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ORIGINAL PAPER

Analysis of propagation of *Bacopa monnieri* (L.) from hairy roots, elicitation and Bacoside A contents of Ri transformed plants

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Abstract *Agrobacterium rhizogenes* mediated transformation has been experimented in leaf explants of the memory herb *Bacopa monnieri* in order to assess the regeneration potential of hairy roots (HR) followed by the elicitation of transformed plants for increased Bacoside A production. Out of the four strains tested, A4 and MTCC 532 derived HR exhibited regrowth in MS basal medium while MTCC 2364 derived HR showed regeneration in MS medium supplemented with suitable phyto hormones. R1000 derived HR possessed no regeneration potential. Comparable to A4, MTCC 532 derived HR displayed maximum regrowth frequency of about 85.71 ± 1.84 % with an increase in biomass to threefold. Therefore, five HR plant lines (MTCC 532 derived) were generated and maintained in MS basal liquid medium in which HR3 topped the others in producing a huge biomass of about 67.09 ± 0.66 g FW. PCR amplification and southern hybridization analysis of *rol A* gene (280 bp) has been performed in order to confirm the transformation process. Moreover, HR3 plant line has accumulated highest total phenolic content of about 165.68 ± 0.82 mg GAE/g DW and highest total flavonoid content of about 497.78 ± 0.57 mg QRE/g DW when compared to other lines and untransformed controls. In addition, HR3 plant extract showed 85.58 ± 0.14 % of DPPH (2, 2-diphenyl-1-picryl hydrazyl) inhibition displaying its reliable anti oxidant potential. Further on elicitation with 10 mg/L chitosan for 2 weeks, HR3 has produced 5.83 % of Bacoside A which is fivefold and threefold increased production when compared to untransformed and transformed unelicited controls respectively. This is the first report on eliciting HR plants for increased metabolite accumulation in *B. monnieri*.

Keywords *Bacopa monnieri* · Bacoside A · Chitosan · Transformed plants

Introduction

Bacosides are a complex mixture of structurally closely related glycosides of either jujubogenin or pseudojujubogenin present in the medicinal plant *Bacopa monnieri* (Murthy et al. 2006). Bacoside A, one among the bacosides of Bacopa, is a major chemical entity shown to be responsible for memory-facilitating action of this plant (Singh and Dhawan 1997). Due to its multipurpose therapeutic uses and availability of many pharmaceutical products based on this plant extract, the need of *B. monnieri* is increasing steeply. Alternative and effective techniques need to be flourished for counter acting the market demand and to preserve the natural resources of this particular plant.

Agrobacterium rhizogenes, a soil bacterium of Rhizobiaceae family induces neoplastic growth of plant cells that differentiate to form "hairy roots" at the sites of infection. Hairy roots are induced by the integration of a segment of bacterial DNA called transfer or T-DNA into the chromosome of the plant cell and its expression (Veena and Taylor 2007). They offer the interesting property of the easy regeneration of whole plants, avoiding callus formation and thus circumventing problems of somaclonal variation in a range of plant species. These plants show different morphological features such as wrinkled leaves, increased branching and rooting, shortened

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internodes, reduced apical dominance and altered flowering (Tepfer 1990). More importantly, regenerated plants from hairy roots can produce higher levels of secondary metabolites. All these peculiar transformed characters are attributed to the expression of four genes *rol* A, B C and D present in the T-DNA of Ri plasmid. Plant regeneration from hairy roots has been carried out in a number of medicinal plants for the purpose of secondary metabolite production. Gangopadhyay et al. (2010) described the regeneration of *Plumbago indica* from hairy roots. Wang et al. (2013) reported spontaneous regeneration of shoots from hairy roots in *Salvia miltiorrhiza* and Mehrotra et al. (2013) carried out the characterization of plants regenerated from hairy root cultures in *Rauwolfia serpentina*. In both the aforementioned studies, significant improvement in the accumulation of particular secondary metabolites has also been witnessed. In addition, this transformation system is ideal for the study of plant secondary metabolism and functional genomics (Sharafi et al. 2013).

The strategy of eliciting the hairy roots which are the store house of phytochemicals has been now achieved for the enhanced production of phytoceuticals. *A. rhizogenes* mediated transformation in combination with elicitation found to be very effective in augmentation of secondary metabolites in a number of medicinal plants (Shilpha et al. 2015). In the case of Bacopa, in vitro production of bacosides was reported using cell suspension cultures and micro propagated plantlets (Rahman et al. 2002; Naik et al. 2010) which were slow growing with a short life span and they gradually lost their capacity to synthesize active molecules over certain period (Srivastava and Srivastava, 2007). Therefore *A. rhizogenes* mediated transformation remains the promising tool for ruling out the drawbacks in the mass production of bacosides.

Hitherto, three reports have been published for *A. rhizogenes* mediated transformation in *B. monnieri*. Majumdar et al. (2011) performed plant regeneration from hairy roots using LBA 9402 and A4 strains and stable hairy root lines were generated by Bansal et al. (2014). Recently Paul et al. (2015) reported about transfer of *cryptogein* gene through Ri plasmid and subsequent plant regeneration. However, elicitation of plants regenerated from hairy roots has not yet been reported for increased saponin production in *B. monnieri*. Hence the present study has been under taken in the view of assessing the regeneration ability of hairy roots induced by four different strains of *A. rhizogenes*, followed by chitosan elicitation of the transformed plants of *B. monnieri* for the first time.

Materials and methods

Plant material

In vitro cultures of *B. monnieri* were initiated by culturing nodes in MS medium supplemented with 1 mg/L 6-benzyl aminopurine (BAP) and 0.1 mg/L α-naphthalene acetic acid (NAA) with 3 % (w/v) sucrose and 0.8 % agar at pH 5.75 based on our previous study. Cultures were maintained at 25 ± 2 °C under 16/8 h photoperiod with light supplied by white cool fluorescent tubes (Phillips, India). Leaf explants from 3 weeks old in vitro shoots were used for transformation. Explants were maintained in MS basal medium supplemented with 100 µM acetosyringone in dark for 1 day prior to infection.

Hairy root induction

Four wild type *A. rhizogenes* strains such as MTCC 532, MTCC 2364, A4 and R1000 were used in this study. Overnight grown bacterial cells of culture O.D 1 was pelleted, mixed with MS basal medium, poured over the wounded explants through a micro pipette and kept in dark for co cultivation. After 3 days, explants were washed thoroughly with MS liquid medium containing a combination of 500 mg/L of ampicillin and cefotaxime. This was followed by placing the explants in MS solid selection medium supplemented with 500 mg/L ampicillin in dark for 2–3 weeks. Control leaves were subjected to same experimentation but wounded explants were poured with MS medium devoid of bacterial cells.

Plant regeneration from hairy roots

Induced hairy roots were excised from the explants and transferred to four different basal solid media such as ½ MS basal, MS basal, ½ B5 basal, B5 basal containing 500 mg/L ampicillin after 20 days and placed in 16/8 h photoperiod for shoot induction along with the control (untransformed roots). During subsequent subculture cycles, concentration of ampicillin was reduced gradually and then it was completely eliminated from the medium. The efficacy of four different strains and four different media on plant regeneration was analyzed based on various parameters such as frequency of shooting response, mean number of shoots and roots and fresh weights (FW) of regenerated plants. As only two strains show regeneration in the above mentioned media, shoot growth potential of other two strains was carried out in MS medium supplemented with different combinations of kinetin (KN) (0.25, 0.5, 0.75 and 1 mg/L) and NAA (0.05, 0.075 and 0.1 mg/L).

Generation of HR plant lines

Five lines of HR plants were generated from five HR clones and maintained in MS basal liquid medium for its mass propagation. After 2 months of culturing, biomass of five HR lines were found out on FW basis. Molecular and

biochemical tests were performed to scrutinize the most efficient HR plant line.

Molecular analysis of Ri transformed plants

In order to confirm the integration of T-DNA into plant cells, PCR amplification of *rol A* gene was performed. Genomic DNA has been isolated from control and five HR plant lines using HiPur A Plant Genomic DNA isolation kit (Hi Media, Mumbai, India). Plasmid DNA isolated from *A. rhizogenes* was taken as positive control. The primer used for the amplification of *rol A* fragment was forward-5′-ACGGTGAGTGTGGTTGTAGG-3′; reverse-5′-GCCACGTGCGTATTAATCCC-3′ (Bonhomme et al. 2000). The 25 µL PCR reaction mixture consisted of 1× reaction buffer, 0.2 mM of each dNTPs (MBI, Fermentas, Lithuania), 1U of Taq DNA polymerase, 0.4 µM of each primer (Sigma, St. Louis, USA) and 50 ng of template DNA. Amplification was carried out in a Thermal Cycler (Eppendorf, Deutschland, Germany) as follows: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min. The last cycle was followed by a final extension at 72 °C for 7 min. Amplified products were resolved in 1.5 % agarose gel in 1× TAE buffer along with 1 kb size marker (MBI, Fermentas, Lithuania), stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc XR, Bio-Rad, Quarry Bay, Hong Kong).

Southern blot hybridization

Southern hybridization analysis was performed by following the standard protocol of Sambrook et al. (1989). 20 µg of DNA from each Ri transformed plant, bacterial plasmid (positive control) and non transformed plant (negative control) were digested with EcoR1 and subjected to electrophoresis on 1 % agarose gel. After denaturing the gel was transferred on to a nylon membrane (Hybond-N+, Amersham Inc) and fixed. PCR products of *rol A* gene was used as probe and was labeled with biotin-11-dUTP using Biotin Deca Label DNA Labeling Kit (Fermentas Life Sciences, USA) by following the manufacturer's instructions. After hybridization and washing the membrane was detected using Biotin Chromogenic Detection kit (Fermentas Life Sciences, USA) and picture was taken.

Estimation of TPC, TFC and DPPH scavenging activity

Total phenolic content (TPC) in methanol extracts of five different HR plant lines were determined by the Folin–Ciocalteu method (Lin et al. 1999) by using gallic acid as standard. On observing the spectroscopic absorbance at 765 nm, result was expressed in gallic acid equivalent per milligram of dry weight (mg GAE/g DW). Total flavonoid content (TFC) assay was measured by the aluminum chloride method (Marinova et al. 2005). Standard curve was prepared using quercetin as standard and absorbance was taken at 510 nm. TFC was expressed as mg quercetin equivalent (mg QRE/g DW) of extract. DPPH (2, 2-diphenyl-1-picryl hydrazyl) radical scavenging activity was measured using the method of Blois (1958). The absorbance was measured at 517 nm using UV–Vis spectrophotometer (Shimadzu, Japan) and Butylated hydroxytoluene (20–100 µg/mL) was used as standard. All the samples were analyzed in triplicates.

Elicitation of transformed plants with chitosan

Chitosan (10 mg/mL) was prepared by adding glacial acetic acid dropwise by placing it at 60 °C for 15 min (final concentration 2 %, v/v), then diluted with de-ionized water, adjusted to pH 5.5 and sterilized by autoclaving (Udomsuk et al. 2011). They were added to culture medium containing 15 days old transformed plants at various concentrations such as 5, 10, 15 and 20 mg/L. Plants were allowed to grow in the presence of elicitor for about 2 weeks and then harvested. Biomass accumulated was found out as fresh weight (FW) and dry weight (DW) at all concentrations.

HPLC analysis

Methanolic extraction of the dry powder was done by following the protocol of Murthy et al. (2006). The quantity of Bacoside A was estimated in a reverse phase HPLC system equipped with a Phenomenex C18 column (25 cm × 4.6 mm i.d.) with 5-µm particle size, LC 8A preparative pumps, CBM-20A Communication Bus Module system controller, SPD-M20A photodiode array detector (Shimadzu, Japan), and LC solution software release 1.24. Bacoside A standard was obtained from Natural Remedies Pvt. Ltd., Bangalore, India.

Statistical analysis

All experiments were repeated thrice with three replicates (each with twelve explants) and the values were expressed as mean ± standard errors. Results were statistically analysed using analysis of variance (ANOVA) and means were compared using Duncan's Multiple Range Test (DMRT) using SPSS 11.0 software version ($P \leq 0.05$).

Results

Hairy root induction was noticed on 5–6 days after transferring the explants to the selection medium (in dark). The nature and morphology of hairy roots induced varied on

using different *A. rhizogenes* strains. On using A4 strain, more than two hairy roots were induced from the cut ends of the explants. Numerous minute root hairs were also noticed along with the roots. The roots induced by the strain MTCC 532 were thick and long when compared to roots induced by other strains. But only one root was seemed to induce from one cut end of the explants. In the case of MTCC 2364, more than two thick, long roots with root hairs were induced from one cut end of the explants. It is interesting to note the shoot bud initiation from the cut ends of the explants infected with A4 strain.

The hairy roots induced by four different strains were cultured on MS basal medium for regeneration. The hairy roots induced from leaves infected with MTCC 532 (Fig. 1a) and A4 have turned to green from white and bulged (Fig. 1b) after 2–3 days on exposure to light. After 10 days, shoot buds were initiated from the hairy root (Fig. 1c) and whole plant with thickened roots was regenerated after 3–4 weeks from the hairy root in *B. monnieri* when cultured under 16/8 h photoperiod (Fig. 1d). MTCC 532 HR plants depicted notable phenotypic changes including increased adventitious roots on stem, reduced apical dominance and increased branching (Fig. 1e). At the same time, roots induced from leaves infected with other two strains (MTCC 2364 and R1000) showed no signs of greening and regrowth.

The efficiency of different media on plant regrowth from hairy root was analysed and results clearly depicted that MS basal medium was more suitable for shoot induction when compared to B5 media for both MTCC 532 and A4 strains. Compared to full strength, half strength media recorded very poor plant regrowth frequency for both the strains and control. In terms of plant regrowth frequency and biomass accumulation, MTCC 532 strain was superior to A4 strain. Moreover, MTCC 532 strain showed highest plant regrowth frequency of about 85.71 ± 1.84 % in MS medium, followed by 71.42 ± 0.75 % in B5 medium while A4 strain showed 57.14 ± 0.45 % in MS medium and 42.85 ± 0.67 % in B5 medium (Fig. 2). In the case of biomass accumulation, MTCC 532 strain has produced a biomass of about 23.25 ± 2.24 g which is three times higher than the FW of the control plant (7.25 ± 1.64 g) on MS basal medium. This has proved that the transformation event has brought an enhancement in biomass producing capacity of the plant. On the other hand, transformation using A4 strain caused about two times increase in FW (14.60 ± 1.53 g) of the plant when compared to the control plant. Biomass generated by both the strains on B5 medium was comparatively lesser than MS medium. Mean number of shoots and roots produced on using different media for both the strains were meticulously counted and depicted in Fig. 3.

The regeneration capacity of other two strains was assessed on MS medium supplemented with four different combinations of auxins and cytokinins. Hairy root induced from R1000 infected leaves were unable to regenerate even in hormonal media also. Shoot bud induction was noticed after 2 weeks (Fig. 4a) and whole plants were regenerated from the root segment induced from leaves infected with MTCC 2364 strain by culturing their hairy roots on medium supplemented with suitable phyto hormones for about 5–6 weeks (Fig. 4b) which displayed weakened stems with wrinkled leaf morphology. Plant regrowth efficiency was assessed by culturing them in different combinations of KN and NAA. MS medium amended with 0.5 mg/L KN and 0.075 mg/L NAA resulted in maximum regrowth frequency of about 52.38 ± 0.57 %. But still frequency of regrowth was very much lesser when compared to HR 532 in MS basal media. Mean number of shoots and roots produced in different combinations of hormones were tabulated in Table 1 which recorded a minimum number in comparison with MTCC 532 strain regenerated shoots and roots. In all hormonal combinations, extensive callus formation was noted but callusing was not that much extensive in 532 strain regenerated plants.

Analysis of regeneration potential of hairy roots induced by 4 different strains of *A. rhizogenes* revealed that MTCC 532 derived HR plants were far superior among others. Therefore, for further experiments MTCC 532 HR plants alone have been selected. Five different lines (named as HR1–HR5) of 532 HR plants have been generated and maintained for 2 months in MS basal liquid medium along with control plants under constant shaking at 100 rpm in an orbitary shaker. Among them, HR3 line exhibited vigorous biomass production of about 67.42 ± 0.85 g (FW) and 8.74 ± 0.45 g (DW) which is fivefold higher than the biomass of control plant (FW—14.28 ± 1.30 g; DW—2.37 ± 0.17 g). FW of other lines were in the range of 45.87–52.92 g (Fig. 5). This unambiguous variation in the biomass produced by HR3 and control plants can be efficiently witnessed from Fig. 6a and the closer view of HR3 line was shown in Fig. 6b.

The transformed status of HR lines was verified through PCR amplification of *rol A* gene using specific primer. The genomic DNA of all five HR lines and the positive control produced a clear and thick band with a size of 280 bp which is the expected size of *rol A*. The band was absent in negative control (Fig. 7a). Further the presence of hybridization bands of *rol A* gene was revealed in southern blot hybridization (Fig. 7b) thus confirming the stable transformation and integration of T-DNA into the genome of transformed plants.

TPC, TFC and DPPH scavenging activity was estimated for five different HR plant lines (HR1–HR5)

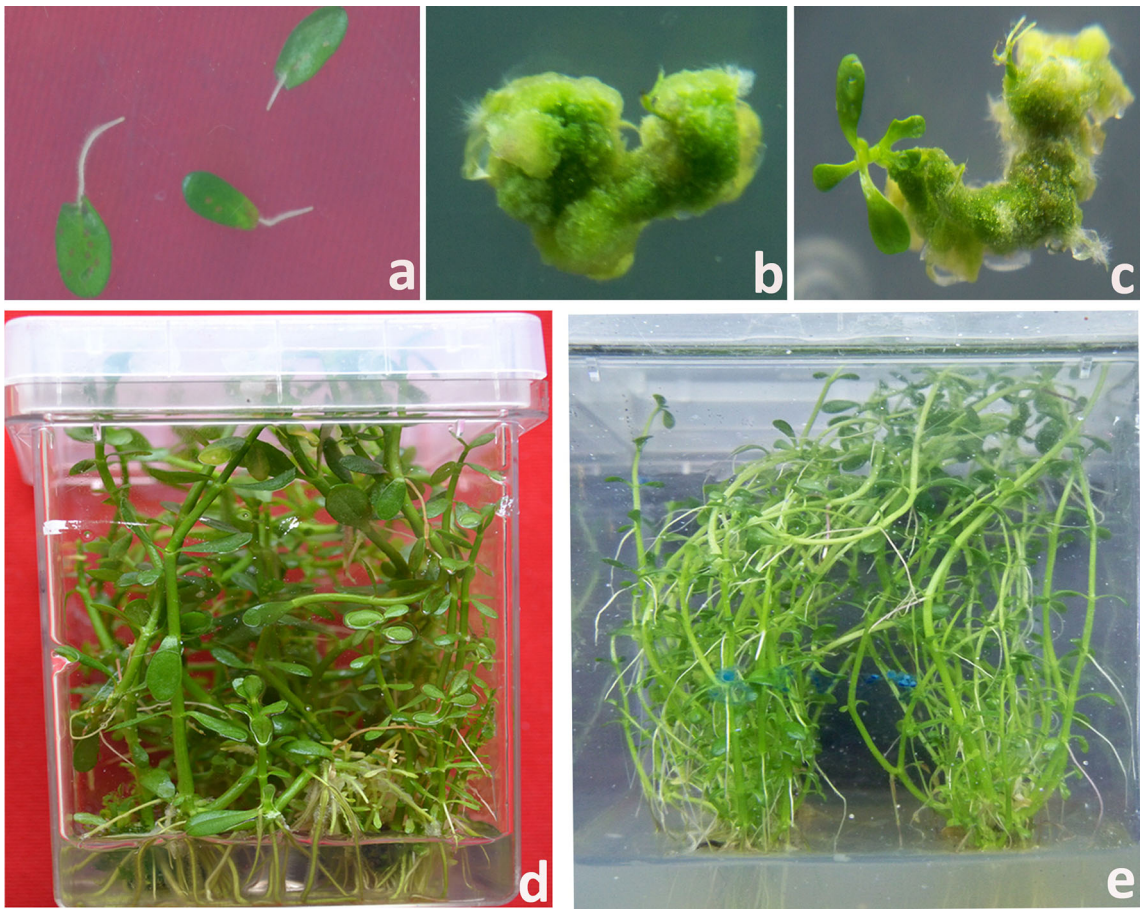

Fig. 1 Plant regeneration from MTCC 532 derived hairy roots of *B. monnieri* on MS basal medium. **a** Hairy roots induced from leaves after 8–10 days of infection. **b** Bulging and greening of hairy roots when placed under 16/8 h photoperiod. **c** Shoot bud induction from hairy root after 10 days. **d** Fully grown HR plantlet showing robust shoots and vigorous thickened roots. **e** Plantlet displaying increased branching and adventitious roots on stems

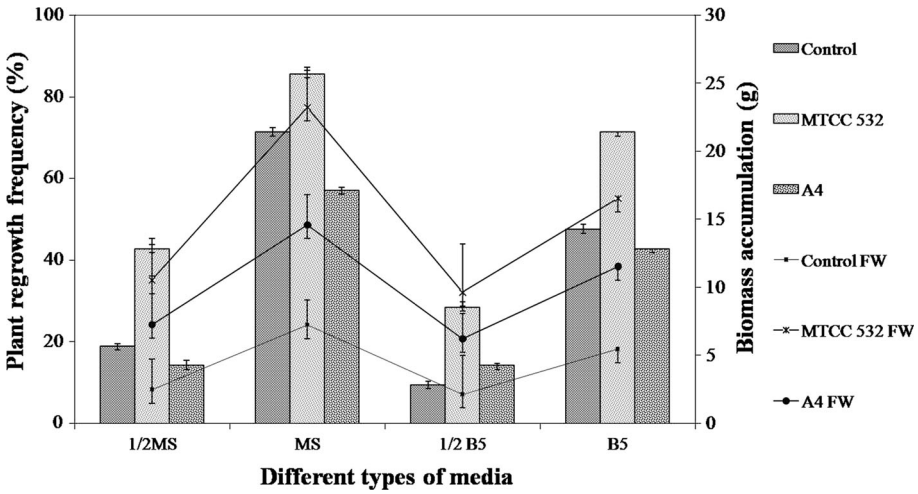

Fig. 2 Plant regrowth potential of A4 and MTCC 532 derived hairy roots of *B. monnieri* on different types of media. Control refers to the regeneration of untransformed roots which had undergone same experimentation but without bacterial culture addition

and untransformed control plant and depicted in Fig. 8. Highest TPC of about 165.68 ± 0.82 mg GAE/g DW was recorded in HR3 and in other lines its range was 125.24 ± 0.62–144.26 ± 0.86 mg GAE/g DW. Compared to untransformed control (51.02 ± 1.23 mg GAE/g DW), the content of total phenolics was

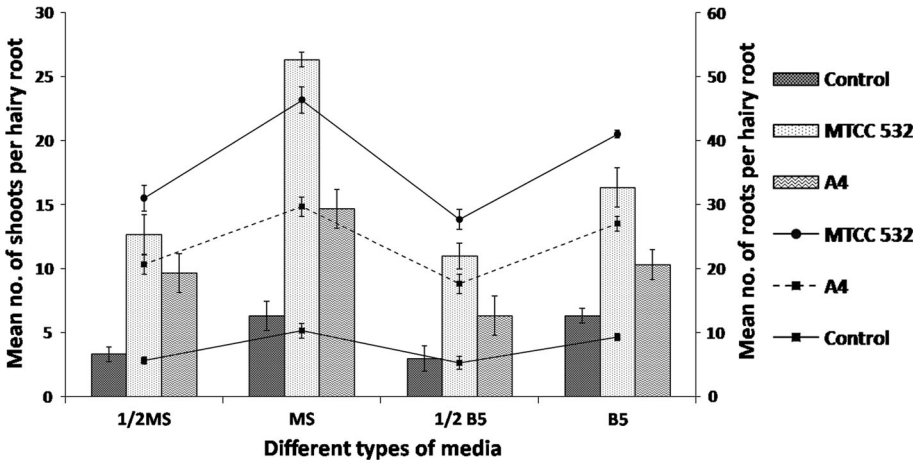

Fig. 3 Efficacy of different types of media on mean number of shoots and roots produced from A4 and MTCC 532 derived hairy roots of *B. monnieri*. Control refers to the regeneration of untransformed roots which had undergone same experimentation but without bacterial culture addition

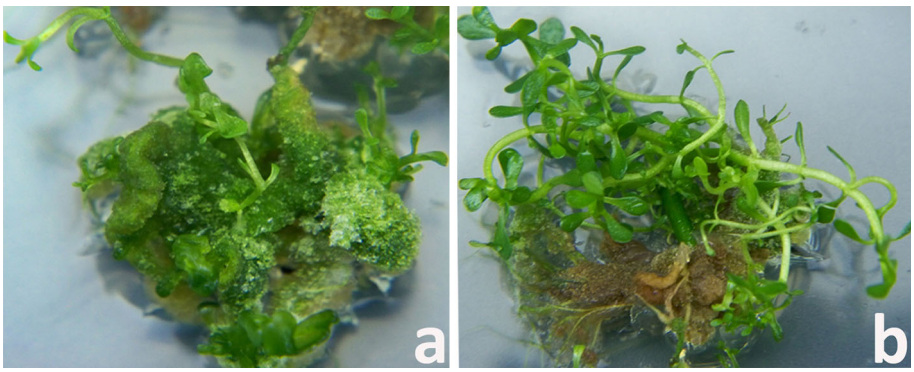

Fig. 4 Plant regeneration from MTCC 2364 derived hairy roots of *B. monnieri* on MS basal medium supplemented with 0.5 mg/L KN and 0.075 mg/L NAA. **a** Shoot buds induction from callus derived from MTCC 2364 derived hairy root after 10 days. **b** Fully grown HR plantlet after 28 days of culture

Table 1 Plant regrowth potential of hairy roots obtained through infection of MTCC 2364 in MS medium supplemented with different combinations of plant hormones

KN (mg/L)	NAA (mg/L)	% of roots forming shoots	Mean no. of shoots/hairy root	Mean no. of roots/hairy root
0.25	0.05	19.04 ± 0.57^{a}	0.67 ± 1.00^{a}	0.33 ± 0.57^{a}
0.5	0.05	33.33 ± 0.45^{c}	1.33 ± 1.15^{c}	1.33 ± 0.57^{b}
0.75	0.05	47.61 ± 1.12^{e}	2.66 ± 0.57^{d}	1.66 ± 1.00^{c}
1	0.05	38.09 ± 0.75^{d}	1.33 ± 0.57^{c}	2.33 ± 1.15^{d}
0.25	0.075	23.80 ± 1.23^{b}	1.00 ± 1.15^{b}	1.33 ± 1.00^{b}
0.5	0.075	52.38 ± 0.75^{f}	4.33 ± 1.15^{e}	2.66 ± 0.57^{e}
0.75	0.075	47.61 ± 0.57^{e}	2.66 ± 1.00^{d}	3.66 ± 1.15^{f}
1	0.075	33.33 ± 0.57^{c}	0.66 ± 0.57^{a}	1.33 ± 1.00^{b}
0.25	0.1	19.04 ± 1.23^{a}	0.66 ± 0.57^{a}	1.33 ± 1.57^{b}
0.5	0.1	47.61 ± 0.75^{e}	2.33 ± 1.15^{d}	3.00 ± 0.57^{e}
0.75	0.1	38.09 ± 1.12^{d}	4.66 ± 1.00^{e}	2.33 ± 1.00^{d}
1	0.1	23.80 ± 0.57^{b}	1.33 ± 0.57^{c}	1.66 ± 1.15^{c}

Means with the same letter within the column are not significantly different at ($P \leq 0.05$) according to DMRT

increased up to threefold. In the case of TFC, HR3 accumulated about 497.78 ± 0.57 mg QRE/g DW which was twofold higher production than the untransformed control. The same tendency has been followed in DPPH scavenging activity also. Highest % of DPPH inhibition was noted in HR3 (85.58 ± 0.14 %) while the other lines inhibited in a range of 57.69 ± 0.57–79.51 ± 0.79 %.

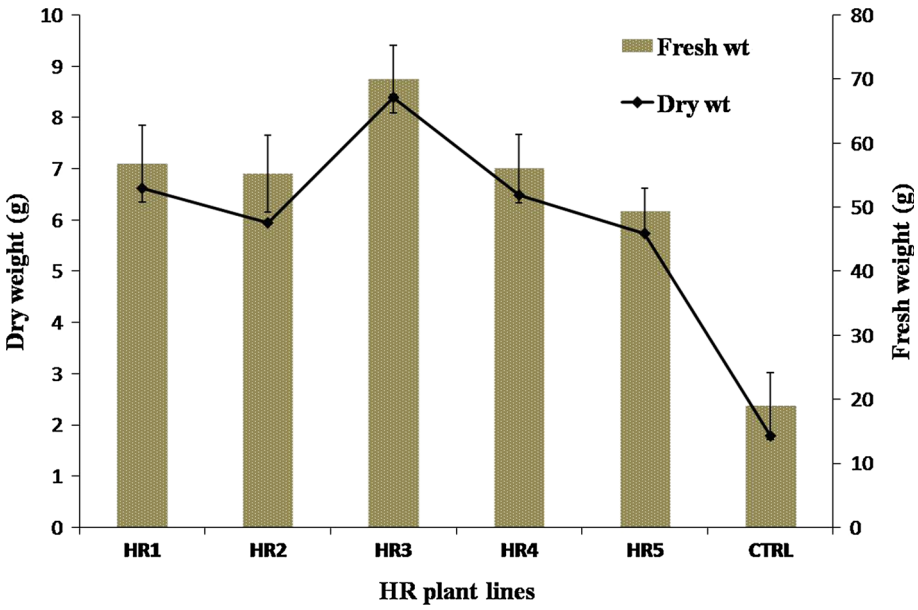

Fig. 5 Biomass accumulation in five different MTCC 532 derived HR plant lines of *B. monnieri* on MS basal liquid medium

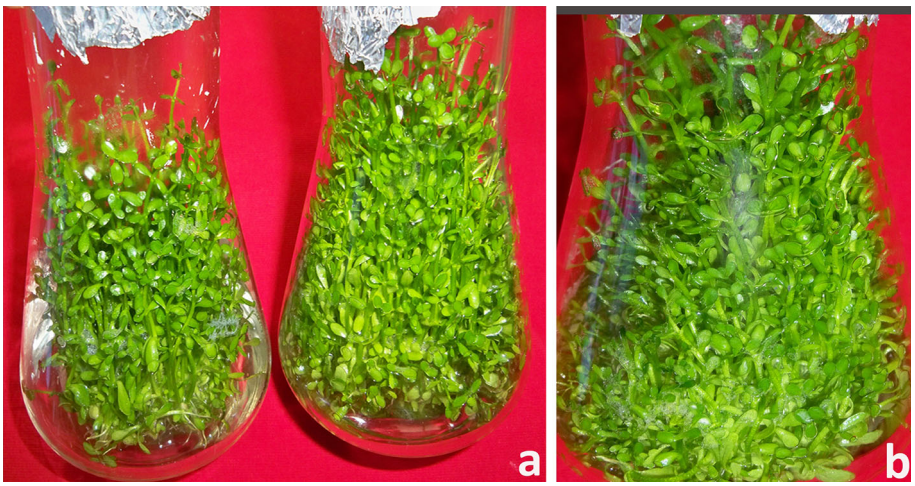

Fig. 6 Mass production of transformed *B. monnieri* on MS basal liquid medium. **a** Comparison of biomass of control and HR3 plantlets in 250 mL conical flasks. **b** Closer view of HR3 plantlets

Based on TPC, TFC, DPPH scavenging activity and biomass production, HR3 line alone has been selected for elicitation experiment. HR3 was elicited with different concentrations of chitosan for about 0–2 weeks. Analysis of FW and DW revealed that concentration is indirectly proportional to biomass accumulation (Table 2). Increased concentration and increased period of exposure of chitosan had detrimental effect on plant growth. For Bacoside A production, different concentrations of chitosan was added to 15 days old HR3 plants for 2 weeks. From HPLC estimation, it was observed that the HR3 plant extract has produced about 3.06 % of Bacoside A which was 2.75 fold higher accumulation when compared to untransformed control plant extract. Previously, A4 transformed plants of *B. monnieri* has resulted in 0.1 and 0.35 % of Bacoside A_3 and Bacopasaponin C (Majumdar et al. 2011) and HR3 plants of this study has produced about 0.6 and 0.97 % of Bacoside A_3 and Bacopasaponin C respectively.

In order to further enhance the Bacoside A levels, the strategy of elicitation has been carried out. On quantification, elicitation with 10 mg/L chitosan has accumulated about 5.84 % of Bacoside A which is two times higher than the content of un elicited cultures (3.06 %) and fivefold higher than the untransformed controls (1.11 %). Apart from 10 mg/L, increased or decreased chitosan concentration produced a lower amount of Bacoside A but higher than the un elicited culture (Table 3). Significant enhancement of all four components of Bacoside A i.e. Bacoside A_3, Bacopaside II, Jujubogenin isomer of Bacopasaponin C and Bacopasaponin C was noticed in elicited HR3 plant extracts. Among the four saponins, Bacopaside II has accumulated maximally (1.92 %) which has

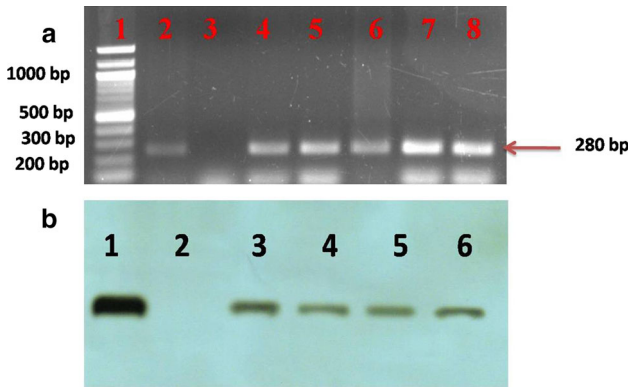

Fig. 7 **a** PCR amplification of *rol A* gene in five HR plant lines of *B. monnieri*. *Lane 1* 100 bp ladder, *lane 2* positive control, *lane 3* negative control (untransformed). *Lanes 4–8* five HR plant lines such as HR1, HR2, HR3, HR4 and HR5. **b** Southern blot hybridization analysis of *rol A* gene in HR plant lines of *B. monnieri*. *Lane 1* positive control (plasmid DNA), *lane 2* negative control (untransformed plant DNA), *lane 3–6* HR plant lines

enhanced two times when compared to unelicited culture (0.98 %). Bacoside A_3, the next highly accumulated saponin has showed 2.5 fold increment (1.66 %) on comparison with unelicited culture (0.6 %). HPLC chromatograms of control and chitosan treated methanolic extracts of HR3 plants of *B. monnieri* were shown in Figs. 9 and 10.

Discussion

The main objective of the study was to determine the regeneration potential of hairy roots of *B. monnieri*. Reduction of nutrients in the medium due to continuous growth and hormonal inequity in transformed tissues can be regarded as factors for shoot regeneration from hairy roots (Mehrotra et al. 2013). It can be light dependent and color change in hairy roots was also noticed as a prelude to

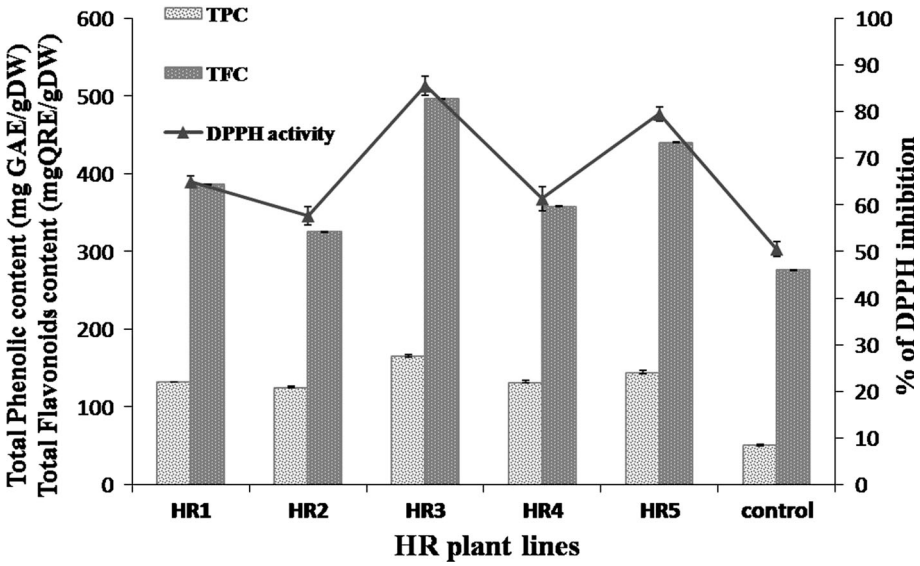

Fig. 8 TPC, TFC and DPPH scavenging activity of control and five HR plant lines of *B. monnieri*

Table 2 Influence of chitosan elicitation on biomass of transformed plants during various time of exposure

Chitosan concentration (mg/L)	Exposure time					
	0 week		1 weeks		2 weeks	
	FW	DW	FW	DW	FW	DW
0	4.52 ± 0.06^{d}	0.64 ± 0.04^{a}	5.63 ± 0.04^{e}	0.81 ± 0.02^{e}	6.47 ± 0.06^{a}	0.88 ± 0.04^{d}
5	4.32 ± 0.06^{b}	0.53 ± 0.04^{a}	4.27 ± 0.07^{d}	0.53 ± 0.03^{d}	3.90 ± 0.05^{c}	0.45 ± 0.01^{c}
10	4.17 ± 0.06^{a}	0.44 ± 0.04^{a}	3.74 ± 0.03^{bc}	0.44 ± 0.02^{c}	3.28 ± 0.02^{bc}	0.30 ± 0.01^{b}
15	4.09 ± 0.06^{e}	0.72 ± 0.04^{a}	3.12 ± 0.04^{b}	0.38 ± 0.01^{b}	2.75 ± 0.04^{b}	0.25 ± 0.03^{b}
20	3.95 ± 0.06^{c}	0.48 ± 0.04^{a}	2.25 ± 0.05^{a}	0.32 ± 0.01^{a}	2.01 ± 0.04^{a}	0.15 ± 0.04^{a}

Means with the same letter within the column are not significantly different at $P \leq 0.05$ according to DMRT

Table 3 Quantification of Bacoside A (based on dry weight) upon elicitation of HR3 with chitosan for about 2 weeks

Concentration of chitosan (mg/L)	Bacoside A_3 (%)	Bacopaside II (%)	Jujubogenin isomer of Bacopasaponin C (%)	Bacopasaponin C (%)	Total Bacoside A (%)
Untransformed (control)	0.29	0.40	0.09	0.33	1.11
Transformed and unelicited	0.6	0.98	0.51	0.97	3.06
5	0.80	1.09	0.66	1.42	3.97
10	1.66	1.92	1.00	1.26	5.84
15	0.77	1.02	0.56	0.91	3.26

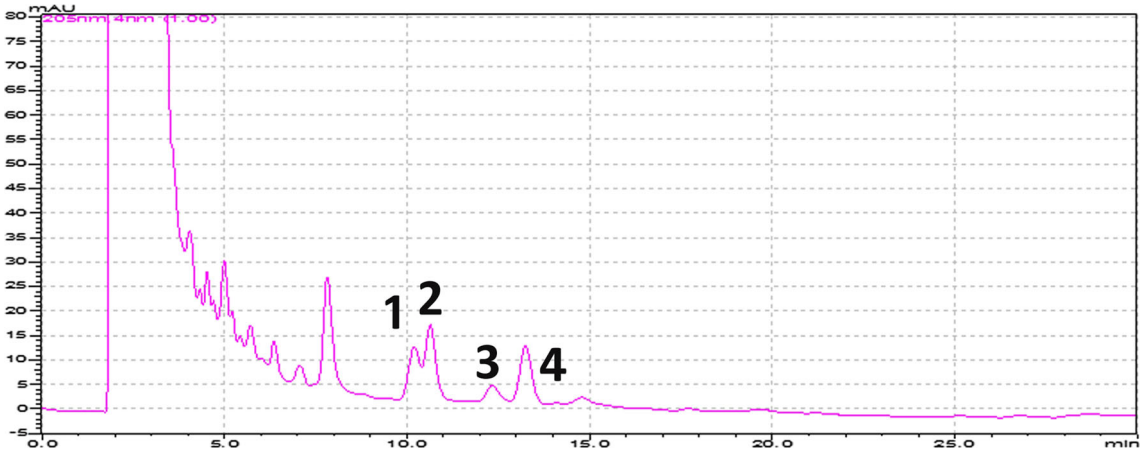

Fig. 9 HPLC chromatogram of methanolic extracts of transformed unelicited HR 3 control of *Bacopa monnieri* (L.). Components of Bacoside A are marked as follows: *1* Bacoside A3, *2* Bacopaside II, *3* Jujubogenin isomer of Bacopasponin C, *4* Bacopasaponin C

regeneration. Ri-transformed root cultures of *Tylophora indica* showed variation in pigmentation when cultured under light. While, the hairy root cultures of *T. indica* remained white when cultured in dark, on exposure to light, they became green to red depending on the light intensity (Chaudhuri et al. 2006). Hairy root turning green on exposure to light was also reported in *Solanum khasianum* (Jacob and Malpathak 2005).

In *B. monnieri*, we could notice the great disparity in regeneration ability of hairy roots. The variation in shoot induction potential among the hairy roots induced by different strains might be due to difference in the virulence of different *A. rhizogenes* strains (Zehra et al. 1999). Besides each transformation event is distinct because of different integration sites and copy numbers of Ri T-DNA and this could be the reason for difference in regeneration potential of different root lines. This was observed in *Lycopersicon* (Peres et al. 2001) and *Plumbago* (Gangopadhyay et al. 2010). Other crucial aspects such as nature of culture medium, its concentration and addition of plant hormones were also dependable for shoot regeneration from hairy roots. Induction of shoot bud directly from the transformed roots in the presence and absence of plant growth regulators were reported in many plants such as *Taraxacum platycarpum* (Lee et al. 2004), *Plumbago rosea* (Satheeshkumar et al. 2009), *S. miltiorrhiza* (Wang et al. 2013), *Hypericum perforatum* (Vinterhalter et al. 2006), *T. indica* (Chaudhuri et al. 2006) and *B. monnieri* (Majumdar et al. 2011) respectively. Recently Piatczak et al. (2015) showed the spontaneous regeneration of *Rehmannia glutinosa* from the A4 hairy roots without plant hormones as like in our case. Concentration of hormones used for effective regeneration varied from plant to plant along with other conditions like, size of transformed root used as explant, concentration of sucrose used, photoperiod at which cultures were maintained, media used for rooting etc.

The vigorous increase in the biomass of transformed plants of *B. monnieri* witnessed the occurrence of transformation event as in the case of many medicinal plants. In *Gentiana macrophylla*, the regenerated plantlets increased the biomass of roots and leaves by fivefold and onefold (Wu et al. 2011). The transformed plants from hairy roots of *Plumbago indica* had a higher root biomass

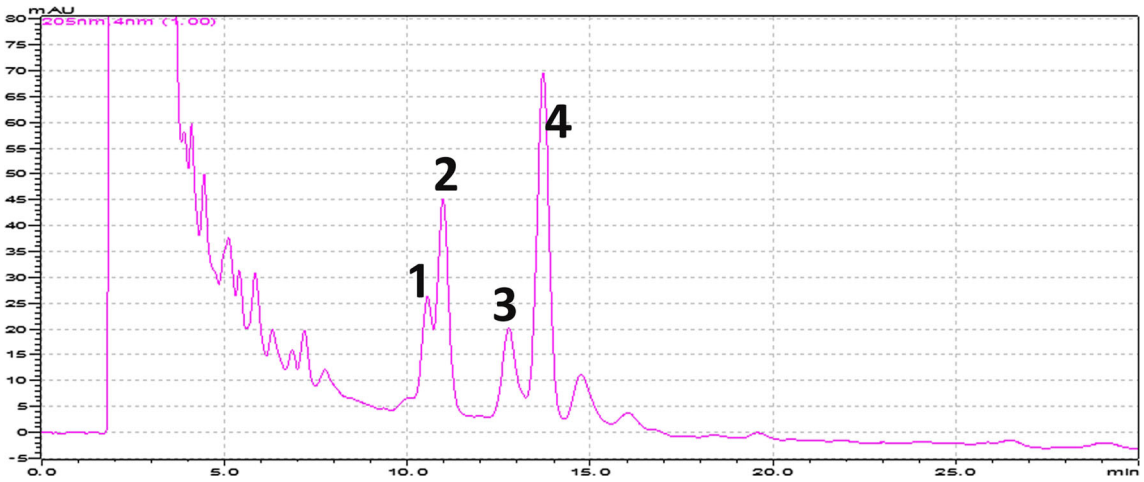

Fig. 10 HPLC chromatogram of methanolic extracts of HR3 of *Bacopa monnieri* (L.) elicited with 10 mg/L chitosan for 2 weeks. Components of Bacoside A are marked as follows: *1* Bacoside A3, *2* Bacopaside II, *3* Jujubogenin isomer of Bacopasponin C, *4* Bacopasaponin C

(Gangopadhyay et al. 2010) when compared to its non transformed counterparts. Chaudhuri et al. (2006) reported about 200–320 % increase in the biomass of plants regenerated from *T. indica* hairy roots and augmented tylophorine content (20–60 %) in the shoots. The increased biomass of transformed plants is because of the expression of *rol C* gene which is responsible for reduced apical dominance, internodes shortening, and increased branching (Nilsson and Olsson 1997). Enhancement of secondary metabolites quantity in Ri transformed plants represents the fact that insertion of T-DNA interfere with the biosynthetic pathway of biologically active secondary metabolites. In the case of Bacopa, we have documented a hike in TPC, TFC and DPPH scavenging activity of transformed plants. Similar study involving the estimation of TPC and DPPH scavenging activity of hairy roots was carried out in *Dracocephalum moldavica* (Jezyna et al. 2013). Recently Vinterhalter et al. (2015) reported higher TPC and DPPH scavenging activity in the hairy roots of *Gentiana dinarica*. The increased DPPH scavenging activity of transformed plants is due to the increased accumulation of TPC and TFC.

Many recent studies have recognized the effects of eliciting hairy roots and plants derived from them for increased production of specific metabolites. As in Bacopa, biomass reduction of adventitious roots of *Withania somnifera* and hairy roots of *Panax ginseng* were reported by Sivanandhan et al. (2012) and Jeong and Park (2005) respectively during elicitation with chitosan. According to Sivanandhan et al. (2012), enhanced production of secondary metabolites depends on genotype and culture conditions like inoculum mass, culture age, elicitor's exposure time and its concentration. Chitosan was proved as a potential elicitor in increasing the syringin production in the suspension cultures of *Saussurea medusa* (Xu et al. 2007), withanolides and valtrate production in adventitious root cultures of *W. somnifera* (Sivanandhan et al. 2012) and in adventitious roots of *Valeriana amurensis* (Cui et al. 2012) respectively.

Conclusions

The present study validated the extensive regeneration potential of MTCC 532 derived hairy roots of *B. monnieri*. On MS basal liquid medium it produced fourfold enhanced biomass of about 63.25 g FW and improved biological activity. It also exhibited a sharp rise in Bacoside A production (5.83 %) when elicited with 10 mg/L chitosan for the first time. Thus the study provides an efficient protocol for increased biomass production and Bacoside A accumulation in HR plants through the combined strategies of transformation and elicitation. This would be beneficial in further scaling up studies for bioreactor based large scale commercial production of biomass and useful metabolites of the plant. In addition, the question of depletion of wild stock to subsidize the ever increasing market demand of the herb would be addressed.

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