Plant Cell, Tissue and Organ Culture (PCTOC) (2021) 146:541–552
https://doi.org/10.1007/s11240-021-02092-5

ORIGINAL ARTICLE

Effects associated with insertion of *rol* genes on morphogenic potential in explants derived from transgenic *Bacopa monnieri* (L.) Wettst

Sayantika Sarkar[1] · **Sumita Jha**[1]

Received: 11 January 2021 / Accepted: 23 April 2021 / Published online: 12 May 2021

Abstract

The present study deals with the establishment of *rolA*-transgenic and *rolB*-transgenic plants for the first time through *Agrobacterium tumefaciens* mediated transformation, exploiting the inherent morphogenic potential of an important medicinal plant, *Bacopa monnieri* (L.) Wettst. The *rolA*-transgenic and *rolB*-transgenic plants showed integration and expression of *rolA* and *rolB* genes respectively, whereas Ri-transformed plants showed integration and expression of *rolA*, *rolB*, *rolC* and *rolD* genes. Morphogenic potential of different types of explants derived from *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants on basal medium was evaluated. Shoot organogenesis was enhanced significantly in leaf (1.6-fold) and internode (1.4-fold) explants derived from *rolA*-transgenic plants, *rolB*-transgenic leaf (2.4-fold) and internode (1.6-fold) explants as well as leaf (5.2-fold) and internode (3.3-fold) explants derived from Ri-transformed plants compared to explants from non-transformed plants. Substantial increase in root organogenesis was also noticed in *rolA*-transgenic leaf (1.7-fold) explants, *rolB*-transgenic leaf (3.6-fold) and internode (1.4-fold) explants as well as leaf (4.1-fold) and internode (1.9-fold) explants derived from Ri-transformed plants compared to non-transformed ones. In addition to this, growth of root tip and shoot regeneration was also noticed from Ri-transformed root explants, but not in *rolA*-transgenic, *rolB*-transgenic and non-transformed roots. Clones of each transgenic plant line harboring rol genes depicted notable phenotypic changes including reduced shoot and internode length, increased number of nodes/plant, leaves/plant and roots/plant. The leaf morphology was altered in *rolB*-transgenic and Ri-transformed plants but not in *rolA*-transgenic plants.

Key message

Insertion of *rol* genes, individual or in combination, of *Agrobacterium rhizogenes* resulted in enhancedmorphogenic potential of excised explants and alteration in phenotype of transgenic *Bacopa monnieri* plantsmaintained in vitro.

Keywords *Bacopa monnieri* · Morphogenesis · Morphology · *rolA* transgenic · *rolB* transgenic · Ri transformed

Introduction

Plant cells are highly totipotent to undergo morphogenesis in vitro through shoot organogenesis and/or somatic embryogenesis (Bhojwani and Razdan 1983). Although both the developmental programmes are regulated by several factors, Skoog and Miller's (1957) classical findings on quantitative auxin/cytokinin ratio plays a pivotal role in morphogenesis. Since then, several pioneer works have concluded that the fate of regenerating organs during morphogenesis can be altered either exogenously by hormone application in the culture media (Tran Than Van 1980) or endogenously within the plant body by introducing *Agrobacterium* T-DNA genes synthesizing auxins and cytokinins (Owens et al. 1988; Wabiko and Minemura 1996).

For centuries, the complex plant-*A. rhizogenes* pathogenic interaction and functions of Ri T-DNA oncogenes have been deciphered (Mauro et al. 2017; Bahramnejad et al.

Communicated by Ali R. Alan.

✉ Sumita Jha
sumitajha.cu@gmail.com; sjbot@caluniv.ac.in

[1] Department of Botany, University of Calcutta, 35, Ballygunge Circular Road, Kolkata, West Bengal 700 019, India

2019). This bacterium induces neoplastic root growth in host plants at infection sites by transferring its T-DNA to produce opines for food and energy (Tepfer and Tempé 1981; Chilton et al. 1982). Molecular analysis has revealed four *ro*oting *l*oci (*rolA*, *rolB*, *rolC*, *rolD*) of T-DNA involved in rhizogenesis by manipulating endogenous hormone metabolism/ sensitivity in transformed plant cells although exact mechanism of action is unknown (White et al. 1985; Mauro et al. 2017). Due to hormonal disequilibrium, developmental programmes are altered, as a side-effect, in transformed plants affecting morphogenesis. The Ri-transformed rhizoclones regenerate spontaneously or induced in hormone-supplemented media into Ri-transformed plants with altered phenotypes through direct or indirect morphogenesis (Roychowdhury et al. 2013). Although Ri-transformed plant regeneration was amply demonstrated, reports on transgenic plant production harbouring individual *rol* genes are scarce which explained the contribution of these genes in morphogenesis (Sarkar et al. 2018). The inter-relationship between *rol* genes and morphogenesis is further confirmed when explants of Ri-transformed or *rol*-transgenic plants were investigated for organogenic response although the number of such reports is very limiting. Among them, *rolB* gene has been extensively studied and is considered as a meristem-inducing gene (Altamura 2004). Casanova et al. (2003) demonstrated dual activities of *rolC* gene due to improved shoot and root regeneration in transgenic *Dianthus caryophyllus* explants in presence of cytokinin and auxin respectively. However, the effects of *rolA* and *rolD* genes on organogenesis has been very less explored. van Altvorst et al. (1992) reported root induction in *rolA* or *rolB* or *rolABC*-expressing tomato leaf explants in presence of auxin but not in controls. But *rolD* gene enhanced flower induction in flowering and rooting media in transgenic tobacco thin cell layer (TCL) explants indicating its commitment towards floral organogenesis (Mauro et al. 1996).

Bacopa monnieri (L.) Wettst. (Plantaginaceae), due to its diverse pharmacological potential, has been widely studied for micropropagation and de novo organogenesis (Saha et al. 2020). We have reported earlier high morphogenic potency of excised leaf and internode explants on basal medium and suggested this species to be an ideal system to study morphogenesis in vitro, circumventing the effects of exogenous phytohormones (Sarkar and Jha 2017).

Induction of Ri-transformed roots followed by Ri-transformed plant regeneration through *A. rhizogenes*-mediated transformation was reported in *B. monnieri* (Majumdar et al. 2011; Paul et al. 2015). Ri-transformed roots, unlike non-transformed roots, regenerated spontaneously either directly or from Ri-transformed calli on basal medium (Majumdar et al. 2011; Paul et al. 2015; Largia et al. 2016), suggesting their higher shoot organogenic competence due to T-DNA oncogenes. As excised *B. monnieri* explants inherently undergo morphogenesis on basal medium, the interaction of *rol* genes with endogenous hormones during in vitro organogenesis can easily be investigated in this species. Reports are available on *A. tumefaciens*-mediated transformation to develop transgenic *B. monnieri* plants (Majumdar et al. 2012; Yadav et al. 2014; Paul et al. 2015). However, attempt to produce transgenic plants expressing individual *rol* genes and to study the role of *rol* genes on morphogenesis in *B. monnieri* has not been made. Therefore, in the present research work, *B. monnieri* was transformed by individual *rolA* and *rolB* genes and also by Ri T-DNA through *Agrobacterium*-mediated transformation. The objectives of the study were to evaluate the effects associated with insertion of *rol* genes, either individual or in combination, on plant phenotype and morphogenic potential of explants from transgenic plants.

Materials and methods

Tissue culture

Axenic cultures of *B. monnieri* were established from shoot tip explants (Sarkar and Jha 2017). The cultures were maintained with regular sub-culture on MS (Murashige and Skoog 1962) basal medium containing 3 % (w/v) sucrose and 0.75 % (w/v) agar (Sigma, India).

Bacterial strains and cultures

Agrobacterium tumefaciens strain GV3101 contains binary vector pMRK10 harbouring *rolA* gene along with plant selectable marker gene, neomycin phosphotransferase (*nptII*) (Slightom et al. 1986). *A. tumefaciens* strain LBA4404 contains binary vector pBIN19 + harbouring only *nptII* gene and served as *nptII* control (Chaudhuri et al. 2009). *A. rhizogenes* strain A4 harbours pRiA4 (Cardarelli et al. 1985). These strains were obtained from Dr. David Tepfer, INRA, Versailles, France.

A. tumefaciens strain LBA4404 contains binary vector MpCAMBIA1301 (Mukherjee et al. 2019) harbouring *rolB* gene along with plant selectable marker gene, hygromycin phosphotransferase (*hptII*). *A. tumefaciens* strain LBA4404 carrying only *hptII* gene was used as *hptII* control. Both the strains were obtained from Dr. Debabrata Basu, Professor, Division of Plant Biology, Bose Institute.

A. tumefaciens strain GV3101-*rolA* was cultured in liquid AP medium (Petit et al. 1983; pH 6.6) supplemented with neomycin (200 mg l^{-1}) and rifampicin (50 mg l^{-1}), strain LBA4404-*nptII* in AP medium with kanamycin (100 mg l^{-1}) whereas strains LBA4404-*rolB* and LBA4404-*hptII* were cultured in LB medium (Miller 1972; pH 7.0) supplemented with kanamycin (50 mg l^{-1}) and rifampicin

(50 mg l^{-1}). *A. rhizogenes* strain A4 was cultured in YMB medium (Hooykass et al. 1977; pH 7.0). Bacterial cultures were kept on a gyratory shaker (Certomat) at 28ºC in dark at 180–200 rpm for 24–48 h. Acetosyringone (200 µM) was added to bacterial suspension (~ 10^{10} cells ml^{-1}) 2 h before infection to improve virulence.

Sensitivity test of antibiotics on shoot organogenesis from explants

Prior to transformation, the amount of kanamycin and hygromycin that inhibit shoot organogenesis in leaf and internode explants of non-transformed plants was tested to select transgenic shoots. The explants were cultured on MS medium containing different concentrations of kanamycin (25, 50, 75, 100 mg l^{-1}), hygromycin (5, 10, 15, 20, 25, 30 mg l^{-1}) and cefotaxime (250, 500 mg l^{-1}). After four weeks, frequency of explants showing shoot organogenesis was recorded. For each experiment, 30 explants of each type were used per treatment and the experiment was repeated thrice.

Transformation procedure

Leaves (1-1.5 cm) and internodes (~ 0.9 cm) excised from 15-days-old micropropagated plants were used as explants. Transformation was done following the protocol of Paul et al. (2015). For each experiment, 50 explants of each type from different plants were infected and the experiment was repeated thrice.

Transformation with *A. tumefaciens* strains

After surface-sterilization, control and infected explants were cultured on MS medium containing cefotaxime (500 mg l^{-1}) and kanamycin (100 mg l^{-1}) for GV3101-*rolA* and LBA4404-*nptII* transformation and MS medium containing cefotaxime (500 mg l^{-1}) and hygromycin (15 mg l^{-1}) for LBA4404-*rolB* and LBA4404-*hptII* transformation.

Transformation with *A. rhizogenes* strain

After surface-sterilization, control and infected explants were cultured on MS medium containing cefotaxime (500 mg l^{-1}). Roots induced at wound sites of infected explants were excised and cultured on the same medium under 16/8 h photoperiod or under complete darkness. Each excised root was propagated as a separate root line. Fast growing axenic root lines were maintained on cefotaxime-free MS medium after six months of culture initiation with regular subculture at 4-weeks interval. Roots induced from uninfected explants (control) were similarly excised and cultured on MS medium containing cefotaxime (500 mg l^{-1}).

Establishment of transgenic plants

Shoot organogenesis and establishment of rolA-transgenic and rolB-transgenic plants

After two months of shoot bud induction from wound sites of infected explants on respective selection media, infected explants with developing micro shoots (< 1.5 cm) were transferred to selection media supplemented with 1.0 mg l^{-1} 6-benzylaminopurine (BA). Micro shoots regenerated from shoot buds induced from wound sites of different explants derived from different plants were propagated as separate clones. After four weeks, excised elongated shoots were cultured on BA-free selection media with sub-culturing at 4-weeks interval (cefotaxime concentration was gradually reduced to zero). Micro shoots were rooted spontaneously to develop complete rooted plantlets. After six months of culture initiation, putatively transgenic plants were cultured on respective liquid bacterial media on a gyratory shaker at 28ºC in dark at 180 rpm for 24–48 h to determine their axenic nature. Finally, clones of *rolA*-transgenic and *nptII*-transgenic plants were established on MS medium containing kanamycin (100 mg l^{-1}) and *rolB*-transgenic plants on MS medium containing hygromycin (15 mg l^{-1}).

Shoots regenerated from leaf and internode explants of non-transformed plants via organogenesis on MS medium and MS containing cefotaxime (500 mg l^{-1}) were maintained separately as control.

Spontaneous regeneration from Ri-transformed roots and establishment of Ri-transformed plants

Shoot buds (~ 1 cm) spontaneously regenerated from Ri-transformed roots were excised and cultured on MS medium containing cefotaxime (500 mg l^{-1}). Micro shoots regenerated from shoot buds induced from different Ri-transformed root lines were multiplied as separate clones. Micro shoots were rooted spontaneously on this medium to develop complete rooted plantlets. Cefotaxime was withdrawn after six months of regular sub-culture at 4-weeks interval and the axenic cultures were finally established on MS basal medium. Excised non-transformed roots were cultured on MS containing BA (0.01 mg l^{-1}) for shoot induction (control).

Confirmation of transformation by PCR and RT-PCR

Genomic DNA was extracted from different transgenic and non-transformed plants after one year of establishment on respective selection media according to Dellaporta et al. (1983). Isolated DNA was analyzed by PCR for *rolA*, *rolB*, *rolC*, *rolD*, *nptII* and *hptII* genes using gene-specific primers (Beck et al. 1982; Diouf et al. 1995; Sevón et al. 1997;

Wang et al. 2001; Christensen et al. 2008). Plasmid DNA isolated from the corresponding bacteria following standard alkali lysis protocol (Sambrook and Russel 2001) was used as positive control, whereas genomic DNA from non-transformed plants was used as negative control. To eliminate the false positive result due to bacterial contamination, *virD1* gene-specific primers (Alpizar et al. 2008) were used. PCR amplicons were resolved by 1.2 % (w/v) agarose gel electrophoresis with a 100 bp plus DNA ladder (Thermo Scientific, USA) and visualized by ethidium bromide staining under UV light. Documentation was done using Gel Doc™ EZ Imager (BioRad). For each transgenic plant, ten replicates per clone were used and the experiment was repeated thrice.

Expression of transgenes at the transcription level was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from fresh tissues of 4-weeks-old transgenic and non-transformed plants using HiPurA™ Plant and Fungal RNA Miniprep Purification Kit (HIMEDIA) following the manufacturer's protocol. RT-PCR reactions were performed as described earlier (Majumdar et al. 2011; Paul et al. 2015).

Maintenance of regenerated transgenic plants

Axenic *rolA*-transgenic, *nptII*-transgenic, *rolB*-transgenic and Ri-transformed plants were maintained on respective selection media for over three years with regular subculture at 8-weeks interval.

Study of in vitro morphogenic potential in explants derived from transgenic plants on basal medium

Excised leaves (~ 0.95 cm), internodes (~ 0.90 cm) and root (1.5-2.0 cm) from 4-weeks-old different transgenic and non-transformed plants were cultured on MS basal medium as reported earlier (Sarkar and Jha 2017). After four weeks, morphogenic responses from different explants were evaluated. For leaf and internode explants, number of shoot buds/ micro shoots induced per explant and number of roots induced per explant were recorded whereas shoot bud development and/or root tip growth were examined for root explants. For each experiment, 30 explants of each type per transgenic clone and non-transformed plant were used and the experiment was repeated thrice.

Analysis of morphology of transgenic plants maintained in vitro

Morphological characterization of different transgenic plants was done based on morphological descriptors reported earlier for Ri-transformed plants (Majumdar et al. 2011). Shoot tips (~ 2 cm) were cultured in culture tubes (15 × 2.5 cm) containing 20 ml MS containing kanamycin (100 mg l^{-1}) for *rolA*-transgenic and *nptII*-transgenic plants, MS with hygromycin (15 mg l^{-1}) for *rolB*-transgenic plants and MS basal medium for Ri-transformed plants under 16/8 h photoperiod. One shoot tip was cultured per culture tube. Shoot tips from non-transformed plants were similarly cultured on MS basal medium (control). After six weeks, the plants were harvested, washed, blotted dry and morphological data were taken. For each experiment, 30 explants were used per transgenic clone and non-transformed plant and the experiment was repeated thrice.

Study of auxin sensitization response and effect of exogenous cytokinins on in vitro morphogenesis in explants derived from transgenic plants

Leaves, internodes and root segments from 4-weeks-old different transgenic and non-transformed plants were cultured on MS media fortified with 0.01, 0.05 and 1.0 mg l^{-1} of filter-sterilized auxin i.e. IAA (indole-3-acetic acid) and cytokinins i.e. 6-benzylaminopurine (BA) and kinetin (KN). After four weeks, number of roots induced per explant in auxin-supplemented media and number of shoot buds/micro shoots induced per explant in cytokinin-supplemented media were recorded for leaf and internode explants whereas shoot bud development and/or root tip growth were examined for root explants in all hormone-treated media. For each hormone concentration, 30 explants of each type per transgenic clone and non-transformed plant were used and the experiment was repeated thrice.

Statistical analysis

All experiments were randomized and were repeated at least thrice. Data were examined by one-way analysis of variance (ANOVA) to detect significant differences ($p \leq 0.05$) in the mean (Sokal and Rohlf 1987). A post hoc mean separation was performed by Tukey B test at the same 5 % probability level using SPSS software (version 22.0). Variability in the data was expressed as the mean ± standard deviation (SD).

Results

Effects of antibiotics on shoot organogenesis: sensitivity test

100 % of leaf and internode explants from non-transformed *B. monnieri* plants cultured on MS medium supplemented with cefotaxime (0, 250, 500 mg l^{-1}) showed shoot regeneration as reported earlier (Majumdar et al. 2012). Shoot induction was observed in excised explants cultured on MS medium containing 25 mg l^{-1} (70–75 %) and 50 mg l^{-1} (40–42 %) kanamycin or 5 mg l^{-1} (60–90 %) and 10 mg

l^{-1} (0–50 %) hygromycin. Total inhibition of shoot induction from explants was observed on MS medium containing 75–100 mg l^{-1} kanamycin or 10–15 mg l^{-1} hygromycin. However, explants were necrosed within four weeks on medium with cefotaxime (500 mg l^{-1}) and kanamycin (100 mg l^{-1}) and medium containing cefotaxime (500 mg l^{-1}) and hygromycin (15 mg l^{-1}).

A. tumefaciens-mediated transformation and establishment of *rolA*-transgenic and *rolB*-transgenic plants on selection media

Direct shoot organogenesis was observed from wound sites of explants infected with *A. tumefaciens* harbouring *rolA* and *nptII* genes and *A. tumefaciens* harbouring *nptII* gene (*nptII* control) within four weeks, whereas control explants showed no organogenesis and necrosed within 6–8 weeks on MS medium containing cefotaxime (500 mg l^{-1}) and kanamycin (100 mg l^{-1}) (Fig. 1a–f). The frequency of explants infected with *A. tumefaciens* harbouring *rolA* and *nptII* genes showing shoot bud induction was recorded 7 % (leaf) and 4 % (internode). The frequency of leaf explants infected with *A. tumefaciens* harbouring *nptII* gene showing shoot bud induction was 6 % whereas infected internode explants showed no shoot organogenesis and necrosed. The *rolA*-transgenic and *nptII*-transgenic plants were established and maintained on MS medium containing 100 mg l^{-1} kanamycin for over three years with regular sub-culture at 4-weeks interval.

Direct shoot organogenesis was observed from wound sites of explants infected with *A. tumefaciens* harbouring *rolB* and *hptII* genes within four weeks, whereas explants infected with *A. tumefaciens* harbouring *hptII* gene (*hptII* control) and uninfected control explants showed no shoot organogenesis and necrosed within 6–8 weeks on MS medium containing cefotaxime (500 mg l^{-1}) and hygromycin (15 mg l^{-1}) (Fig. 1 g–l). The frequency of infected leaf explants showing shoot bud development was 5 % whereas internode explants did not respond and necrosed. The *rolB*-transgenic plants were finally established and maintained on MS medium containing 15 mg l^{-1} hygromycin for over three years with regular sub-culture at 4-weeks interval. However, *hptII*-transgenic plants could not be established in the present study.

Establishment of Ri-transformed root culture and development of Ri-transformed plants following infection with *A. rhizogenes* strain A4

Roots were induced from wound sites of infected leaf and internode explants (30 %) within four weeks on MS medium containing cefotaxime (500 mg l^{-1}). The roots induced were thin, light-green and fast-growing with lateral branches. Each putatively transformed primary root induced at each wound site was maintained as root line. Shoot buds (5 per explant) were regenerated spontaneously in Ri-transformed root lines (60 %) within 10 days when excised and cultured on the same medium. Micro shoots regenerated from shoot

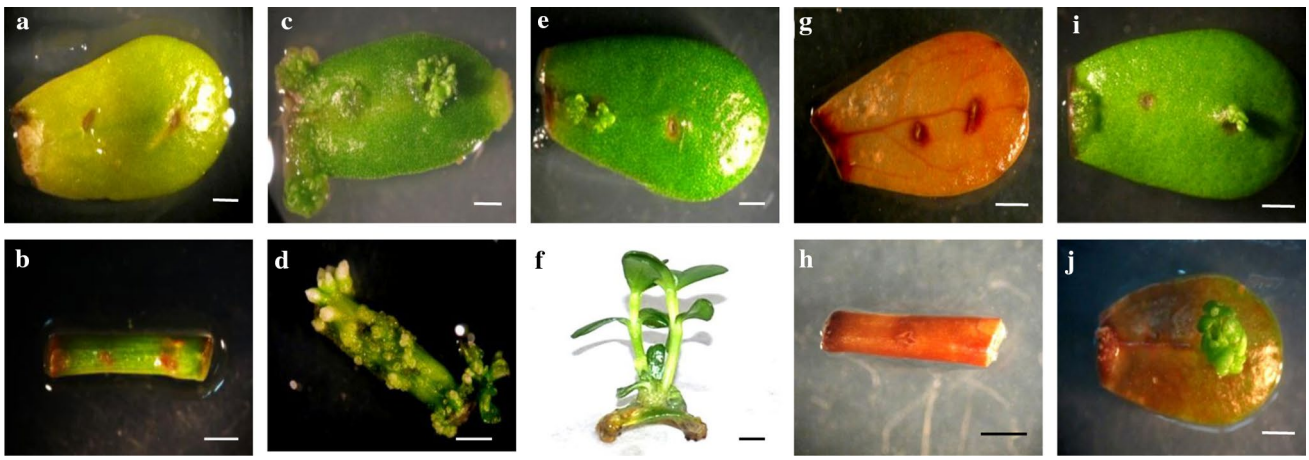

Fig. 1 Shoot bud induction following infection of *B. monnieri* explants with different *A. tumefaciens* strains on respective selection media. **a, b** uninfected (control) leaf and internode explants after four weeks on MS + $Cefo_{500}$ + Kan_{100}, **c, d** shoot bud induction from wound sites of leaf and internode explants following transformation with *A. tumefaciens* strain GV3101 harbouring *rolA* gene on MS + $Cefo_{500}$ + Kan_{100}, **e, f** shoot bud induction from leaf explants infected with *A. tumefaciens* strain LBA4404 harbouring *nptII* gene on MS + $Cefo_{500}$ + Kan_{100},, **g, h** uninfected (control) leaf and internode explants after four weeks on MS + $Cefo_{500}$ + $Hygro_{15}$, **i, j** shoot bud induction from wound site of leaf explant infected with *A. tumefaciens* strain LBA4404 harbouring *rolB* gene on MS + $Cefo_{500}$ + $Hygro_{15}$. Scale bar = 10mm

buds induced from different Ri-transformed root lines were maintained as separate clones.

Roots induced from uninfected control explants necrosed within four weeks on MS medium containing cefotaxime (500 mg l^{-1}). Shoots were induced from excised roots of non-transformed plants on MS medium containing BA (0.01 mg l^{-1}) within four weeks and were used as control.

Confirmation of transformation

PCR and RT-PCR analysis showed integration and expression of *rolA* and *nptII* genes in *rolA*-transgenic plant clones, *nptII* gene in *nptII*-transgenic plant clones, *rolB* and *hptII* genes in *rolB*-transgenic plant clones and *rolA*, *rolB*, *rolC*, *rolD* genes in Ri-transformed plant clones (Fig. S1). The amplified products were of expected size and identical to respective positive controls. Absence of *virD1* gene amplification in transgenic plants confirmed no bacterial contamination. No amplification was observed in the genomic DNA of non-transformed plants.

Comparative morphology of transgenic plants harbouring *rolA*, *rolB* and Ri T-DNA genes

In the present study, *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants of *B. monnieri* displayed notable changes in vegetative morphology compared to respective controls.

The *rolA*-transgenic plants were shorter with reduced internode length, higher number of nodes/plant and leaves/plant than *nptII*-transgenic and non-transformed plants (Fig. 2). The number of roots/plant and root length were also increased in *rolA*-transgenic plants. However, *rolA*-transgenic plants were similar to *nptII*-transgenic and non-transformed plants in leaf length and shape (oblanceolate) with no axillary shoot formation (Fig. S2). The morphological characteristics of *nptII*-transgenic plants were similar to that of non-transformed plants with respect to shoot and internode length, number of nodes/plant, leaves/plant, roots/plant and leaf morphology. However, roots induced from nptII-transgenic plants were shorter than the roots induced from non-transformed plants.

The *rolB*-transformants were dwarf due to decreased shoot and internode length, with greater number of nodes/plant and leaves/plant than non-transformed plants (Fig. 2). Leaves of *rolB*-transgenic plants were smaller due to alteration in leaf shape (roundish) (Fig. S2b). These transgenic plants produced higher number of roots/plant and longer roots. Axillary shoots were also noted in *rolB*-transgenic plant clones unlike in non-transformed plants.

Insertion of Ri T-DNA had a pleiotropic effect producing highly aberrant Ri-transformed plants with reduced internode and shoot length, increased number of nodes/plant and leaves/plant than non-transformed plants (Fig. 2). Leaves were also smaller and round in shape (Fig. S2b). Ri-transformed plants had increased rooting and the roots were long. Reduced apical dominance in these plants resulted in the formation of axillary shoots.

Therefore, each of *rolA* and *rolB* genes seemed to cause phenotypic alteration in *B. monnieri*, although the degree varied. Additionally, both *rolB*-transgenic and Ri-transformed plants showed characteristic morphological changes associated with *A. rhizogenes*-mediated transformation. The morphology of *rolB*-transgenic plants was found to be more or less similar to Ri-transformed plants, suggesting that *rolB* gene has a pronounced effect in altering plant phenotype in this species.

Morphogenic potential of explants derived from transgenic plants on basal medium

Organogenesis from leaf, internode and root explants derived from rolA-transgenic plants

In the present study, excised leaf and internode explants of *rolA*-transgenic and *nptII*-transgenic plants showed shoot and root organogenesis within two weeks on MS basal medium as observed in explants of non-transformed plants (Fig. 3). However, after four weeks, the rates of shoot and root organogenesis were enhanced significantly in explants of *rolA*-transgenic plants than explants of *nptII*-transgenic and non-transformed plants (Fig. 4). The number of shoot buds/micro shoots induced per explant was 1.5-fold higher in both leaf and internode explants of *rolA*-transgenic plants than *nptII*-transgenic explants whereas it was 1.6-fold (leaf) and 1.4-fold (internode) higher than non-transformed explants. The number of roots induced per explant was 2.0-fold and 1.7-fold greater in leaf explants of *rolA*-transgenic plants than explants of *nptII*-transgenic and non-transformed plants respectively. But, internode explants of *rolA*-transgenic plants were found to be less responsive. No significant differences in the rate of shoot and root organogenesis was observed between leaf and internode explants of *nptII*-transgenic and non-transformed plants. Excised root explants of *rolA*-transgenic, *nptII*-transgenic and non-transformed plants showed no morphogenic response and eventually died within six-eight weeks on MS basal medium (Fig. 3).

Organogenesis from leaf, internode and root explants derived from rolB-transgenic plants

Shoot and root organogenesis were observed from both leaf and internode explants of *rolB*-transgenic plants within two weeks on MS basal medium similar to explants of non-transformed plants (Fig. 3). However, after four weeks, shoot organogenic potential was 2.4-fold and 1.6-fold higher in

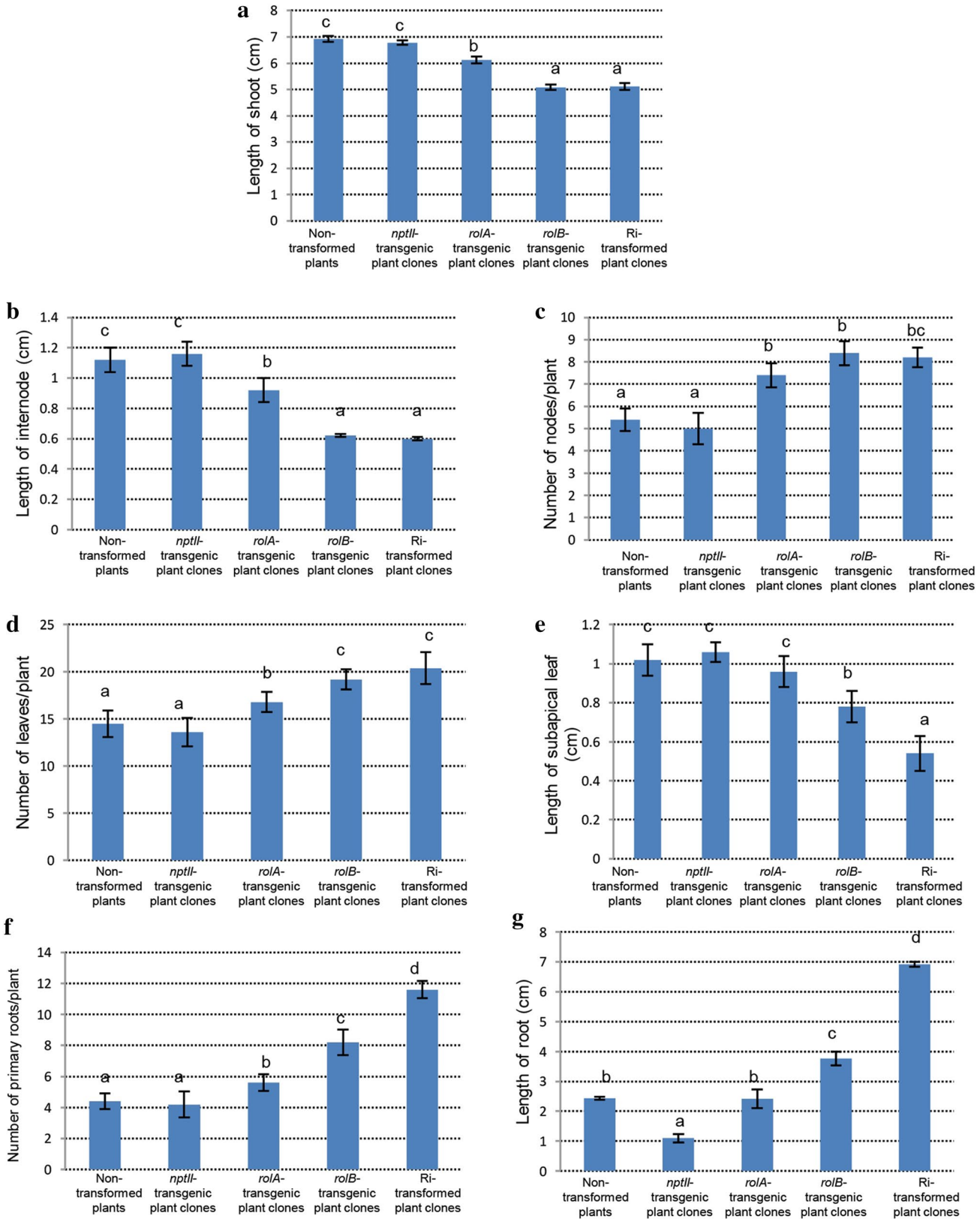

Fig. 2 Morphological characterization of in vitro grown different transgenic and non-transformed ones based on **a** shoot length (cm), **b** internode length (cm), **c** number of nodes/plant, **d** number of leaves/plant, **e** length of sub-apical leaf (cm), **f** number of primary roots/plant, and **g** length of root (cm). Values represent mean ± SD. Means marked with different letters are significantly different ($p \leq 0.05$)

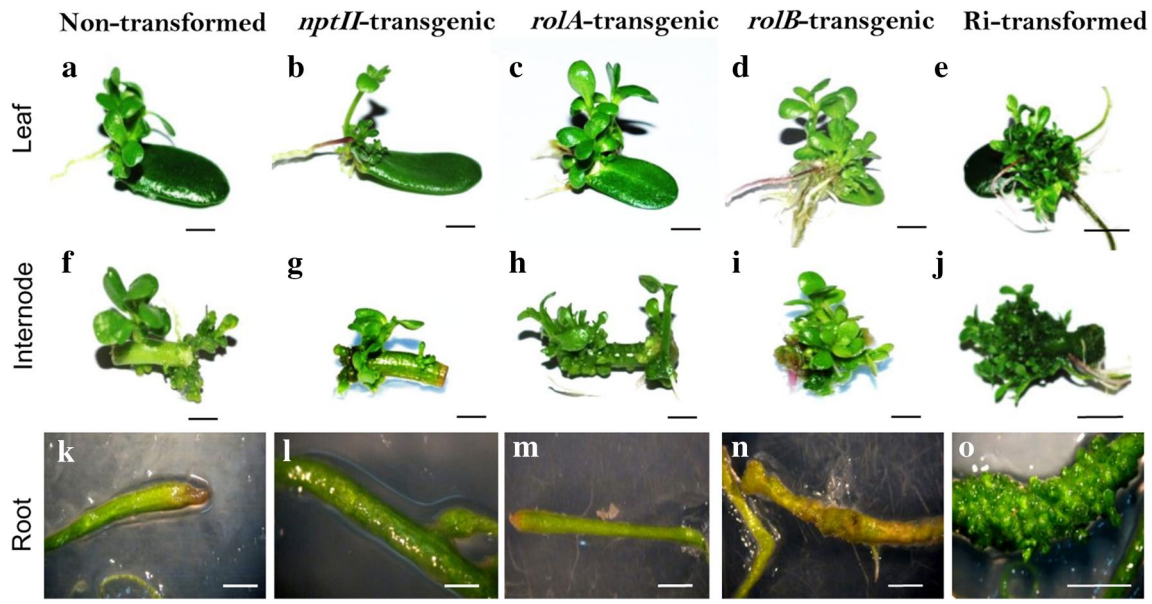

Fig. 3 Morphogenic responses from excised explants derived from different transgenic and non-transformed plants after four weeks of culture on MS basal medium. Scale bar = 2mm

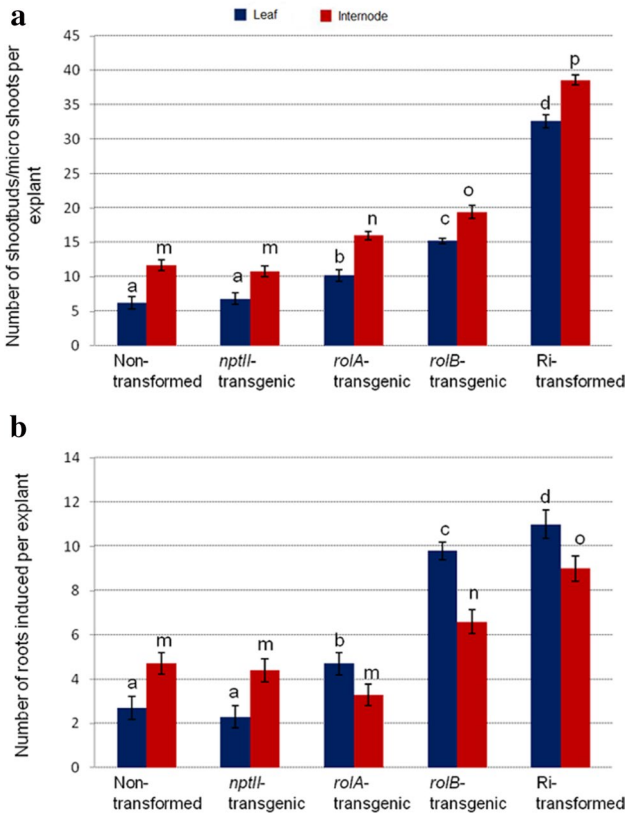

Fig. 4 Bar graph showing **a** shoot and **b** root regeneration potential of explants excised from different transgenic and non-transformed plants after four weeks on MS basal medium. Values represent mean ± S.D. Means marked with different letters are significantly different ($p \leq 0.05$)

leaf and internode explants of *rolB*-transgenic plants than explants of non-transformed plants respectively (Fig. 4a). The *rolB*-expressing explants exhibited 3.6-fold (leaf) and 1.4-fold (internode) increase in root organogenesis compared to explants of non-transformed plants (Fig. 4b). Excised root explants of *rolB*-transgenic plants showed no morphogenesis and necrosed within six-eight weeks on MS basal medium (Fig. 3).

Organogenesis from leaf, internode and root explants derived from Ri-transformed plants

Leaf and internode explants of Ri-transformed plants developed adventitious shoots and roots within two weeks on MS basal medium similar to explants of non-transformed plants (Fig. 3). However, after four weeks, shoot organogenesis was significantly enhanced in leaf (5.2-fold) and internode (3.3-fold) explants of Ri-transformed plants compared to explants of non-transformed (Fig. 4a). Root regeneration potential was also enhanced in Ri-transformed leaf (4.1-fold) and internode (1.9-fold) explants than non-transformed ones (Fig. 4b). Root explants of Ri-transformed plants showed shoot regeneration and extensive root tip growth (4-6 cm) along with primary laterals formation within four weeks on MS basal medium (Fig. 3).

Table 1 Adventitious root regeneration in leaf and internode explants derived from *rolA*-transgenic, *rolB*-transgenic, Ri-transformed and non-transformed plants of *B. monnieri* after four weeks of culture on MS medium in presence of exogenous auxin

Concentration of IAA (mg l^{-1})	Plant Lines				
	nptII-transgenic	*rolA*-transgenic	*rolB*-transgenic	Ri-transformed	Non-transformed
Leaf explants					
0.0	2.5 ± 0.75^{a}	4.8 ± 0.83^{b}	10.0 ± 0.70^{c}	11.4 ± 0.54^{d}	2.6 ± 0.74^{a}
0.01	2.5 ± 0.53^{a}	4.6 ± 0.51^{b}	7.4 ± 0.51^{d}	5.7 ± 0.71^{c}	2.5 ± 0.53^{a}
0.05	3.0 ± 0.53^{a}	6.6 ± 0.91^{b}	8.1 ± 0.83^{c}	6.5 ± 0.92^{b}	2.7 ± 0.71^{a}
1.0	7.1 ± 0.99^{a}	13.4 ± 1.06^{c}	9.1 ± 1.24^{b}	7.7 ± 0.71^{a}	7.6 ± 1.30^{a}
Internode explants					
0.0	4.0 ± 0.63^{a}	3.8 ± 0.83^{a}	6.8 ± 0.44^{b}	8.7 ± 0.50^{c}	4.5 ± 0.54^{a}
0.01	4.3 ± 0.81^{a}	6.4 ± 0.54^{b}	9.2 ± 0.83^{c}	5.5 ± 0.57^{b}	4.2 ± 0.75^{a}
0.05	4.6 ± 0.79^{a}	7.0 ± 0.71^{b}	8.2 ± 0.83^{b}	5.2 ± 0.95^{a}	4.7 ± 0.75^{a}
1.0	2.2 ± 0.41^{b}	2.0 ± 0.71^{b}	2.8 ± 0.44^{b}	0.0 ± 0.0^{a}	2.0 ± 0.63^{b}

Values represent mean ± SD. Means with different letters in a row were significantly different ($p \leq 0.05$) according to ANOVA and Tukey B's multiple comparison test

Auxin sensitization response of different explants derived from transgenic plants

Root organogenesis was enhanced significantly in both leaf and internode explants derived from *rolA*-transgenic and *rolB*-transgenic plants than respective controls in presence of IAA (Table 1). Leaf explants derived from Ri-transformed plants showed significantly higher root organogenesis while internode explants showed variable response. Moreover, in presence of IAA, root explants derived from *rolA*-transgenic and *rolB*-transgenic plants produced callus near cut ends and lateral branching, thereby showing increased auxin sensitivity. Root explants from Ri-transformed plants showed root tip elongation extensively (6–7 cm) along with shoot organogenesis (4–6 shoot buds/explant) and regeneration of whole plants. Root explants derived from non-transformed and *nptII*-transgenic plants showed slow growth without any other distinct change in presence of IAA.

Table 2 Adventitious shoot regeneration from leaf and internode explants derived from *rolA*-transgenic, *rolB*-transgenic, Ri-transformed and non-transformed plants of *B. monnieri* after four weeks of culture on MS medium containing exogenous cytokinins

Concentration of cytokinins (mg l^{-1})		Plant Lines				
BA	KN	*nptII*-transgenic	*rolA*-transgenic	*rolB*-transgenic	Ri-transformed	Non-transformed
Leaf explants						
0.0	0.0	6.0 ± 0.75^{a}	10.4 ± 0.55^{b}	15.0 ± 0.71^{c}	33.0 ± 0.71^{d}	6.6 ± 1.14^{a}
0.01	0.0	15.1 ± 0.83^{a}	16.5 ± 0.92^{b}	18.1 ± 0.64^{c}	54.2 ± 1.16^{d}	15.4 ± 0.91^{a}
0.05	0.0	17.2 ± 0.46^{a}	18.2 ± 0.70^{a}	22.4 ± 0.74^{b}	52.2 ± 1.91^{c}	17.6 ± 0.92^{a}
1.0	0.0	42.0 ± 1.19^{b}	53.9 ± 1.64^{c}	26.6 ± 0.91^{a}	71.6 ± 1.30^{d}	41.2 ± 1.38^{b}
0.0	0.01	10.2 ± 1.39^{a}	13.7 ± 0.88^{b}	16.5 ± 0.53^{c}	24.5 ± 0.92^{d}	10.7 ± 1.16^{a}
0.0	0.05	10.5 ± 1.07^{a}	15.7 ± 1.38^{b}	24.9 ± 1.45^{d}	22.1 ± 1.12^{c}	11.0 ± 0.75^{a}
0.0	1.0	18.0 ± 1.07^{a}	25.5 ± 0.92^{b}	34.6 ± 1.18^{c}	26.4 ± 0.74^{b}	18.5 ± 0.92^{a}
Internode explants						
0.0	0.0	10.7 ± 0.96^{a}	16.6 ± 0.55^{b}	19.6 ± 0.54^{c}	39.2 ± 0.44^{d}	11.6 ± 0.89^{a}
0.01	0.0	29.0 ± 0.82^{c}	15.2 ± 0.95^{b}	12.5 ± 1.0^{a}	> 70	41.2 ± 1.50^{d}
0.05	0.0	31.2 ± 0.96^{b}	17.0 ± 0.81^{a}	17.5 ± 0.57^{a}	> 70	43.2 ± 0.50^{c}
1.0	0.0	41.7 ± 0.50^{a}	> 70	> 70	> 70	> 70
0.0	0.01	16.5 ± 0.58^{b}	18.2 ± 0.50^{b}	8.0 ± 0.81^{a}	> 70	27.0 ± 1.82^{c}
0.0	0.05	22.2 ± 0.96^{b}	28.7 ± 0.50^{d}	16.2 ± 0.50^{a}	> 70	26.0 ± 0.81^{c}
0.0	1.0	39.5 ± 0.58^{a}	40.7 ± 0.95^{a}	54.0 ± 1.15^{b}	> 70	39.7 ± 0.95^{a}

Values represent mean ± S.D. Means with different letters in a row were significantly different ($p \leq 0.05$) according to ANOVA and Tukey B's multiple comparison test

Shoot organogenesis in different explants derived from transgenic plants on cytokinin supplemented media

Shoot organogenesis was enhanced in leaf explants of *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants compared to respective controls in presence of BA and KN (Table 2). The interaction between transgenic explants and media showed that BA was superior to KN. On the other hand, internode explants of Ri-transformed plants induced uncountable shoot buds/micro shoots whereas internode explants derived from *rolA*-transgenic and *rolB*-transgenic plants showed variable response in presence of exogenous cytokinins used (Table 2). Root explants of Ri-transformed plants showed shoot regeneration on BA and KN supplemented media at all concentrations whereas *rolA*-transgenic root segments induced shoot buds only in presence of 1.0 mg l^{-1} BA and KN. However, root explants from *rolB*-transgenic plants did not respond in any medium and necrosed within eight weeks. Non-transformed and *nptII*-transgenic root explants showed shoot regeneration on medium containing BA only.

Discussion

Ri-transformed roots and Ri-transformed plants were established in *B. monnieri* following *A. rhizogenes* infection to demonstrate the effect of T-DNA genes on growth, biomass accumulation and bacosides production in transformed tissues (Majumdar et al. 2011; Largia et al. 2016). Attempts were also made on *A. tumefaciens*-mediated transformation where hygromycin/ kanamycin resistant transgenic *B. monnieri* plants were produced (Majumdar et al. 2012; Yadav et al. 2014). But, to our knowledge, transgenic *B. monnieri* plants harbouring individual *rol* genes have not been developed. Therefore, the present study reports for the first time on successful establishment of *rolA*-transgenic and *rolB*-transgenic plants in *B. monnieri* through *A. tumefaciens* mediated transformation. Ri-transformed plants harbouring Ri T-DNA were also regenerated from *A. rhizogenes* induced Ri-transformed roots to investigate the effects associated with integration and expression of *rol* genes, individually or synergistically, on plant morphology and morphogenesis in vitro.

In the present study, *rolA* and *rolB* genes, individually, causes shortening of internodes with increased number of nodes, leaves and roots per plant in *B. monnieri*. Additionally, *rolB*-transgenic plants exhibited certain transgenic characters due to which their morphology closely resembled Ri-transformed plants. This result indicates that *rolB* gene is more potent in causing developmental abnormalities in *B. monnieri* as reported earlier in other species (Bettini et al. 2016; Kodahl et al. 2016). The abnormal phenotypic traits caused by individual *rol* genes became exaggerated in Ri-transformed plants, probably due to the combined expression of *rol* genes since each of these genes is associated with specific phenotypic alterations (Sarkar et al. 2018).

The inherent morphogenic ability of excised leaf and internode explants of *B. monnieri* on MS basal medium was demonstrated earlier (Sarkar and Jha 2017) and was again confirmed in the present study. Enhanced shoot and root formation in explants of *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants therefore indicate a positive correlation between *rol* genes and organogenic competence of this plant species. According to Skoog and Miller (1957), plant organogenesis can be controlled by auxin/ cytokinin ratio. Decades-long study of *A. rhizogenes*-plant pathogenic interaction has revealed that *rol* genes of Ri T-DNA perturb endogenous hormone metabolism/sensitivity, primarily auxin and cytokinin, in transformed cells (Delbarre et al. 1994; Faiss et al. 1996) which could explain enhanced morphogenesis in transgenic explants. However, maximum morphogenic responses in Ri-transformed explants were probably due to the synergistic action of *rol* genes on endogenous auxin/cytokinin ratio. In addition to this, growth of root tips and shoot regeneration in Ri-transformed root explants, but not in *rolA* transgenic, *rolB*-transgenic and non-transformed roots denoted that *rolA* or *rolB* gene alone is not sufficient for morphogenesis in excised *B. monnieri* roots.

The fact that *rol* genes affect hormone-controlled morphogenesis in transformed cells became more prominent when exogenous auxin and cytokinin were added to the culture medium in the present study. Enhanced rhizogenesis in explants derived from *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants of *B. monnieri* in presence of auxin is supposed to be the consequence of increased auxin sensitivity in *rol*-transgenic cells. This also explains the formation of lateral branching and callus induction in *rolA*-transgenic and *rolB*-transgenic root explants and root tip growth in Ri-transformed root explants in IAA-supplemented medium. According to van Altvorst et al. (1992), root formation in leaf explants of RolA, RolB and RolABC tomato plants in NAA-supplemented medium but not in control could be due to enhanced auxin sensitivity of *rol*-expressing tissues. Furthermore, inhibition of root formation by oligogalacturonides in *rolB*-transgenic tobacco leaf explants and protoplasts by inhibiting auxin-dependent *rolB* gene expression makes the idea of involvement of *rolB* gene in auxin signaling pathway more apparent (Bellincampi et al. 1996).

Enhanced shoot organogenesis in explants derived from *rolA*-transgenic and *rolB*-transgenic plants of *B. monnieri* in presence of cytokinin also describes the possibility of increased cytokinin sensitivity rendered by *rolA* and *rolB* genes in the present study. Altamura et al. (1998) also predicted the presence of a positive interaction between *rolB*

gene and exogenous cytokinin due to the promotion of shoot formation in *rolB*-transgenic tobacco TCLs and leaf explants in presence of very low cytokinin concentrations although the mechanism is unclear. Later on, *rolB* is hypothesized to cause meristem formation triggered by increased auxin sensitivity in transformed cells which can develop into different types of organs depending on local hormone level and cell's organogenic competency (Altamura 2004). Recently, Bulgakov et al. (2018) showed promising results in the modification of both auxin/cytokinin signaling pathways by *rolB*-transformed cells of *Arabidopsis thaliana* Columbia (Col-0). The *rolC* gene is also found to affect shoot and root organogenesis in presence of cytokinin and auxin respectively in *D. caryophyllus* leaf explants (Casanova et al. 2003). Therefore interaction of *rol* genes, in combination, with hormonal metabolism/sensitivity offers a plausible explanation for maximum morphogenic response in explants of Ri-transformed plants of *B. monnieri* in hormone-supplemented media. However, further investigations are needed to clarify expression level of rol genes, integration sites, their copy number and interaction of these genes with other genes involved in morphogenic pathway to establish the exact correlation between *rol* genes and morphogenesis.

Conclusions

In conclusion, the present study demonstrated successful establishment of *rolA*-transgenic and *rolB*-transgenic plants of *B. monnieri* for the first time through direct shoot organogenesis following *A. tumefaciens* mediated transformation. Ri-transformed plants harbouring Ri T-DNA were spontaneously regenerated from Ri-transformed roots induced by *A. rhizogenes* infection. Respective transgenes were integrated and expressed in all transgenic plants, confirming their transgenic nature. The *rol* genes, either singly or in combination, played significant role in substantial increase in shoot and root organogenic potential on hormone unsupplemented medium by interacting positively with inherent morphogenic potential of this species. Each of the *rol* genes is found to be associated with the alteration of plant morphology in *B. monnieri*, although their degree varied. Furthermore, enhanced morphogenic response in transgenic explants containing *rolA* or *rolB* or Ri T-DNA genes even in presence of low concentrations of exogenous auxin and cytokinin might therefore be a manifestation of increased auxin and cytokinin sensitivity respectively although the exact mode of action of these genes needs further investigations.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11240-021-02092-5.

Acknowledgements SS acknowledges Department of Biotechnology, GOI, for Senior Research Fellowship. S.J. is thankful to the National Academy of Sciences (NASI, Allahabad, India), for award of "Senior Scientist, NASI" and providing the financial support to continue the research. The authors thank the Head, Department of Botany, University of Calcutta for facilities provided.

Author contributions SS and SJ conceived and designed research. SS conducted this research, analyzed the results and wrote the manuscript. All authors read and approved the manuscript.

Declarations

Conflict of interest The authors declare no conflict of interests.

References

Alpizar E, Dechamp E, Lapeyre-Montes F et al (2008) *Agrobacterium rhizogenes*-transformed roots of coffee (*Coffea arabica*): conditions for long-term proliferation, and morphological and molecular characterization. Ann Bot 101:929–940

Altamura MM (2004) *Agrobacterium rhizogenes rolB* and *rolD* genes: regulation and involvement in plant development. Plant Cell Tiss Organ Cult 77:89–101

Altamura MM, D'Angeli S, Capitani F (1998) The protein of *rolB* gene enhances shoot formation in tobacco leaf explants and thin cell layers from plants in different physiological stages. J Exp Bot 49:1139–1146

Bahramnejad B, Naji M, Bose R et al (2019) A critical review on use of *Agrobacterium rhizogenes* and their associated binary vectors for plant transformation. Biotechnol Adv 37:1–14

Beck E, Ludwig G, Averswald EA et al (1982) Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene 19:327–336

Bellincampi D, Cardarelli M, Zaghi D et al (1996) Oligogalacturonides prevent rhizogenesis in *rolB*-transformed tobacco explants by inhibiting auxin-induced expression of the *rolB* gene. Plant Cell 8:477–487

Bettini PP, Marvasi M, Fani F et al (2016) *Agrobacterium rhizogenes rolB* gene affects photosynthesis and chlorophyll content in transgenic tomato (*Solanum lycopersicum* L.) plants. J Plant Physiol 204:27–35

Bhojwani SS, Razdan MK (1983) Plant tissue culture: theory and practice. Amsterdam

Bulgakov VP, Vereshchagina YV, Bulgakov DV et al (2018) The *rolB* plant oncogene affects multiple signaling protein modules related to hormone signaling and plant defense. Sci Rep 8:2285

Cardarelli M, Spano L, Mariotti D et al (1985) Identification of the genetic locus responsible for non polar root induction by *Agrobacterium rhizogenes* Ri plasmid. Plant Mol Bio 5:385–391

Casanova E, Zuker A, Trillas MI et al (2003) The *rolC* gene in carnation exhibits cytokinin- and auxin-like activities. Sci Hortic 97:321–331

Chaudhuri K, Das S, Bandyopadhyay M et al (2009) Transgenic mimicry of pathogen attack stimulates growth and secondary metabolite accumulation. Transgenic Res 18:121–134

Chilton MD, Tepfer DA, Petit A et al (1982) *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. Nature 295:432–434

Christensen B, Sriskandarajah S, Serek M et al (2008) Transformation of *Kalanchoe blossfeldiana* with *rol*-genes is useful in molecular breeding towards compact growth. Plant Cell Rep 27:1485–1495

Delbarre A, Muller P, Imhoff V et al (1994) The *rolB* gene of *Agrobacterium rhizogenes* does not increase the auxin sensitivity of tobacco protoplasts by modifying the intracellular auxin concentration. Plant Physiol 105:563–569

Dellaporta SL, Woods J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19–21

Diouf D, Gherbi H, Prin Y et al (1995) Hairy root nodulation of *Casuarina glauca*: a system for the study of symbiotic gene expression in an actinorhizal tree. Mol Plant Microbe Interact 8:532–537

Faiss M, Strnad M, Redig P et al (1996) Chemically induced expression of the *rolC*-encoded β-glucosidase in transgenic tobacco plants and analysis of cytokinin metabolism: *rolC* does not hydrolyze endogenous cytokinin glucosides in planta. Plant J 10:33–46

Hooykass PJJ, Klapwjik PM, Nuti MP et al (1977) Transfer of the *A. tumefaciens* Ti plasmid to avirulent *Agrobacteria* and *Rhizobium* ex planta. J Gen Microbiol 98:477–484

Kodahl N, Müller R, Lütken H (2016) The *Agrobacterium rhizogenes* oncogenes *rolB* and *ORF13* increase formation of generative shoots and induce dwarfism in *Arabidopsis thaliana* (L.) Heynh. Plant Sci 252:22–29

Koul A, Sharma A, Gupta S et al (2014) Cost effective protocol for micropropagation of *Bacopa monnieri* using leaf explants. Int J Sci Res 3:210–212

Largia MJV, Satish L, Johnsi R et al (2016) Analysis of propagation of *Bacopa monnieri* (L.) from hairy roots, elicitation and Bacoside A contents of Ri transformed plants. World J Microbiol Biotechnol 32:131–141

Majumdar S, Garai S, Jha S (2011) Genetic transformation of *Bacopa monnieri* by wild type strains of *Agrobacterium rhizogenes* stimulates production of bacopa saponins in transformed calli and plants. Plant Cell Rep 30:941–954

Majumdar S, Garai S, Jha S (2012) Use of the *cryptogein* gene to stimulate the accumulation of bacopa saponins in transgenic *Bacopa monnieri* plants. Plant Cell Rep 31:1899–1909

Mauro ML, Costantino P, Bettini PP (2017) The never ending story of *rol* genes: a century after. Plant Cell Tiss Organ Cult 131:201–212

Mauro ML, Trovato M, Paolis AD et al (1996) The plant oncogene *rolD* stimulates flowering in transgenic tobacco plants. Dev Biol 180:693–700

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor, New York

Mukherjee A, Mazumder M, Jana J et al (2019) Enhancement of ABA sensitivity through conditional expression of the ARF10 gene in *Brassica juncea* reveals fertile plants with tolerance against *Alternaria brassicicola*. MPMI 32:1429–1447

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Owens LD, Cohen JD, Seelke R (1988) Gene introduction to induce morphogenesis. Hort Sci 23:520–525

Paul P, Sarkar S, Jha S (2015) Effects associated with insertion of *cryptogein* gene utilizing Ri and Ti plasmids on morphology and secondary metabolites are stable in *Bacopa monnieri*-transformed plants grown in vitro and ex vitro. Plant Biotechnol Rep 9:231–245

Petit A, David C, Dahl GA et al (1983) Further extension of the opine concept: plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. Mol Gen Genet 190:204–214

Roychowdhury D, Majumder A, Jha S (2013) *Agrobacterium rhizogenes*-mediated transformation in medicinal plants: Prospects and challenges. In: Chandra S, Lata H, Varma A (eds) Biotechnology for medicinal plants: micropropagation and Improvement. Springer, Verlag, Berlin, pp 29–68

Saha PS, Sarkar S, Jeyasri R et al (2020) In vitro propagation, phytochemical and neuropharmacological profiles of *Bacopa monnieri* (L.)Wettst.: a review. Plants 9:411–435

Sambrook J, Russel DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbour Press, Cold Spring Harbour, New York

Sarkar S, Jha S (2017) Morpho-histological characterization and direct shoot organogenesis in two types of explants from *Bacopa monnieri* on unsupplemented basal medium. Plant Cell Tiss Organ Cult 130:435–441

Sarkar S, Ghosh I, Roychowdhury D et al (2018) The effects of *rol* genes of *Agrobacterium rhizogenes* on morphogenesis and secondary metabolite accumulation in medicinal plants. In: Kumar N et al (eds) Biotechnological approaches for medicinal and aromatic plants. Springer, Singapore, pp 27–51

Sevón N, Dräger B, Hiltunen R et al (1997) Characterization of transgenic plants derived from hairy roots of *Hyoscyamus muticus*. Plant Cell Rep 16:605–611

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp Soc Exp Biol 11:118–131

Slightom JL, Durand-Tardif M, Jouanin L et al (1986) Nucleotide Sequence Analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. J Biol Chem 261:108–121

Sokal RR, Rohlf FJ (1987) Introduction to biostatistics. WH Freeman, New York

Tepfer D, Tempé J (1981) Production of d'agropine par des racines transformes sous I'action *d'Agrobacterium rhizogenes* souche A4. Comptes rendus de l'Académie des sciences 292:153–156

Tran Than Van K (1980) Control of morphogenesis by inherent and exogenously applied factors in thin cell layers. Int Rev Cytol Suppl 11A:175–194

van Altvorst AC, Bino RJ, van Dijk AJ et al (1992) Effects of the introduction of *Agrobacterium rhizogenes rol* genes on tomato plant and flower development. Plant Sci 83:77–85

Wabiko H, Minemura M (1996) Exogenous phytohormone-independent growth and regeneration of tobacco plants transgenic for the 6b gene of *Agrobacterium tumefaciens* AKE10. Plant Physiol 112:939–951

Wang YM, Wang JB, Luo D et al (2001) Regeneration of plants from callus cultures of roots induced by *Agrobacterium rhizogenes* on *Alhagi pseudoalhagi*. Cell Res 11:279–284

White FF, Taylor BH, Huffman GA et al (1985) Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. J Bacteriol 164:33–44

Yadav S, Sharma P, Srivastava A et al (2014) Strain specific *Agrobacterium*-mediated genetic transformation of *Bacopa monnieri*. J Genet Eng Biotechnol 12:89–94