Efficient shoots regeneration and genetic transformation of *Bacopa monniera*

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Abstract *Bacopa monniera* is an important source of metabolites with pharmaceutical value. It has been regarded as a valuable medicinal plant and its entire commercial requirement is met from wild natural population. Recently, metabolic engineering has emerged as an important solution for sustained supply of assured and quality raw material for the production of active principles. Present report describes efficient in vitro multiplication and transformation method for genetic manipulation of this species. MS medium supplemented with 2 mg l⁻¹ BA and 0.2 mg l⁻¹ IAA was found optimum for maximum shoot regeneration (98.33 %) from in vitro leaves with 2–3 longitudinal cuts. *Agrobacterium tumefaciens*-mediated transformation method was used for generating transgenic *B. monniera* plants. Putative transformants were confirmed by GUS assay and PCR based confirmation of *hptII* gene. DNA blot analysis showed single copy insertion of transgene cassette. An average of 87.5 % of the regenerated shoots were found PCR positive for *hptII* gene and GUS activity was detected in leaves of transgenic shoots at a frequency of 82.5 %. The efficient multiple shoots

regeneration system described herein may help in mass production of *B. monniera* plant. Also, the high frequency transformation protocol described here can be used for genetic engineering of *B. monniera* for enhancement of its pharmaceutically important metabolites.

Keywords *Agrobacterium tumefaciens* · *Bacopa monniera* · Genetic transformation

Abbreviations

X- Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid
Gus	β-Glucuronidase
BA	6-benzylaminopurine
IAA	Indole-3-acetic acid
<i>hptII</i>	Hygromycin phosphotransferase
YEB	Yeast extract broth
MS	Murashige and Skoog's
A ₆₀₀	Absorbance at 600 nm

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Introduction

Bacopa monniera (L.) Wettst. is an important medicinal plant which has been ranked second for its medicinal importance, commercial value and potential for further research and development (Anonymous 1997). It is a member of family *Scrophulariaceae*, commonly known as Brahmi, jal-brahmi or Neera-Brahmi. This small prostate herb which grows in wet, damp and marshy areas and has been extensively studied for its chemical constituents (Darokar et al. 2001). In traditional system of Indian medicine, it is considered as a drug to improve intelligence, memory function and counteract the effects of mental stress. It is effective in cases of anxiety and neurosis and is a part of ayurvedic formulations like

Brahmighrtam and Sarasvatarism (Shrivastava and Rajani 1999). It contains different types of saponins such as bacosides A, B, C, and D, which are the active triterpenoid principles and known as “memory chemicals” (Sivaramakrishna et al. 2005). The plant also contains several alkaloids, such as nicotine, brahmine, herpestine, several other pharmaceutically important compounds like stigmastanol, β -sitosterol and stigma sterol (Basu and Walia 1994).

Triterpenoids, like bacosides are built up from C5 compound isopentenyl diphosphate (IPP). IPP is synthesized via two pathways named cytosolic mevalonic acid (MVA) pathway and plastidial methylerythritol phosphate (MEP) pathway. Triterpenoids are biosynthesized via MVA pathway and regulated by several enzymes. The first branchpoint step in triterpenoid biosynthesis is the cyclization of 2,3-oxidosqualene catalyzed by oxidosqualene cyclases (OSCs) to give triterpenoid skeletons (Sawai and Saito 2011). The triterpenoid backbone then undergoes various modifications (oxidation, substitution and glycosylation), mediated by cytochrome P450-dependent monooxygenases, glycosyltransferases and other enzymes.

The recent discovery of the memory-enhancing property of the bacosides has enhanced the demand of this plant and resulted in its extensive use in several commercial productions. Almost the entire commercial requirement is met from the wild natural populations. Thus *B. monnierea* has become a rank holder in the list of threatened plant by the International Union for Conservation of Nature and National Resources (Tiwari et al. 2001). With an increasing world-wide demand for plant derived medicines and formulations, there has been a concomitant increase in the demand for raw material. Hence, there is a need to develop approaches for ensuring the availability of raw material of a consistent quality from regular and viable sources, which may also reduce pressure on its natural population (Shrivastava and Rajani 1999).

To improve and modify secondary metabolite contents of such medicinal and aromatic plants, metabolic engineering is emerging as one of the important approaches (in vitro approaches). Because triterpenoids are naturally produced in very low quantities, purification of such metabolites suffers from low yields and depletion of large amount of resources. Successful genetic transformation technology is thought to be one of the most reasonable approaches to enhance the production of secondary metabolites through genetic manipulation of biosynthetic pathway. Hence, establishment of an efficient transgenic system is a prerequisite for genetic improvement of the valuable medicinal plant, rich in therapeutically active secondary metabolites (Nisha et al. 2003).

The present study describes the efficient regeneration protocol for *B. monnierea* and the efficient transformation using *Agrobacterium*-mediated transformation system.

Material and methods

Establishment of aseptic culture and multiple shoot regeneration

The disease free, healthy young nodal segments of *B. monnierea* were collected from greenhouse at National Chemical Laboratory, Pune. They were washed under running tap water for 30 min in order to remove the external contaminants and treated with 10 % (v/v) savlon for 1 min followed by washing with sterile distilled water 2–3 times. They were treated with 70 % (v/v) ethanol for 1 min in laminar air flow cabinet. Ethanol was quickly decanted and explants were washed thoroughly with sterile distilled water and then treated with 0.01 % (w/v) mercuric chloride (HgCl_2) solution for 1 min followed by thorough rinsing with sterile distilled water 3–4 times. Explants were prepared by cutting nodal segments into pieces of 1–2 cm length containing 2–3 nodes and inoculated on semi solid MS (Murashige and Skoog 1962) supplemented with 0.5 mg l^{-1} BA and kept for 2–3 weeks. The cultures were then maintained by regular subculture on shoot proliferation medium (PM, hormone free MS basal medium) after every 30 days. Longitudinal cuts (2–3) were made on leaves and placed on shoot regeneration medium (SRM; MS basal medium containing $1\text{--}2 \text{ mg l}^{-1}$ BA and $0\text{--}0.2 \text{ mg l}^{-1}$ IAA). After 20 days, multiple shoots regenerated from leaf surface were transferred to proliferation medium for root induction. Rooted shoots were planted in 1:1:1 (soil-sand-peat) mixture and hardened plants were established in greenhouse.

All media were supplemented with 30 g l^{-1} sucrose and solidified with 8 g l^{-1} agar. All cultures were incubated under 16 h photoperiod, $60 \mu\text{mol m}^{-2} \text{ s}^{-1}$ light intensity at $25 \pm 1^\circ \text{C}$. Each treatment contained a minimum of 36 explants and each experiment is repeated three times.

Histological studies

The leaf explants, incubated on shoot regeneration medium, were collected at regular interval and fixed in 5: 5: 90 (v/v) ratio of formalin/glacial acetic acid/ethanol solution. Fixed tissues were dehydrated through an ethanol-xylene series (3:1, 1:1, and 1:3 v/v). Tissues were infiltrated with paraffin wax (melting point $58\text{--}60^\circ \text{C}$) initially at room temperature for 24 h followed by hot infiltration by keeping the samples in wax at 65°C in hot air oven for 48–60 h till xylene was replaced by wax completely in the tissues. Paraffin blocks were made by embedding the tissues in paraffin wax and allowed to solidify. Sections of $15 \mu\text{m}$ thickness with a microtome (Leica RM 2155) were cut and stained with 1 % (w/v) safranin and 1 % (w/v) light green prior to microscopic observation. Observations were taken under Axioplan microscope (Carl Zeiss) and APO 11 microscope (Carl Zeiss). The digital photomicrography was done using Axiocam MRC5 system.

Plasmid vector and *Agrobacterium* strain

Agrobacterium tumefaciens, strain GV2260 harboring binary vector pCambia1301 (Fig. 1) was used for genetic transformation. This vector contains a reporter gene, namely β -Glucuronidase (*gus*) and selectable marker gene hygromycin phosphotransferase (*hptII*), imparting resistance to hygromycin B under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter.

Agrobacterium-mediated transformation

Agrobacterium tumefaciens harboring pCambia1301 was inoculated in YEB medium (0.05 g l⁻¹ peptone, 0.01 g l⁻¹ yeast extract, 0.05 g l⁻¹ beef extract, 0.05 g l⁻¹ sucrose and 2 mM MgSO₄) containing 50 mg l⁻¹ rifampicin and 50 mg l⁻¹ kanamycin and incubated at 28 °C for overnight with constant agitation at 175 rpm. The bacterial culture (*A*₆₀₀ of 0.5) was used for the genetic transformation of leaf explants of *B. monniera*. Leaf explants, excised from in vitro shoots were made longitudinal cuts (2–3) and co-cultivated with overnight grown culture of *A. tumefaciens* strain GV2260 harboring binary vector pCambia1301 for 15 min. Explants were subsequently blotted on a sterile Whatman No.1 filter paper to remove excess of the bacterial suspension and transferred to SRM. Co-cultivation was carried out for 2 days under dark at 28 °C.

Transgenic plant selection

After 2 days of co-cultivation with *A. tumefaciens*, explants were washed with sterile distilled water containing 200 mg l⁻¹ cefotaxime to remove the bacteria, blotted dry on sterile Whatman No. 1 filter paper and cultured on selection medium (SRM supplemented with 200 mg l⁻¹ cefotaxime and 10 mg l⁻¹ hygromycin B) for 10–15 days and cultured under the same conditions. After 4 weeks on selection medium, hygromycin B resistant shoots from *A. tumefaciens* was transferred to PM. The transformation was confirmed by GUS assay as described by Jefferson et al. (1987) in the leaves of transgenic shoots. Transgenic shoots of *B. monniera* were transferred to PM containing 10 mg l⁻¹ hygromycin B for rooting as mentioned above. The rooted shoots were shifted to soil-sand-peat mixture and hardened as mentioned above.

Molecular characterization of transgenic events

Total genomic DNA was extracted from the young leaves of transformed plant and non-transformed control plant by using plant DNA extraction kit (HiMedia, Hipura Plant Genomic Purification kit, India). Putative transformants were screened by PCR for the presence of *hptII* gene. The *hptII* gene forward and reverse primer sequences were 5'-TCCTGCAAGCTCCG GATGCCTC-3' and 5'-CGTGCACAGGGTGTACAGTT GC-3'. The components of PCR reaction mixture were: template DNA, 0.2 mM of dNTPs, *hptII* gene specific forward and reverse primers (500 nM each), 1 U of *Taq* DNA polymerase and 1X reaction buffer, in a total volume of 20 μ l. The PCR reaction was carried out in a thermal cycler (C1000 BIORAD) with following conditions, initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min 20 s, and a final extension cycle of 72 °C for 5 min. For checking bacterial contamination in *A. tumefaciens* transformed plants, PCR amplification of above extracted DNA was carried out using 16S rRNA primer pair 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-ACGGGCGGTG GTTTC-3' (Aggarwal et al. 2011) specific to bacterial 16S rRNA. Bacterial genomic DNA was used as positive control under same amplification conditions as mentioned above. The PCR amplified products were analyzed by 1 % agarose gel electrophoresis and visualized by ethidium bromide staining.

Southern blot analysis

The insertion of the transgene in putative transformants further confirmed by DNA blot analysis. Good quality genomic DNA was isolated using HiPurA™ Plant Genomic DNA Purification Kit (HiMedia, India). Two transformed *B. monniera* plants along with non-transformed plant genomic DNAs were restriction digested using two enzymes *EcoRI* and *NdeI*. Standard protocol as described by Sambrook et al. (1989), was followed for the Southern blot experiment. In brief, Digested DNA was electrophoretically separated on 0.8 % agarose gel and transferred to a Nylon Hybond-N membrane (Amersham, USA). Membrane was prehybridized for 8 h at 62 °C in hybridization buffer (1 % BSA; 0.5 M Na₂HPO₄·2H₂O, pH-7.2; 7 % SDS and 1 mM EDTA, pH-8.0) (Sambrook et al. 1989). Hybridization was performed at 62 °C for 18 h in the

Fig. 1 T-DNA map of plant transformation vector pCambia1301. T-DNA region indicating the locations of the *gus* gene (β -Glucuronidase) and *hptII* gene (Hygromycin phosphotransferase). 35S pro (cauliflower

mosaic virus 35S promoter), LB (left border), RB (right border), NOS ter (nopaline synthase terminator)

same buffer containing radiolabeled probe (α' -P³²). PCR amplified *hptII* gene fragment was used to prepare radiolabeled probe using Amersham's random labelling kit. The blot was washed with moderate wash buffer (2X SSC and 0.1 % SDS) before exposing to Storage Phosphor Screen (GE, Healthcare) for 4 h. The Phosphor Screen was scanned at 200 μ m resolution in Typhoon Trio+(GE).

Statistical analysis

The data were analyzed by Analysis of Variance (ANOVA) and the treatment means were compared by Duncan's multiple range test (Duncan 1955).

Results and discussion

In vitro plant regeneration and de novo shoot regeneration

The axillary buds of nodal explants incubated on the initiation medium showed shoot formation after 5–6 days of incubation. About 3–4 shoots were formed from each of these explants. These cultures were maintained by regular subculture on PM.

Leaf explants, inoculated on MS basal medium with various concentrations of BA and IAA, showed maximum proliferation of multiple shoots after about 2 weeks of incubation (Fig. 2a). The shoots appeared from the cut regions of leaf lamina (Fig. 2A. g). Excised leaves of *B. monniera* with longitudinal cuts showed very high frequency of shoot induction as compared to uninjured

Fig. 2 **A.** Multiple shoot regeneration on SRM from leaf explants of *B. monniera*. **(a)** Control leaf (without injury). **(b)** Control leaf shows shoot formation at petiole. **(c)** Leaf with longitudinal cuts on the lamina. **(d)** Leaf explants after 6 days of incubation. **(e)** Leaf explants after 9 days of incubation. **(f)** Leaf explants after 12 days of incubation. **(g)** Leaf explants after 15 days of incubation, showing multiple shoot formation on surface of leaf. **(h)** Leaf explants after 3 weeks incubation. **(i, j)** Multiple shoot showed root formation on PM medium. Multiple shoots formation is indicated by arrows. **B.** Histological sections showing multiple shoot regeneration in various stages of development. **(I)** Control. **(II)** After 6 days of incubation. **(III)** After 9 days of incubation. **(IV)** After 12 days of incubation. **(V)** After 15 days of incubation. *Arrows* indicate the initiation of shoot regeneration

leaf which showed shoot formation at only petiole end (Fig. 2a. a, b, f). After 20 days of growth, multiple shoots produced from leaves were transferred to PM for root induction (Fig. 2a i, j). Among the various treatment combinations, explants cultured on MS basal medium supplemented with 2 mg l⁻¹ BA and 0.2 mg l⁻¹ IAA showed the highest frequency of shoot regeneration (98.33 %) (Table 1). This medium composition was further used for the transformation experiments.

Efficient regeneration protocol is necessary for successful *Agrobacterium*-mediated transformation and fast recovery of transformants. Therefore, a protocol for high frequency regeneration of shoots from leaf explants was standardized to obtain maximum frequency of transformants. There are earlier reports on shoot induction in *B. monniera* where the leaf explants was more responsive than other explants (Shrivastava and Rajani 1999; Tiwari et al. 1998, 2000, 2001; Ceasar et al. 2010). Recently, Mahender et al. (2012) reported 90 % shoot regeneration frequency of *B. monniera* using leaf explants. In our study, shoot regeneration frequency from excised leaves of *B. monniera* with 2–3 longitudinal cuts on the lamina was found to be much higher than the previous reports, where uninjured leaf was used.

Histology of the leaves from 0 to 15 days of incubation on SRM showed the emergence of shoot buds from leaf explants on the 6th day of incubation (Fig. 2b).

Table 1 Effect of BA and IAA on shoot regeneration frequency from leaf explants of *B. monniera*

Growth regulator (mg l ⁻¹)		Shoot regeneration frequency (%) ^a
BA	IAA	
1.0	0.00	6.67 ^g
1.0	0.05	17.67 ^f
1.0	0.10	32.67 ^c
1.0	0.15	45.67 ^d
1.0	0.20	90.33 ^b
2.0	0.00	7.67 ^g
2.0	0.05	20.00 ^f
2.0	0.10	51.67 ^d
2.0	0.15	71.67 ^c
2.0	0.20	98.33 ^a

^aLeaf explants were cultured on shoot regeneration medium (SRM) for 2 weeks and then transferred to proliferation medium (PM). Shoot regeneration frequency (%) was recorded after 1 week on PM. Values are means of three independent experiments. Values in the last column denoted by same letter are not significantly different according Duncan's multiple range test ($p=0.01$)

Transformation, selection and analysis of putative transgenic plants

The leaf explants were co-cultivated with *A. tumefaciens* strain GV2260 harboring binary vector pCambia1301 in an experiment. After 15 days of culture on selection medium, leaf explants showed direct shoot development without an intervening callus phase on SRM selection medium (Fig. 3A. a). The hygromycin B resistant shoots were transferred to fresh SRM selection medium and allowed for further proliferation (Fig. 3A. b). After 15 days, these proliferated shoots were again transferred to PM for rooting. After another 10 days, the rooted transformed shoots were shifted to the plastic pots containing sterile soil, hardened for a week and then transferred to green house for further acclimatization (Fig. 3a. c, d). Approximately 75 % of transferred plants survived in green house. Leaf samples from these plants were used for subsequent analysis.

Histochemical GUS assay

In this experiment the bacterial *gus* gene was used as a reporter gene. Transient expression of *gus* gene was histochemically visualized, that resulted in a uniform and intense blue staining in lamina of the leaf and no GUS activity was visualized in control plant leaf (Fig. 3b). In *A. tumefaciens* mediated transformation, among the 40 plants tested, 33 (82.50 %) were found GUS positive.

Molecular analysis

For molecular confirmation of transgenic plants, genomic DNA was extracted from putative transformants and PCR was performed using *hptII* gene specific primers. In *A. tumefaciens* mediated transformation, out of 40 putative transformants 35 (87.50 %) were found to be *hptII* gene PCR positive. The PCR amplification of DNA extracted from leaves of putative transformants using *hptII* specific primers showed a ~500 bp band while control plant did not show any amplification (Fig. 3c). Amplification of DNA specific to 16S rRNA of DNA extracted from young leaves of transformed plants were not observed, indicating the complete abolition of *A. tumefaciens* contamination from these tissues while positive control (Bacterial DNA) showed an amplification of ~1500 bp 16S rRNA fragment (data not shown).

Southern hybridization analysis was performed in order to estimate the number of inserted loci. Figure 3D showed the Southern hybridization pattern obtained with *hptII* probe. The probe used had no restriction site for *EcoRI* and one restriction site for *NdeI*. Careful observation revealed single insertion of transgene cassette in transformed *B. monniera*. No band was observed in the non-transformed control plant (Data not shown). Single or low-copy integration of transgenes through

Fig. 3 **A.** Transgenic *B. monniera* plants obtained from *Agrobacterium*-mediated transformation (**a**) Hygromycin B resistant shoots obtained after 15 days of transformation. (**b**) Transgenic plants on selection medium. (**c**) Transgenic plants in pot. (**d**) Transgenic plants in the greenhouse. **B.** Histochemical GUS assay of *B. monniera*. (**I**) Control leaf. (**II**) Leaf of transformed *B. monniera* showing expression of *gus* gene. **C.** PCR

analysis of putative transgenic lines using *hptII* gene specific primers and genomic DNA as a template. Lane M, Low range DNA ruler; Lane 1–17, putative transformed plants; Lane 18, non-transformed plant (negative control); Lane 19 plasmid pCAMBIA1301 (positive control). **D.** Southern blot analysis. DNA blot hybridization of two transformed *B. monniera* plants genomic DNA using *hptII* gene fragment as a probe

A. tumefaciens have been observed in several plant species (Tsai et al. 2012; Ho et al. 2013).

In the present report, we describe successful genetic transformation of *B. monniera* mediated by *A. tumefaciens* using the *hptII* gene as a selectable marker. *A. tumefaciens* (A_{600} of 0.5–1) harboring binary vector pCAMBIA1301 with co-cultivation time of 15 min was found to be most suitable method for transformation using leaf explant. There are three previous reports of successful *Agrobacterium*-mediated transformation of *B. monniera* (Mahender et al. 2012; Ramesh et al. 2011; Nisha et al. 2003). Nisha et al. (2003) observed 60 % transformation efficiencies using callus while it was found to be 68.8 % by Ramesh et al. (2011) where nodal explant was used for transformation. The transformation efficiency achieved by Mahender et al. (2012) was 70.6 %, with

an average of 10.4 ± 0.15 transgenic plantlets per leaf explants. In our study, we have achieved stable transformation efficiency of 82.50 % accounted on basis of GUS assay which was much higher than previous reports. The high transformation efficiency observed in the present study may be attributed to the highly efficient regeneration response (98.33 %) (Table 1).

Conclusions

In conclusion, this study describes efficient multiple shoot regeneration system and reproducible efficient genetic transformation method. This protocol developed for *B. monniera*, may be utilized for quick multiplication of the species for commercial production of drugs. This efficient transformation

protocol will also assist to produce transgenic *B. monniera* with altered secondary metabolic profiles by using terpenoids specific metabolic pathway engineering.

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Competing interests The authors declare that they have no competing interests.

Authors' contributions Prof. B. M. Khan and MVS designed and reviewed the manuscript. Uma Kumari and RKV performed experiments. Uma and RKV wrote the manuscript. Neha Gupta and Ruby helped in analyzing data and proofreading of the manuscript. All authors read and approved the final manuscript.

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