter × 9 cm height) containing autoclaved garden soil + sand mixture (1:1) and placed in a glasshouse (25 ± 5°C; humidity 80 ± 10%; maximum PFD = 200 µmol m^{-2} s^{-1}). After one month acclimatization, plantlets were transferred to the earthen pots (28 cm diameter × 30 cm height) containing garden soil in a glasshouse and subsequently transplanted to the field (18-32°C; humidity 55-80%; maximum PFD = 400 µmol m^{-2} s^{-1}) after one month's growth. All plantlets were watered three times a day with tap water.

Antibiotic sensitivity

Stock solutions of antibiotics were prepared by dissolving them in sterile double distilled water and filter sterilized with Millex® GV 0.22 µm Durapore® PVDF membrane syringe driven filter (Millipore, Carrighwahill, Co. Cork, Ireland). All antibiotics were added in pre-sterilized shoot regeneration medium (SRM; MS + 1.0 mg/L BA) at several concentrations (cefotaxime (Cef): 250, 500, 750, 1000 mg/L; kanamycin (Kan): 25, 50, 75, 100, 125, 150 mg/L and hygromycin (Hyg): 5, 10, 15, 20, 25 mg/L) separately and poured in sterile Petri dish (10 cm diameter, Axygen, New Delhi, India). SRM without any antibiotic served as the control. Ten nodal explants were inoculated per Petri dish and 4 Petri dishes were used for each concentration. The Petri dishes were sealed

Table 1 Effect of BA, Kin and TDZ on shoot regeneration in leaf and nodal explants of *B. monnieri*.

Cytokinins (mg/L)	Explants			
	Leaf		Node	
	Shoot regeneration frequency (%)	No. of shoot buds/explant	Shoot regeneration frequency (%)	No. of shoot buds/explant
BA				
0	72	08.7 ± 0.4	71	08.3 ± 0.6
0.1	85	39.7 ± 0.5 j	82	38.6 ± 0.4 i
0.2	94	53.8 ± 0.3 h	93	51.7 ± 0.6 h
0.3	100	61.2 ± 0.3 g	100	59.9 ± 0.8 g
0.4	100	74.9 ± 0.5 d	100	73.2 ± 0.2 d
0.5	100	82.1 ± 0.2 c	100	81.2 ± 0.3 c
1.0	100	95.4 ± 0.1 a	100	90.7 ± 0.7 a
2.0	100	86.9 ± 0.6 b	100	84.3 ± 0.9 b
3.0	100	71.3 ± 0.6 e	100	69.8 ± 0.5 e
4.0	100	64.8 ± 0.7 f	100	61.6 ± 0.7 f
5.0	100	42.7 ± 0.4 i	100	39.6 ± 0.2 i
Kin				
0.1	73	07.9 ± 0.5 i	79	06.3 ± 0.3 h
0.2	89	09.4 ± 0.3 h	88	08.6 ± 0.5 g
0.3	95	14.3 ± 0.5 e	97	13.2 ± 0.7 e
0.4	100	18.2 ± 0.1 d	100	17.6 ± 0.3 d
0.5	100	23.7 ± 0.6 b	100	21.9 ± 0.2 b
1.0	100	27.9 ± 0.8 a	100	25.4 ± 0.6 a
2.0	100	24.7 ± 0.5 b	100	21.6 ± 0.7 b
3.0	100	21.5 ± 0.3 c	100	19.9 ± 0.4 c
4.0	100	19.8 ± 0.9 d	100	17.3 ± 0.6 d
5.0	100	12.1 ± 0.3 g	100	10.9 ± 0.2 f
TDZ				
0.1	100	87.3 ± 0.7 a	100	85.6 ± 0.2 a
0.2	100	79.4 ± 0.5 b	100	76.8 ± 0.6 b
0.3	100	75.2 ± 0.3 c	100	72.1 ± 0.7 c
0.4	100	64.3 ± 0.7 d	100	60.9 ± 0.4 d
0.5	100	42.6 ± 0.2 e	100	38.7 ± 0.3 e
1.0	96	29.5 ± 0.7 f	94	25.1 ± 0.2 f
2.0	87	23.9 ± 0.4 g	89	21.4 ± 0.4 g
3.0	74	17.5 ± 0.8 h	73	15.7 ± 0.6 h
4.0	56	13.2 ± 0.6 i	58	11.9 ± 0.7 i
5.0	43	09.6 ± 0.3 j	47	07.8 ± 0.7 j

BA: 6-benzyladenine; Kin: kinetin; TDZ: thidiazuron. The values represent the mean ± SE calculated on three independent experiments, each based on a minimum of 21 replicates. Means followed by the same letters within columns are not significantly different at the 5% level (DMRT). DMRT was applied to BA, Kin and TDZ separately.

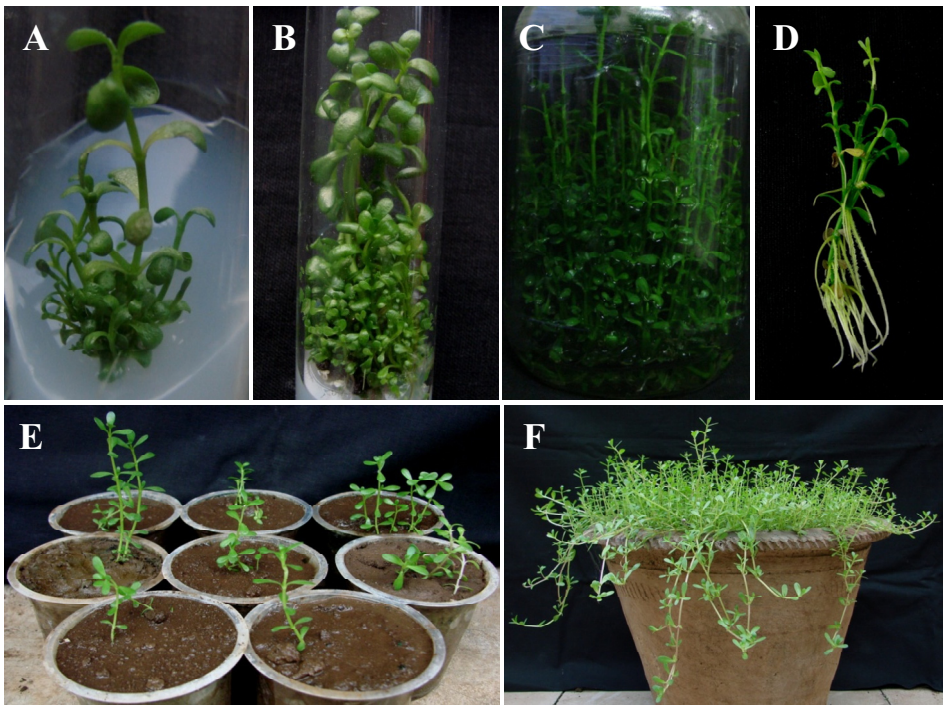

Fig. 1 ***In vitro*** **shoot regeneration from nodal explants of** ***Bacopa monnieri.*** (**A**) Nodal explants on MS + 1.0 mg/L BA after 28 days of inoculation. (**B**) Nodal explants on MS + 1.0 mg/L BA after second subculture at 28 days. (**C**) Shoot culture maintained on liquid MS + 1.0 mg/L BA in culture bottles. (**D**) Rooted shoots on MS + 0.2 mg/L NAA. (**E**) *In vitro* raised plantlet in plastic glass after one month growth in glass house. (**F**) *In vitro* raised plant in earthen pot in natural condition after 4 months growth.

with Parafilm "M" (Pachiney, Chicago, USA) and placed under controlled conditions as described earlier. Necrosis of tis-sues was recorded for 28 days after culture initiation at 7-day intervals.

Data analysis

A completely randomized design (CRD) was used in all experiments. The experiments were repeated at least thrice. Data were subjected to analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) at $P \leq 0.05$.

RESULTS AND DISCUSSION

Shoot regeneration

Rapid progress in the area of crop biotechnology is mainly because of the development of efficient regeneration and suitable *Agrobacterium*-mediated transformation protocols for different crop plants (Hiei *et al*. 1997). Similar success can be achieved in medicinal plants by developing efficient regeneration and *Agrobacterium*-mediated transformation protocols, which in turn could be used for the enhancement of their secondary metabolite content. The shoot regeneration response of leaf and nodal explants cultured on MS basal medium supplemented with different concentrations of BA, Kin and TDZ is depicted in **Table 1**. MS medium without supplementation of plant growth regulators induced shoots from leaf and nodal explants within 21 days of incubation. Supplementation of cytokinins in medium accelerates shoot bud induction from both the explants.

The variable degree of shoot regeneration response was observed on different type and concentrations of cytokinins in medium, among which BA showed most prominent response for shoot regeneration. Similar results were obtained by earlier workers in *B. monnieri* (Tiwari *et al*. 1998; Joshi *et al*. 2010; Sharma *et al*. 2010). BA was previously reported to be important for shoot regeneration in several plants (Stefaan *et al*. 1994). Kin was also found suitable for shoot regeneration; however, the response was comparatively poorer than BA. In contrast to this Naik *et al*. (2010) reported the maximum shoot regeneration was achieved on Kin containing medium in *B. monnieri*. Among the different concentrations of BA, supplementation of 1.0 mg/L BA was found to be optimum for shoot bud induction and elongation. About 95.4 ± 0.1 shoots were obtained after 28 days of incubation from leaf explants and 90.7 ± 0.7 shoot from nodal explants (**Table 1; Fig. 1A, 1B**). Lower and higher concentrations of BA resulted in decrease in shoot number per explant. Similarly, Ahire *et al*. (2011) reported the decrease in shoot number at lower and higher concentrations than optimum concentration. Shoots obtained were sub-cultured after 28 days on fresh medium resulted in the vigorous growth. Tiwari *et al*. (2001) obtained an average of 129 shoots after 3rd subculture on BA containing medium from leaf explants of *B. monnieri*. However, in the present study similar number of shoots was obtained on BA containing medium only after second subculture. There are some reports on shoot regeneration in *B. monnieri*, but most of the reports require two step culture methods with combination of cytokinins and auxin treatment.

TDZ, a substituted phenylurea is a potent bioregulant for *in vitro* morphogenesis. TDZ induces high rates of shoot organogenesis in variety of woody plant species (Fiola *et al*. 1990; Malik and Saxena 1992). In the present investigation, TDZ induces shoot buds from leaf as well as nodal explants, but induced shoot buds failed to elongate on the fresh parental medium. Similarly, Tiwari *et al*. (2001) reported an average of 93 shoot buds on TDZ containing medium but shoot elongation was not observed even after subculture on fresh parental medium or with lower concentration of TDZ.

For maintenance of shoots, SRM solid and liquid was evaluated of which liquid SRM was found to be superior. On SRM, shoot cultures were maintained over a period of 2 year after sub-culturing at 28 days interval (**Fig. 1C**). The shoot regenerative capacity of nodal and leaf explants obtained from the maintained shoots was remained similar as that of the fresh explants even after 2 years.

Rooting of shoots and acclimatization

For rooting of *in vitro* grown shoots, we tried solid and liquid MS medium without plant growth regulator. Among solid and liquid; liquid MS medium was found suitable for the rooting of shoots. About 95% shoots produced roots on

Table 2 Effect of NAA and IAA on rooting in *in vitro* regenerated shoots of *B. monnieri*.

Auxins (mg/L)	Rooted shoots (%)	Mean no. of roots/shoot	Mean root length (cm)	Degree of swelling and callusing	Survival (%)
MS	95	06.6 ± 0.4	11.0 ± 0.2	-	93
NAA					
0.1	100	12.2 ± 0.3 c	13.4 ± 0.5 b	-	100
0.2	100	15.5 ± 0.3 a	13.7 ± 0.2 a	-	100
0.3	100	13.1 ± 0.4 b	12.2 ± 0.3 c	-	100
0.4	100	10.8 ± 0.1 d	10.4 ± 0.7 d	-	94
0.5	100	09.1 ± 0.4 e	09.3 ± 0.5 e	*	88
IAA					
0.1	100	10.1 ± 0.3 b	12.1 ± 0.2 a	-	95
0.2	100	12.7 ± 0.2 a	11.4 ± 0.1 b	-	91
0.3	100	09.5 ± 0.2 c	10.9 ± 0.2 c	*	84
0.4	100	07.1 ± 0.4 d	09.6 ± 0.2 d	*	71
0.5	100	05.9 ± 0.3 e	07.9 ± 0.3 e	*	65

NAA: α-naphthaleneacetic acid; IAA: indole-3-acetic acid. Values are mean ± SE of 21 replicates after four weeks of culture. Means followed by the same letters within columns are not significantly different at the 5% level (DMRT). DMRT was applied separately to NAA and IAA. * = Slight swelling followed by rooting.

transfer to liquid MS medium without any swelling. On an average 6.6 ± 0.4 roots were produced per shoot with an average length of 11.0 ± 0.2 cm (**Table 2**).

Inclusion of different concentrations of IAA and NAA (0.1–0.5 mg/L) in liquid MS medium resulted in 100% rooting of shoots within two weeks culture. However, among different types and concentrations of auxins, 0.2 mg/L NAA showed better performance in terms of number of roots per shoot (15.5 ± 0.3) and average root length (13.7 ± 0.2 cm) (**Table 2; Fig. 1D**). Increase in the concentration of NAA resulted in decrease in mean number of roots and mean root length. Higher concentrations of NAA and IAA resulted in the swelling and callusing at the cut ends of shoots. Tiwari *et al*. (1998) reported the rooting of *in vitro* regenerated shoot on full- or half-strength MS medium with or without 0.5–1.0 mg/L IBA or 0.5–1.0 mg/L NAA. Rooting was achieved in microshoots on half-strength MS liquid medium containing IBA (2 μM) and 1% sucrose (Joshi *et al*. 2010). Sharma *et al*. (2010) reported the rooting of shoots on MS medium supplemented with 0.15 mg/L IBA.

The rooted shoots, on transfer to plastic containers containing a mixture of garden soil + sand (1:1) (**Fig. 1E**), could acclimatize within 14 days under glasshouse conditions. The acclimatized *in vitro*-grown plants showed 100% survival after transfer to soil in earthen pots (**Fig. 1F**). There was no detectable variation among the micropropagated plants with respect to morphological and growth characteristics.

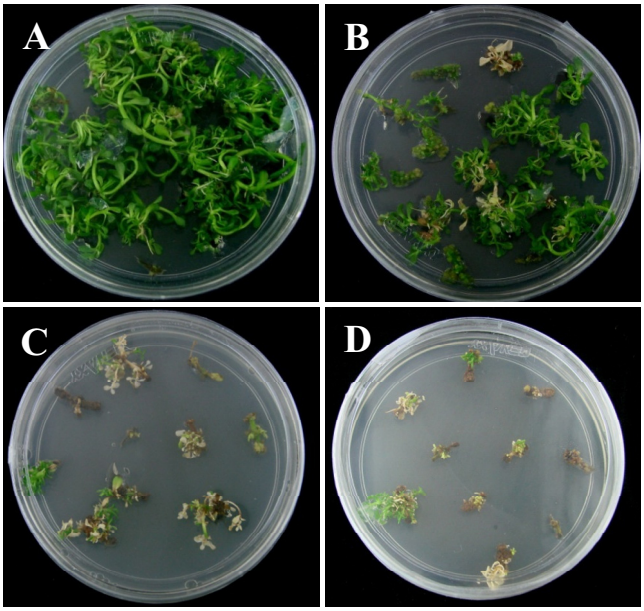

Fig. 2 Antibiotic selection of nodal explants of *B. monnieri*. (**A**) Nodal explants on MS + 1.0 mg/L BA (control). (**B**) Nodal explants on MS + 1.0 mg/L BA + 500 mg/L cefotaxime. (**C**) Nodal explants on MS + 1.0 mg/L BA + 100 mg/L kanamycin. (**D**) Nodal explants on MS + 1.0 mg/L BA + 15 mg/L hygromycin.

Antibiotic selection

In most of the plant genetic transformation studies cefotaxime is used to control the bacterial overgrowth. Optimum dose of Cef, to control bacterial overgrowth without inhibiting shoot regeneration was determined by culturing the nodal explants on SRM with different concentration of Cef (0–1000 mg/L). Nodal explants did not show obvious difference for shoot regeneration on medium with 250 mg/L Cef versus control (no Cef). About 93.0% (**Fig. 3**) explants showed shoot regeneration response and regenerated shoots were healthy. Fifty percent (**Fig. 3**) explants showed shoot regeneration on SRM supplemented with 500 mg/L Cef (**Fig. 2B**). Drastic decline in shoot regeneration frequency was observed with increasing Cef concentration. About 23.0% explants showed bleaching after 15 days of incubation on SRM containing 750 mg/L Cef and only 12.0% of explants responded for shoot regeneration at 1000 mg/L Cef (**Fig. 3**). About 75 and 90.0% of explants showed browning and bleaching on media supplemented with 750 and 1000 mg/L Cef, respectively after 28 days of incubation (**Fig. 3**). In the present study, optimum concentration of Cef was found to be 500 mg/L whereas Nisha *et al*. (2003) used 300 mg/L Cef (for 6 weeks) to control the bacterial growth in *B. monnieri* during transformation studies using *Agrobacterium tumefaciens* strain EHA105 with binary plasmid pBE2113. Ramesh *et al*. (2011) used Cef at 250 mg/L (for 45 days) to control the bacterial growth during transformation study with *A. tumefaciens* strain EHA105 harbouring binary vector pCAMBIA 1301. Similar to our results, Murthy *et al*. (2003) also used 500 mg/L Cef in selection medium to check the bacterial growth after co-cultivation in *Guizotia abyssinica* using seedling explants; Sonia *et al*. (2007) for *Vigna radiata* using cotyledonary node as an explants and Chaudhry and Rashid (2010) for tomato using leaf disc and hypocotyl explants.

Aminoglycoside antibiotics are often used as selective agents in plant genetic transformation for selection of putative transformants (Teixeira da Silva *et al*. 2003). Kan is an aminoglycoside derivative antibiotic and widely used to select *nptII* transformed cells (Terakami *et al*. 2007). Kan is one of the most frequently used selective agent for selection of putative transformants using *nptII* as a selective marker gene (Qin *et al*. 2011). Kan has negative effects on plant organogenesis and an optimum concentration is the key to successful plant genetic transformation (Perós *et al*. 1998). The impact of Kan on shoot regeneration was determined by culture of nodal explants on SRM containing increasing amount of Kan (0, 25, 50, 75, 100, 125 and 150 mg/L) in order to determine the optimum concentration for selection of transgenic shoots (**Fig. 4**). Kan negatively affected shoot regeneration efficiency. Shoot regeneration frequency decreased as Kan increased from 0 to 150 mg/L. Shoot

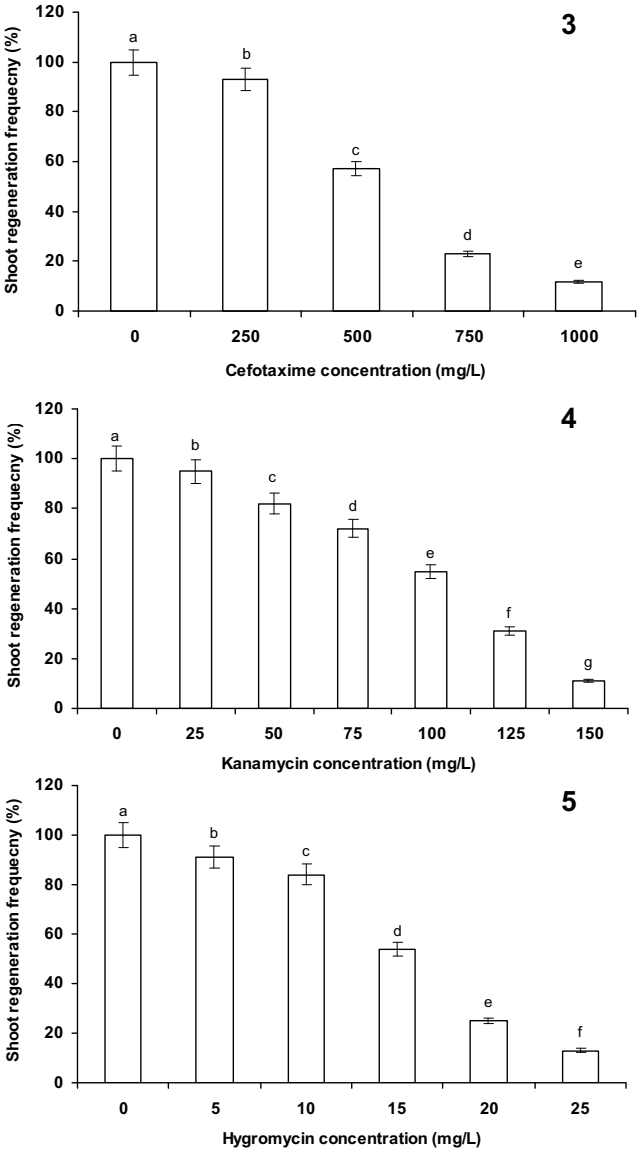

Fig. 3 Effect of cefotaxime doses in media on shoot regeneration frequency in nodal explants of *B. monnieri*. Ten nodal explants were inoculated per Petri dish and 4 Petri dishes were used for each concentration. **Fig. 4** Effect of kanamycin doses in media on shoot regeneration frequency in nodal explants of *B. monnieri*. Ten nodal explants were inoculated per Petri dish and 4 Petri dishes were used for each concentration. **Fig. 5** Effect of hygromycin doses in media on shoot regeneration frequency in nodal explants of *B. monnieri*. Ten nodal explants were inoculated per Petri dish and 4 Petri dishes were used for each concentration.

regeneration frequency was 100% on SRM without supplementation of Kan (control). By increasing Kan concentration shoot regeneration was significantly inhibited and resulted in extensive necrosis. Lower concentrations (25 and 50 mg/L) of Kan did not show obvious difference in shoot regeneration (**Fig. 4**). LD_{50} (55.0%) was obtained on SRM supplemented with Kan at 100 mg/L after 15 days of culture (**Fig. 2C**). Shoot regeneration was inhibited by 150 mg/L Kan or more. The explants which did not induce shoots were white and became necrotic after subsequent culture. From this study, the optimum dose of Kan was determined as 100 mg/L. In contrast to this Nisha *et al*. (2003) used only 15 mg/L Kan to select the transgenic events in *B. monnieri*. Similar results were recorded for other plant species where Kan at 100 mg/L were used to select the putative transformants such as *Lens culinaris* (Akcay *et al*. 2009); in ginger (Suma *et al*. 2008); in *Lathyrus sativus* (Barik *et al*. 2004).

Hyg B is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus*, which kills bacteria, fungi and higher eukaryotic cells by inhibiting protein synthesis. It also interferes with translocation and to cause mistranslation at 70s ribosome (González *et al*. 1978). It is most widely used antibiotic selection agent, as it kills non transformed cells more quickly and only resistant or in other words, transformed cells survives. Hyg B has been successfully applied to a number of crop plants, including dicots and monocots as selective agent. Negative effects on the rates of shoot regeneration and explant browning were observed at all Hyg concentrations tested. Shoot regeneration was significantly reduced as Hyg concentrations increased (**Fig. 5**). Linear decreased in shoot regeneration was observed as Hyg concentration increases. The LD_{50} for nodal explants (54%) was obtained with Hyg at 15 mg/L (**Fig. 2D; Fig. 5**). Negative effect of Hyg led to delayed shoot induction and substantial reduction in shoot regeneration rate. Ramesh *et al*. (2011) used Hyg at 10 mg/L concentration to select the putative transformants. Similarly, 10-15 mg/L Hyg was used as selection agent in safflower (Ying *et al*. 1992; Orlikowska *et al*. 1995; Rao and Rohini 1999; Sri Shilpa *et al*. 2010) and *Agrobacterium*-mediated genetic transformation of cotton (Meng *et al*. 2007).

CONCLUSION

The present study demonstrates an efficient and reproducible method for direct organogenesis in an important medicinal plant *Bacopa monniera*. Hundred percent rooting of shoots and acclimatization of the plantlets was achieved. The protocol developed may work as a key for *Agrobacterium*-mediated genetic transformation studies and genetic manipulation for secondary metabolite production. In addition to identifying antibiotics that had least phytotoxic effects on shoot regeneration frequency and shoot growth of *B. monnieri* were explored. The results obtained in the present investigation could serve as an indicator for the use of antibiotics to control *Agrobacterium* overgrowth and selection of putative transformants.

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