

Somatic embryogenesis and *Agrobacterium*-mediated transformation in *Bixa orellana* L.

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Abstract Establishment, maintenance, regeneration, and transformation of somatic embryos by both direct and indirect means (callus-mediated) was achieved for *Bixa orellana*, a tropical plant whose seeds produce commercially edible ‘annatto pigment,’ which mainly constitutes an apocarotenoid called bixin. Callus-mediated methodology was found to be efficient in producing a greater number of embryos in a short time. The maximum of 28 somatic embryos were produced in 16–18 weeks when immature zygotic embryonic stalks were inoculated onto Murashige and Skoog (MS) medium containing B5 vitamins supplemented with 0.44 μM benzyladenine (BA), 0.054 μM α -naphthaleneacetic acid (NAA), 2.89 μM gibberellic acid (GA_3), 0.02 μM triiodobenzoic acid (TIBA), and 0.011 μM triacontanol (TRIA). Callus initiation from hypocotyl explants was obtained on MS medium supplemented with 1.07–2.14 μM NAA and 10.2 μM BA. In 3 months, somatic embryos were produced when callus was inoculated onto MS medium supplemented with 4.44 μM BA, 40 μM AgNO_3 , and 0.011 μM TRIA. Somatic embryos were efficiently regenerated on MS basal solid and liquid media supplemented with 0.44–4.4 μM BA, 0.54–2.69 μM NAA, 4.92 μM 2iP, 2.1 μM calcium D-pantothenate, 0.21 μM biotin, 227.7 μM cysteine HCl monohydrate, and 108.6 μM adenine sulfate. *Agrobacterium tumefaciens* GV 3101 harboring pCAMBIA 1305.2 binary vector-mediated stable transformation of somatic

embryos exhibited a transformation frequency of 2.56%. As somatic embryogenesis in any perennial system is useful in terms of both commercial and scientific nature, this somatic embryo-based transformation protocol for the commercially important dye-yielding tropical plant *B. orellana* is useful for its improvement through genetic engineering.

Keywords *Agrobacterium tumefaciens* · Annatto · Regeneration · Rooting · Triacontanol

Abbreviations

BA	Benzyladenine
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
MS	Murashige and Skoog
NAA	α -Naphthaleneacetic acid
PGR	Plant growth regulator
TIBA	2,3,5-Triiodobenzoic acid
TRIA	Triacontanol

Introduction

Somatic embryos are the major tool for any plant transformation studies, either by *Agrobacterium* or by gene gun—the two major plant transformation methodologies—since the beginning of biotechnology in agricultural crops involving gene regulation through genetic engineering approaches. Similarly, in plant tissue culture, somatic embryos play a more significant role than any mode of organogenesis. Annatto pigment constituting mainly of bixin and norbixin is formed on the aril portion of seeds of

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Bixa orellana L. ($2n = 2x = 14$). They are also referred to as oxygenated carotenoids. These two principal coloring components of annatto pigment are the liposoluble diapocarotenoid 9'-*cis* bixin, a monomethyl ester of norbixin, and the dicarboxylic acid water-soluble 9'-*cis* norbixin. Based on structural characteristics, they are classified as isoprenoid derivatives (Hari et al. 1994). The European Union has authorized 43 colorants as food additives, with each colorant being assigned a different "E number," of which 17 are synthetic and the remaining are natural or nature-derived compounds (Downham and Collins 2000). Annatto pigment has the E-number or EEC (European Economic Community) number E160b.

The standardization of somatic embryogenesis helps to maintain and enhance the multiplication of elite clones of interest for higher productivity and economic benefits, and also for the establishment and utility of a transformation protocol for genetic engineering studies to regulate the biosynthetic pathway (Kumar et al. 2006, 2007). In *B. orellana*, due to low seed viability (20%) and poor germination (5%), *in vitro* studies possess commercial value (D'Souza and Sharon 2001). Some plant growth regulators (PGRs), such as 2,4-D, are known to play important roles in the induction and establishment of somatic embryos in numerous plant species. Other PGRs such as triacontanol (TRIA) and 2,3,4-triiodobenzoic acid (TIBA) have been reported to induce somatic embryogenesis in a few species, such as *Coffea* spp. (Giridhar et al. 2004) and *Carica papaya* (Bhattacharya and Khuspe 2000), respectively. In this study, the influence of some of these PGRs on the induction of somatic embryogenesis and regeneration in annatto has been investigated. Moreover, somatic embryos were used for developing an *Agrobacterium*-mediated transformation protocol for annatto.

Materials and methods

Explant preparation and establishment of somatic embryos

Immature fruits (50–60 days old) of *B. orellana* L. (Bixaceae) were collected during September 2006 from 5-year-old plants at the Plant Cell Biotechnology Department, Central Food Technological Research Institute (CFTRI), Mysore, India. The collected fruits were soaked in 70% ethanol for 5 min and all of the remaining procedures were carried out under sterile conditions using laminar air flow. Developing seeds were taken out from immature fruits and rinsed in 70% ethanol for 1 min and washed twice with sterile distilled water and blot dried. These immature zygotic embryos were used as whole or the embryonic stalk

alone as the explants for the induction of primary direct somatic embryogenesis.

Induction of primary somatic embryos

Two types of explants, viz., immature zygotic embryos and immature embryonic stalk (devoid of cotyledonary portion), were inoculated onto Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with B5 vitamins (Gamborg et al. 1968), 0.22–4.44 μM benzyladenine (BA), 0.027–0.54 μM α -naphthaleneacetic acid (NAA), 2.89 μM gibberellic acid (GA_3), 0.02 μM triiodobenzoic acid (TIBA) and 0.011 μM triacontanol (TRIA) to induce primary somatic embryos. Besides growth regulators, MS medium also consisted of sucrose (3% w/v) and 0.01% w/v myo-inositol, and the pH of the medium adjusted to 5.7 and solidified using agar (0.68%). Forty ml of medium was dispensed into a 150-ml Erlenmeyer flask or glass jar and autoclaved at 1.21 kg cm^{-2} pressure and 121°C for 20 min. One hundred immature zygotic embryos (ten per flask) and 100 immature embryonic stalks (ten per flask) were used for the experiment.

Establishment and maintenance of secondary somatic embryos

Primary somatic embryos obtained from embryonic stalk were sub-cultured onto fresh MS basal medium supplemented with BA (0.44 μM), NAA (0.054 μM), GA_3 (2.89 μM), TIBA (0.02 μM), and TRIA (0.011 μM) with and without agar for solid and liquid medium, respectively, for the production of secondary somatic embryos. Hereafter, this PGR combination will be referred to as RBANGT.

Indirect somatic embryogenesis

Callus cultures were established by inoculating hypocotyls from *in vitro* raised seedlings onto MS medium supplemented with 1.07–2.14 μM NAA and 10.2 μM BA or 0.45–0.90 μM 2,4-D and 10.2 μM BA. Fifty explants (ten per plate) for each PGR combination as given in Table 2 were used for this experiment. Four-week-old soft friable callus obtained from MS basal medium supplemented with 1.07 μM NAA and 10.2 μM BA were further sub-cultured onto the same medium for maintenance. Hence, obtained calli were used for the production of callus-mediated somatic embryos. Callus cultures that are established and maintained as mentioned above were sub-cultured onto MS basal liquid medium supplemented with BA (0.44–4.44 μM), AgNO_3 (40 μM), and TRIA (0.011 μM) for callus-mediated somatic embryogenesis; this medium composition is referred to as BAT.

In vitro rooting of somatic embryos

Secondary somatic embryos were sub-cultured onto MS basal liquid medium supplemented with BA (0.44 μM), NAA (0.11 μM), GA_3 (2.89 μM), TIBA (0.02 μM), and TRIA (0.011 μM). The only difference between RBANGT and the rooting medium is that the auxin (NAA) concentration has been doubled. Fifty embryonic clumps (~ 4 embryos per clump) were used for the induction of in vitro rooting.

Regeneration of plantlets from rooted somatic embryos

Rooted embryogenic plantlets were inoculated onto MS basal solid and liquid media supplemented with BA (0.44–4.4 μM), NAA (0.54–2.69 μM), 2iP (4.92 μM), calcium D-pantothenate (2.1 μM), biotin (0.21 μM), cysteine HCl monohydrate (227.7 μM), and adenine sulfate (108.6 μM) for their further growth.

Agrobacterium-mediated transformation in vitro

Somatic embryos were inoculated onto RBANGT medium containing different concentrations of hygromycin (2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 mg l^{-1}) for the antibiotic sensitivity test. Filter-sterilized hygromycin stock of 20 mg ml^{-1} were used to prepare RBANGT medium containing different concentrations of hygromycin (Duchefa, The Netherlands) as mentioned above. Cultures were maintained in the dark for an initial period of 1 week.

Preparation of bacterial culture for transformation

A. tumefaciens GV3101 strain harboring the binary vector pCAMBIA 1305.2 was used for standardization of the transformation protocol. Overnight-grown culture was used to inoculate 25 ml of LB containing kanamycin and was kept at 28°C and 120 rpm (Gyrotory Shaker SI-600R, Jeiotech, Lab Companion, Korea) for growth until the absorbance at 600 nm was 1.0 O.D. These cultures were used for transformation with somatic embryos.

Co-cultivation of somatic embryos

Somatic embryos grown to 1.0 O.D. were co-cultivated with 25 ml of *A. tumefaciens* GV3101 (growth density 1.0 O.D.) and 100 μM of acetosyringone solution (3',5'-Dimethoxy-4'-hydroxy-acetophenone, Sigma-Aldrich, USA) for 45 min with intermittent shaking once every 10 min. These co-cultivated somatic embryos were blot dried using sterile filter papers under a laminar flow and inoculated onto RBANGT medium containing 10 μM of acetosyringone and

incubated in the dark for 2 days. Later, they were kept under a normal photoperiod as mentioned in the tissue culture section. Once overgrowth of bacterium becomes visible, they were washed with sterile dH_2O containing cefotaxime (Duchefa, The Netherlands) at 500 mg l^{-1} concentration. Hence, washed somatic embryos were blot dried using sterile filter paper and inoculated onto the RBANGT medium containing hygromycin (10 mg l^{-1}) and cefotaxime (250 mg l^{-1}), and incubated in the step-in growth chamber for selection.

Selection and confirmation of transformants

For confirmation of transformants, molecular confirmation by polymerase chain reaction (PCR) using *gus* and *hptII* primers were done and, simultaneously, histochemical GUS assay using X-glcA was also made to confirm the transgenic nature of embryos. Details of the molecular confirmation and histochemical confirmation are given next.

Molecular confirmation of transformation using PCR and Southern analysis

Genomic DNA from the secondary somatic embryos that are grown in the selection medium was used for PCR analysis to confirm the presence of T-DNA in plant genome. Forward and reverse primers specific to partial hygromycin phosphotransferase (*hptII*) and glucuronidase (*gus*) genes were designed using Primer3 software (Rozen and Skaletsky 2000) and synthesized from Sigma-Aldrich, USA. The primer sequences are as follows:

HptII F: 5'-GAT GTT GGC GAC CTC GTA TT-3'
 HptII R: 5'-GTG TCA CGT TGC AAG ACC TG-3'
 Gus F: 5'-CCG TCC CAA GCA GTT ACA AT-3'
 Gus R: 5'-TTC GGA ATC TCC ACG TTA CC-3'

Amplification reactions were in volumes of 25.0 μl , each containing 16.0 μl of sterilized double distilled water, 2.5 μl of $10\times$ assay buffer with 15 mM MgCl_2 , 1.0 μl of dNTP (2.5 mM each in the dNTP mix), 2.0 μl each of forward and reverse primers, 0.5 μl (~ 2.5 U) of *Taq* polymerase (Bangalore Genei, Bangalore, India), and 1.0 μl of template DNA. Amplification was performed using 0.2-ml PCR tubes (Axygen Inc.) in an Eppendorf Mastercycler Personal (Eppendorf, Germany) programmed for 30 cycles consisting of 30 s at 94°C, 30 s at 55°C, and 1 min 30 s at 72°C for denaturing, annealing, and extension, respectively. These cycles were preceded by an initial denaturation of 2 min at 94°C. After 30 cycles, there was a final extension for 5 min at 72°C and then it was maintained at 4°C until electrophoresis. Amplified products were resolved based on their molecular weight by running

the products on a 1.0% agarose gel matrix using a submarine electrophoresis (Consort E861, Germany) with $1 \times$ TAE buffer at 5 V cm^{-1} . A 3-kbp ladder (MBI Fermentas, Germany) was used to identify the presence of amplicons at the desired size. Gels were stained and viewed on a UV transilluminator. The intercalating agent ethidium bromide fluoresces orange upon UV illumination (310–320 nm). The gel image has been documented using a Herolab documentation unit (Herolab E.A.S.Y. 442 K, Germany).

Genomic DNA (approx. 40 μg in quantity) from putative transformants was digested with *EcoRI* enzyme for Southern blot analysis as reported by Sambrook et al. (1989). BioBond-Plus nylon membrane (Sigma, USA) was used and hybridized with the 600-bp *gus* gene probe. The probe for the *gus* gene was prepared using a Psoralen-Biotin Labeling Kit (Ambion, USA). Hybridization signals were detected using a Nonisotopic BioDetect Kit (Ambion, USA).

Histochemical confirmation using GUS assay

Secondary somatic embryos that were grown on selection medium were used for the histochemical GUS assay. GUS assay solution was prepared with slight modifications from Jefferson et al. (1987); instead of sodium phosphate buffer, potassium phosphate buffer was used to prepare the GUS assay solution. GUS assay solution with putative secondary somatic embryos were incubated at 37°C for 2 days in a hybridization oven (1004-2E, Shel Lab, USA). After 2 days, the somatic embryos were transferred to destaining solution to visualize the blue color clearly by avoiding the background effect of chlorophyll and other components. They were photographed using a Nikon digital camera.

Statistical analysis

Experiments were repeated thrice. The mean of five observations was taken as the replicate value and data were analyzed using analysis of variance (ANOVA) (Steel and Torrie 1980). Mean \pm standard error of the mean (SE) values of the results are presented in the tables in this paper.

Results and discussion

In the present study, a highly reproducible and efficient protocol for direct somatic embryogenesis from the immature zygotic embryo stalk and callus-mediated somatic embryo formation from hypocotyls was developed, along with a stable transformation protocol for *B. orellana*.

Direct somatic embryogenesis

Direct somatic embryos induction was noticed from the stalk of immature zygotic embryos (from 50–60-day-old fruits) when cultured on optimized PGR combination. Sixteen to eighteen weeks after inoculation, on RBANGT medium comprising MS salts, B5 vitamins supplemented with $0.44 \mu\text{M}$ of BA, $0.054 \mu\text{M}$ of NAA, $2.89 \mu\text{M}$ of GA_3 , $0.02 \mu\text{M}$ of TIBA, and $0.011 \mu\text{M}$ of TRIA, these immature zygotic embryonic stalks produced a maximum of 28 primary somatic embryos when compared to other PGR concentrations (Table 1; Fig. 1a, b). Based on our preliminary studies, these PGR combinations were optimized. Similarly, the TRIA ($0.011 \mu\text{M}$) was used based on our

Table 1 Direct somatic embryogenesis from immature zygotic embryonic stalk explants after 16 weeks of in vitro culturing

Plant growth regulator concentrations (μM)		Mean number of somatic embryos formed
BA	NAA	
0.22	0.027	4.3 ± 0.20
0.44	0.027	$17.4 \pm 0.73^{**}$
0.88	0.027	4.1 ± 0.18
1.11	0.027	4.8 ± 0.12
2.22	0.027	3.3 ± 0.11
4.44	0.027	$7.6 \pm 0.13^*$
0.22	0.054	$12.4 \pm 0.59^{**}$
0.44	0.054	$28.6 \pm 0.98^{**}$
0.88	0.054	$23.2 \pm 0.77^{**}$
1.11	0.054	$21.4 \pm 0.86^*$
2.22	0.054	$18.9 \pm 0.49^*$
4.44	0.054	–
0.22	0.216	3.2 ± 0.10
0.44	0.216	3.8 ± 0.13
0.88	0.216	4.7 ± 0.15
1.11	0.216	2.1 ± 0.09
2.22	0.216	–
4.44	0.216	–
0.22	0.54	–
0.44	0.54	–
0.88	0.54	–
1.11	0.54	–
2.22	0.54	–
4.44	0.54	–

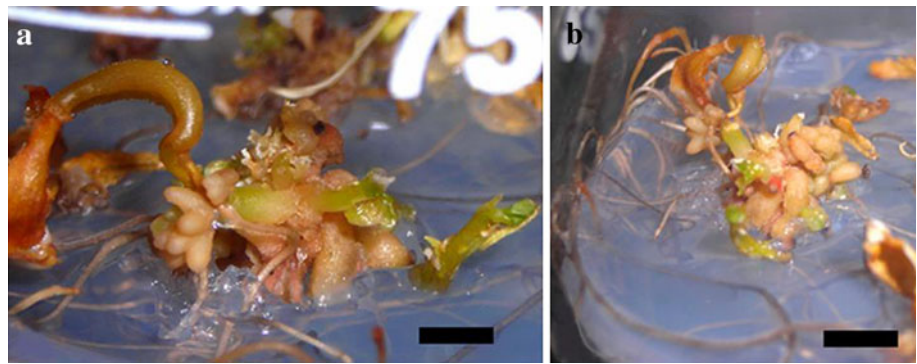
Along with different concentrations of BA and NAA, $2.89 \mu\text{M}$ of GA_3 , $0.02 \mu\text{M}$ of TIBA, and $0.011 \mu\text{M}$ of TRIA were incorporated for all treatment concentrations

Values represent mean \pm standard error of the mean (SE) (values are the mean of five replicates)

**Significant at $P < 0.01$

*Significant at $P < 0.05$

Fig. 1a, b Primary somatic embryo formation from immature zygotic embryonic stalk explants of *Bixa orellana*. **a** Horizontal view of the explant producing primary somatic embryos (bar 15 mm). **b** Elevated view of the same explant producing primary somatic embryos (bar 30 mm)



earlier studies of TRIA's influence on somatic embryogenesis in *Coffea* spp. (Giridhar et al. 2004).

The use of immature zygotic embryos is considered as a reliable source of explants (Dunstan et al. 1995) for somatic embryogenesis. The combination of 2,4-D and kinetin is generally recommended for somatic embryogenesis of several angiosperms (Dunstan et al. 1995) and, accordingly, the earlier studies on *B. orellana* somatic embryogenesis were based on 2,4-D and kinetin (de Pavia Neto et al. 2003a). When mature zygotic embryos were used as explants, somatic embryogenesis was not noticed, but most of them germinated with thin long stem, leaves, and root. Probably, the developmental stage of zygotic embryo is very important for somatic embryogenesis (de Pavia Neto et al. 2003a). The advantage of BA, NAA rather than 2,4-D, kinetin and activated charcoal as used by de Pavia Neto et al. (2003a) was that there were no abnormalities such as fluted and fused somatic embryos.

The presence of TIBA is an important factor for the production of somatic embryos because TIBA is a well-known inhibitor of auxins. Since the presence of auxins inhibits the formation of somatic embryos, TIBA regulates the formation of somatic embryogenesis (Ranjan et al. 2003) and it is exhibited in *B. orellana*. The significance of TIBA in somatic embryo production in our study was further confirmed by similar reports in other plants (Bronsema et al. 2001; Ramarosandratana and Van Staden 2004). A very small quantity of NAA at 0.054 μM is added to maintain endogenous levels of auxins for growth.

Primary somatic embryos obtained from immature zygotic embryonic stalk were used to produce secondary somatic embryos by inoculating them onto the same RBANGT medium in both liquid and solid form. Secondary somatic embryos were formed in 6 weeks and are seen to be very healthy and about 10–12 secondary somatic embryos were formed from each primary somatic embryo (Fig. 2a) under RBANGT liquid medium. However, in case of the primary somatic embryos that are inoculated onto RBANGT solid medium, they produced more than 35 secondary somatic embryos (Fig. 2b). This difference, in spite of the same medium composition, may be due to the continuous

shaking, whereby newly formed embryos detach and form as separate clumps and proliferate further to produce secondary somatic embryos (Fig. 2). Secondary somatic embryos that are produced from RBANGT medium were maintained by sub-culturing onto the same medium every 6–8 weeks. The initiation of secondary somatic embryo formation mostly occurred near the apex portion than the root portion of the primary somatic embryo, as could be seen in Fig. 2c.

Indirect somatic embryogenesis

Callus induction

Callus initiation from hypocotyl explants on MS medium supplemented with 1.07–2.14 μM NAA and 10.2 μM BA was evident by 1 week of culturing and further callus growth was prominent by 2 weeks (Table 2; Fig. 3a). They were sub-cultured once every 4 weeks to obtain soft and friable callus. The incorporation of 1.07 μM NAA along with 10.2 μM BA in MS medium induced brown color callus with pale red tinge from hypocotyl explants (Fig. 3a), whereas the callus obtained from the same explants when inoculated onto the MS medium supplemented with 0.45–0.9 μM 2,4-D and 10.2 μM BA gave callus (Fig. 3b) that were not as good as the ones from NAA. Callus formed from 1.07 μM NAA and 10.2 μM BA showed pigmented spots when observed under the microscope (Fig. 3c). A possible reason for the formation of pigment in MS medium supplemented with NAA alone and not in 2,4-D might be the different mechanism through which the different PGRs acts. As reported earlier by Ranjan et al. (2003), the production of pigments will be inhibited under the influence of 2,4-D due to the fact that it is basically a herbicide that possesses the property of auxins at different concentrations.

Callus-mediated indirect somatic embryogenesis

Callus cultures that were maintained on MS medium supplemented with 1.07 μM NAA and 10.2 μM BA were used to establish somatic embryos. MS liquid medium was supplemented with 0.44–4.4 μM BA, 40 μM AgNO_3 , and

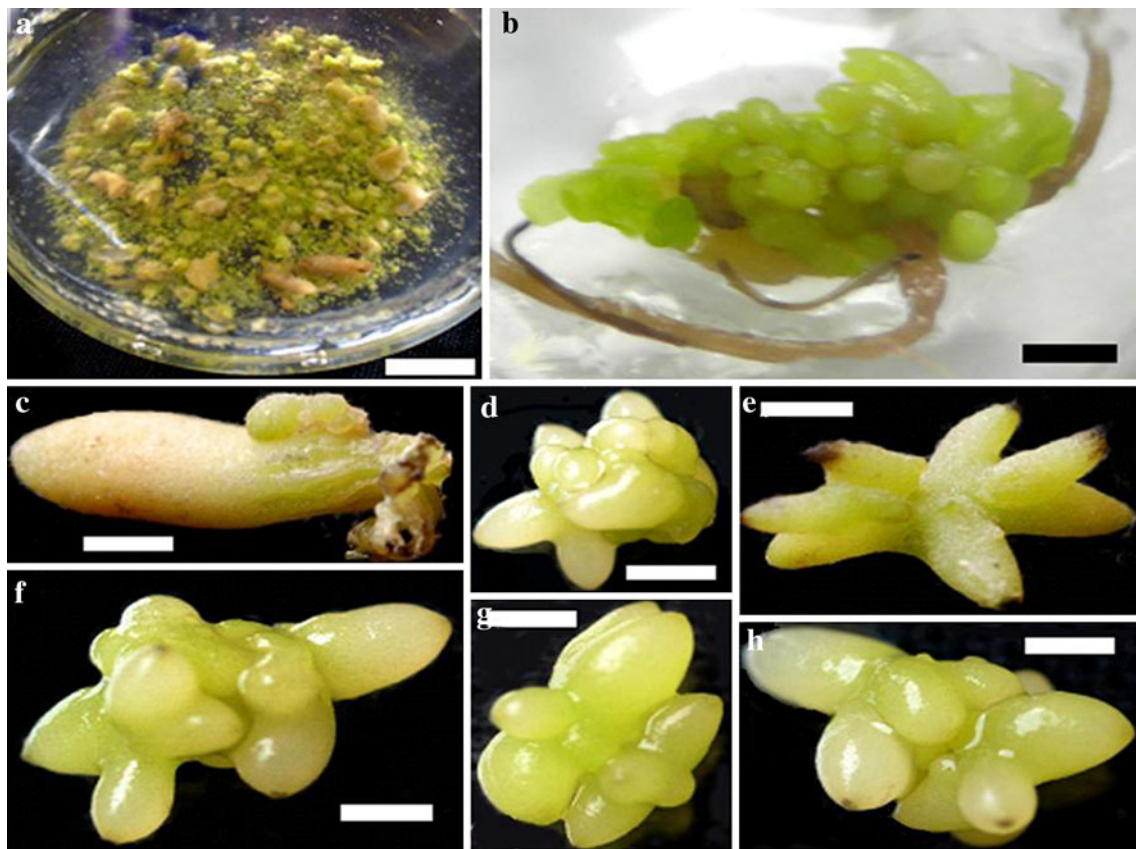


Fig. 2a–h Secondary somatic embryo formation from primary somatic embryos. **a** 10–12 secondary somatic embryo formation in liquid RBANGT medium (*bar* 10 mm). **b** 35–40 secondary somatic embryo formation in solid RBANGT medium (*bar* 5 mm). **c** Initiation

of secondary somatic embryo from a primary somatic embryo (*bar* 1 mm). **d–h** Single clump of secondary somatic embryos in liquid RBANGT medium (*bar* 3 mm)

0.011 μM TRIA for the optimization of concentrations of growth regulators for the production of somatic embryos. Globular stage somatic embryos were formed on medium supplemented with MS salts and vitamins, 4.44 μM BA, 40 μM AgNO_3 , and 0.011 μM TRIA in 3 months (Fig. 4a, b). This medium composition is herein referred to as BAT medium. These somatic embryos that were produced from callus were sub-cultured once every 6–8 weeks onto the same medium for the maintenance of secondary somatic embryos. Microscopic observation of the liquid cultures revealed that, in 1 ml of culture, 164–220 embryos were found and most of them are of globular stage and their subsequent transfer to the same medium with agar lead to torpedo-shape embryos and further regeneration. The combination of silver nitrate and BA in the presence of auxins stimulates somatic embryogenesis along with organogenesis, as reported in *C. canephora* (Fuentes et al. 2000; Sridevi et al. 2010). In case of the initial establishment of somatic embryos from callus, they were slightly brown in color, whereas they turned green when sub-cultured onto the same medium for the production and maintenance of secondary somatic embryos (Fig. 4c, d).

Table 2 Callus induction using hypocotyl explants of *Bixa orellana*

Plant growth regulator (μM)			Frequency of explants inducing callus
NAA	2,4-D	BA	
1.07	0	10.2	100
2.14	0	10.2	86
0	0.45	10.2	72
0	0.9	10.2	69

These globular green somatic embryos, when further sub-cultured onto the same medium, produced embryos of torpedo stage in 2 months. At this stage, they were sub-cultured either in RBANGT medium or in BAT medium for further maintenance or its further use in regeneration and also for transformation in vitro.

In vitro rooting of somatic embryos

Secondary somatic embryos that are established from immature zygotic embryonic stalk or callus were used for rooting. In vitro rooting of somatic embryos was achieved

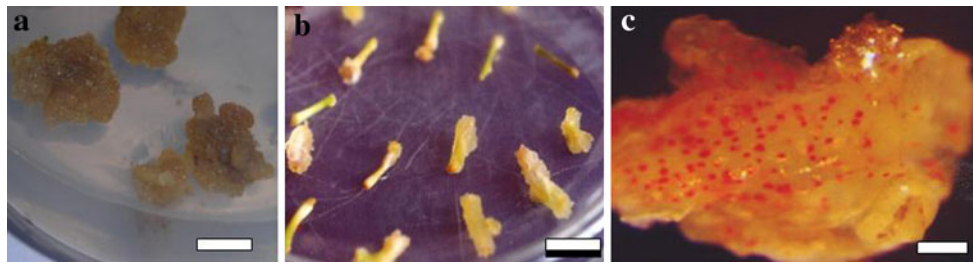


Fig. 3a–c Callus induction using NAA and 2,4-D from hypocotyl explants of *B. orellana*. **a** Callus induction from hypocotyls using 1.07 μM NAA and 10.2 μM BA (*bar* 10 mm). **b** Callus induction

from hypocotyls using 0.45 μM 2,4-D and 10.2 μM BA (*bar* 15 mm). **c** Pigment production on callus obtained from 1.07 μM NAA and 10.2 μM BA as observed under a light microscope (*bar* 500 μm)

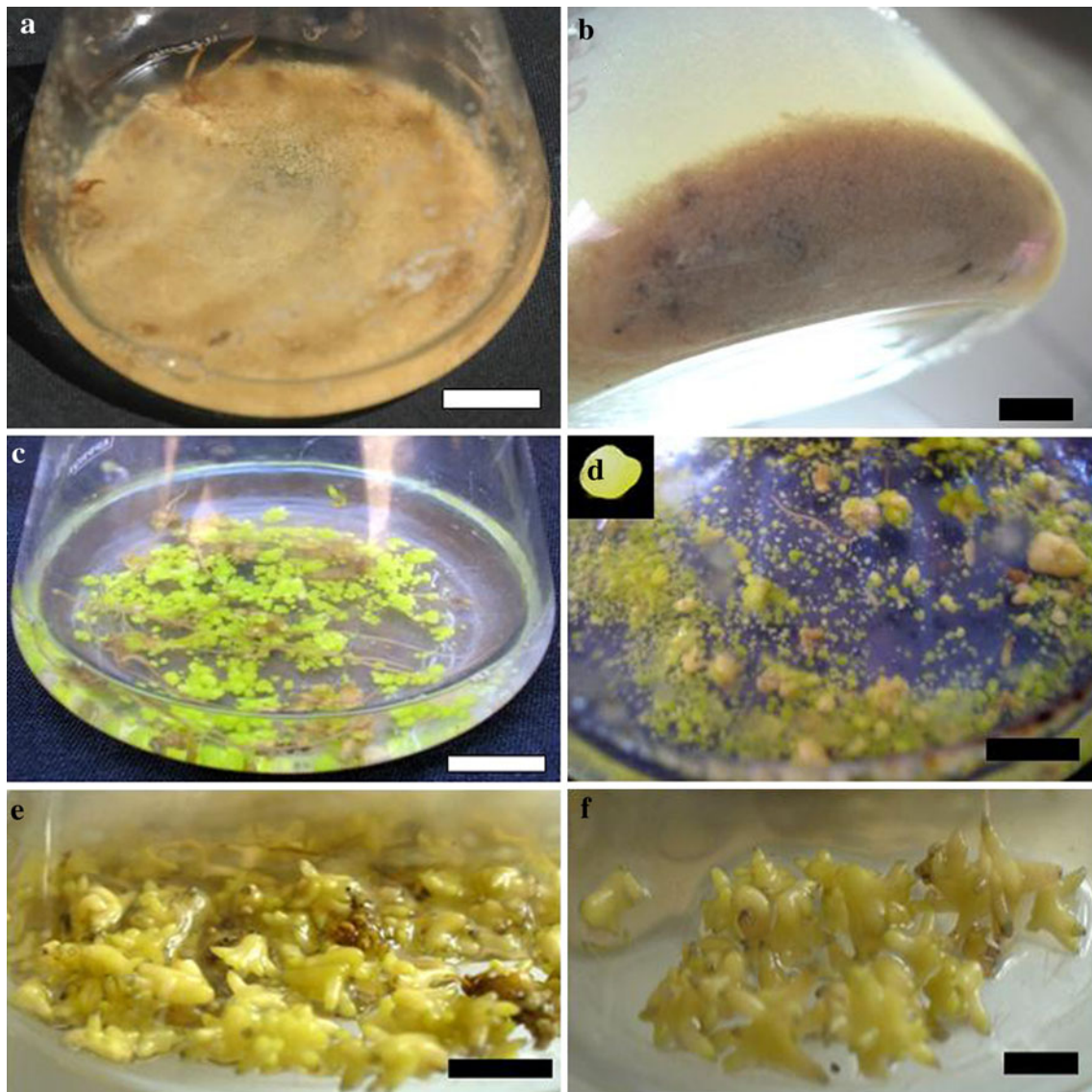


Fig. 4a–f Callus-mediated indirect somatic embryogenesis. **a** Somatic embryo induction on BAT medium using cell suspensions (*bar* 10 mm). **b** Somatic embryo induction on BAT medium in globular stage (*bar* 10 mm). **c** Production of secondary somatic

embryos on BAT medium (*bar* 10 mm). **d** Maintenance of somatic embryos (*inset* single globular somatic embryo) (*bar* 5 mm). **e**, **f** Somatic embryos at torpedo stage (*bar* 8 mm)

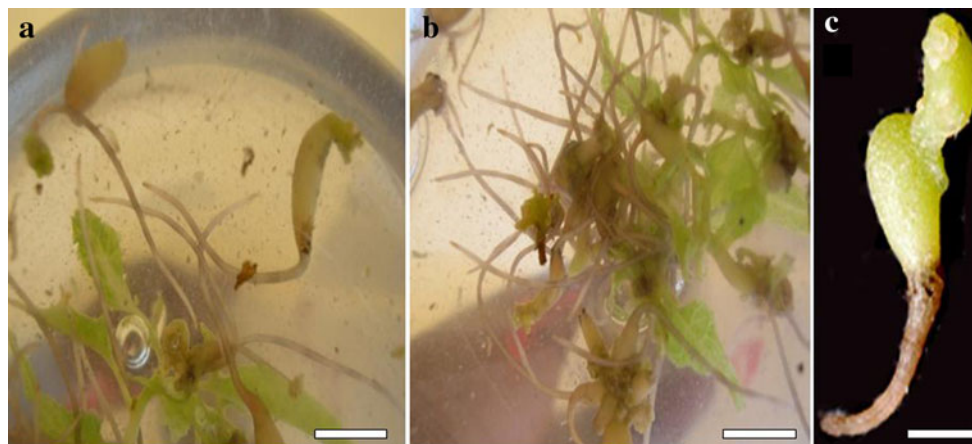


Fig. 5a–c In vitro rooting of somatic embryos. **a, b** Induction of rooting from somatic embryos (*bar* 10 mm). **c** Single somatic embryo showing the formation of tap root in vitro (*bar* 7 mm)

Table 3 Regeneration of rooted somatic embryos of *B. orellana* in 6 weeks

Plant growth regulator concentration (μM)		Mean number of shoots	Mean shoot length (cm)
BA	NAA		
0.44	0.54	–	–
0.88	0.54	–	–
2.22	0.54	–	–
4.44	0.54	1.4 ± 0.01	1.8 ± 0.02
0.44	1.07	–	–
0.88	1.07	–	–
2.22	1.07	$24.8 \pm 0.16^{**}$	$7.3 \pm 0.05^{**}$
4.44	1.07	$17.6 \pm 0.11^{**}$	$4.2 \pm 0.03^{**}$
0.44	2.69	–	–
0.88	2.69	–	–
2.22	2.69	–	–
4.44	2.69	$12.7 \pm 0.08^{**}$	$4.1 \pm 0.03^{**}$

Along with different concentrations of BA and NAA, 2iP ($4.92 \mu\text{M}$), calcium D-pantothenate ($2.1 \mu\text{M}$), biotin ($0.21 \mu\text{M}$), cysteine HCl monohydrate ($227.7 \mu\text{M}$), and adenine sulfate ($108.6 \mu\text{M}$) were incorporated for all treatment concentrations

Values represent mean \pm standard error of the mean (SE) (values are the mean of five replicates)

**Significant at $P < 0.01$

*Significant at $P < 0.05$

by doubling the NAA concentration in RBANGT medium. In vitro rooting was obtained in 2 weeks (Fig. 5a–c). In the RBANGT medium, the NAA concentration was $0.054 \mu\text{M}$, whereas for the rooting of somatic embryos, the NAA concentration was increased to $0.11 \mu\text{M}$.

Regeneration of plantlets from rooted somatic embryos

Rooted somatic embryos as mentioned above were used to obtain regenerated plantlets as a part of the regeneration protocol exclusively for somatic embryogenesis. Of the different PGR concentrations used, rooted somatic embryos that are inoculated onto the liquid medium

comprising MS salts and vitamins, supplemented with BA ($2.22 \mu\text{M}$), NAA ($1.07 \mu\text{M}$), 2iP ($4.92 \mu\text{M}$), calcium D-pantothenate ($2.1 \mu\text{M}$), biotin ($0.21 \mu\text{M}$), cysteine HCl monohydrate ($227.7 \mu\text{M}$), and adenine sulfate ($108.6 \mu\text{M}$), herein referred to as ABI medium, produced regeneration along with multiple shoots from the rooted secondary somatic embryos (Table 3; Fig. 6) in 6 weeks. Calcium D-pantothenate acts as vitamin B5 and biotin acts as vitamin B7, which are water-soluble vitamins important for plant growth, as demonstrated earlier by us (Giridhar et al. 2001). Adenine sulfate possesses cytokinin-like activity, as has been proved earlier in *C. arabica* and *C. canephora* for the regeneration of somatic embryos (Samson et al. 2006).

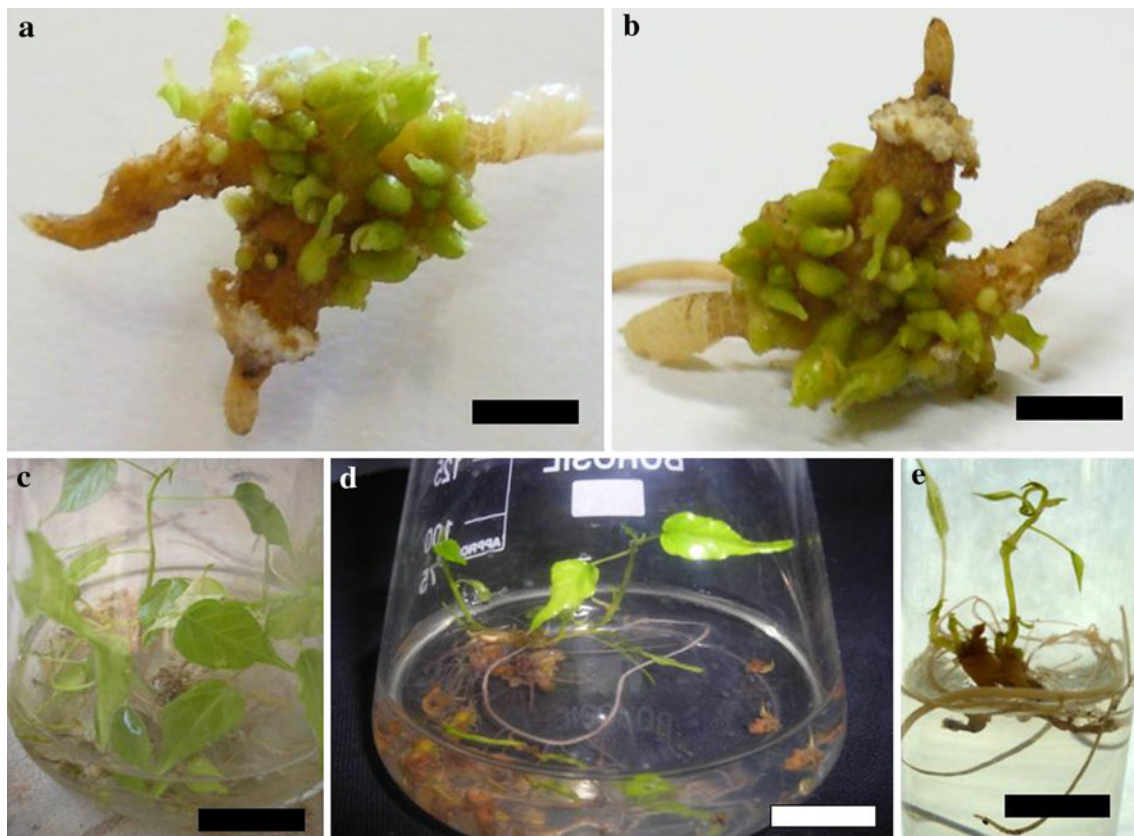
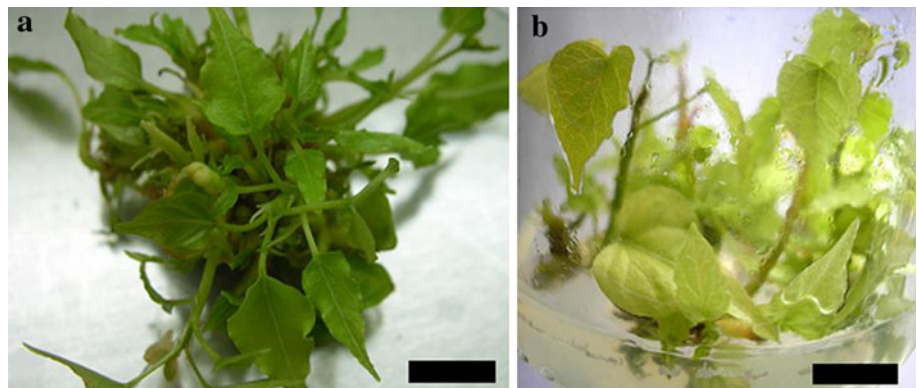


Fig. 6a–e Regeneration of rooted somatic embryos. **a** Rooted somatic embryos producing micro shoots on ABI medium (*bar* 10 mm). **b** Elevated view of rooted somatic embryos producing micro shoots on ABI medium (*bar* 10 mm). **c** Shoot elongation of regenerated somatic embryos on ABI medium (*bar* 20 mm). **d** Shoot

elongation of regenerated somatic embryos on medium with 4.44 and 0.54 μM of BA and NAA, respectively, along with the other components (*bar* 10 mm). **e** Shoot elongation of rooted somatic embryos in solid ABI medium (*bar* 10 mm)

Fig. 7a, b Regeneration of somatic embryos on BAB medium. **a** Multiple shoots from regenerated somatic embryo clump (*bar* 15 mm). **b** Elongated shoots from multiple shoots of regenerated embryos (*bar* 20 mm)



In our present study, we have standardized another medium (BAB) for regeneration to minimize the components of the ABI medium. Since there are two cytokinins used in ABI medium, the cytokinin 2iP is eliminated from it and also the auxin (NAA), thereby, reducing the concentration of BA. The revised medium was tested at 1.11 μM BA along with increased concentrations of calcium D-pantothenate and biotin proportionately from ABI

medium. Concentrations of cysteine HCl monohydrate and adenine sulfate are maintained as in the case of ABI medium. The somatic embryos that are inoculated onto solid as well as liquid BAB medium comprising MS salts and vitamins, supplemented with BA (1.11 μM), calcium D-pantothenate (42.0 μM), biotin (4.1 μM), cysteine HCl monohydrate (227.7 μM), and adenine sulfate (108.6 μM) regenerated in 1 month (Fig. 7a, b) and are sub-cultured in

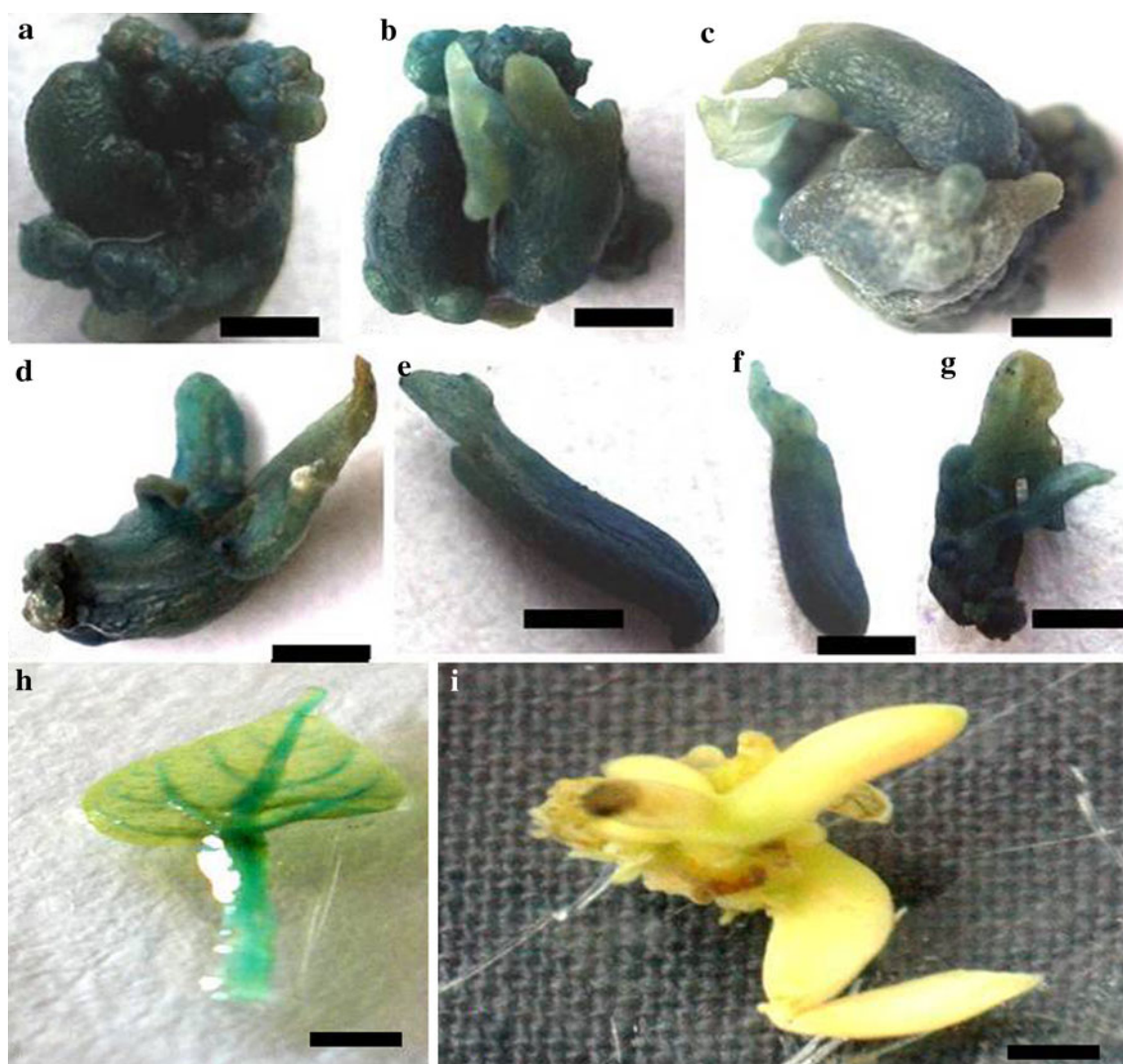


Fig. 8a–i GUS expression from the regenerated somatic embryos in selection medium after 2 months (RBANGT medium with hygromycin 10 mg l^{-1} and cefotaxime 250 mg l^{-1}). **a** Clump of secondary somatic embryos (at globular stage) regenerated from the somatic embryo explant (*bar 1 mm*). **b** Torpedo stage of somatic embryo regenerated from somatic embryo explant under selection medium (*bar 1 mm*). **c** Initiation of regeneration from secondary somatic

embryos (*bar 2 mm*). **d** Secondary somatic embryos under regeneration stage (*bar 2 mm*). **e** Single somatic embryo with initiation of leaves (*bar 2.5 mm*). **f** Side view of single somatic embryo with GUS expression also on the initiating leaves (*bar 3 mm*). **g** Regeneration from somatic embryo (*bar 4 mm*). **h** Leaf of regenerated plant from transformed somatic embryo (*bar 8 mm*). **i** Non-transformed somatic embryo (*bar 5 mm*)

the same medium once in a month until hardening. The same medium responded for normal organogenesis from nodal shoot tip explants or from regenerated plantlets of somatic embryos.

Agrobacterium-mediated transformation in vitro

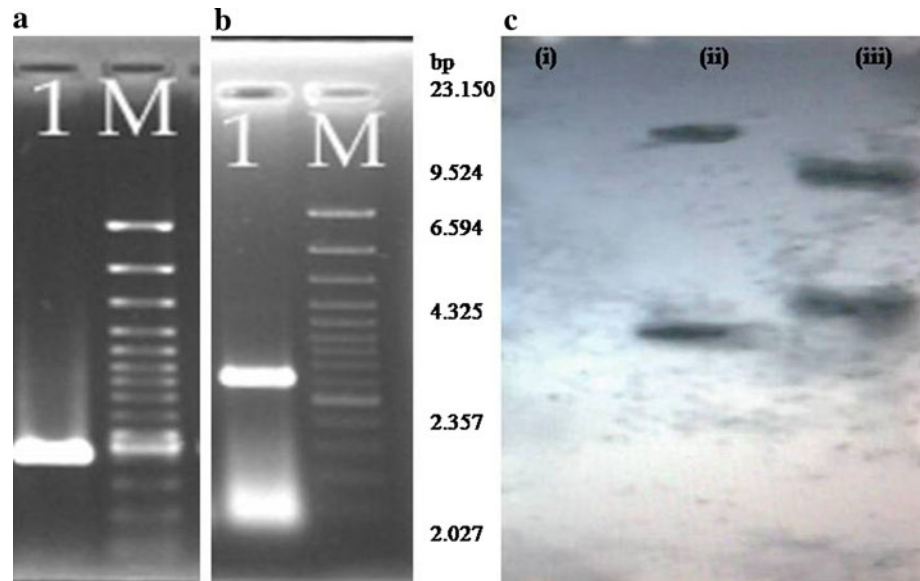
Established somatic embryos (either from embryonic stalk or callus) were used for transformation studies in vitro. Sensitivity studies for somatic embryos with hygromycin indicated that, of the different concentrations tested ($2\text{--}20 \text{ mg l}^{-1}$), it was found that 10 mg l^{-1} was the minimal concentration at which somatic embryos started

browning in 4 weeks and completely died off in 6 weeks. However, all of the cultures were maintained for 2 months for confirmation.

Co-cultivation and selection

A. tumefaciens strain GV 3101 harboring pCAMBIA 1305.2 vector was used for transformation. Using a bacterial density of 1.0 ($\text{OD}_{600\text{nm}}$) for infection, the frequencies of transient *gus* expression was 86.7%, as 13 of 15 tested samples showed blue color. The presence of aceto-syringone in co-cultivation medium had enhanced the transformation efficiency as reported earlier (Humara et al.

Fig. 9a–c **a** PCR amplification of partial *hptII* gene. Lane 1 407-bp amplicon of partial *hptII* gene, Lane M 3-kb DNA ladder. **b** PCR amplification of partial *gus* gene. Lane 1 589-bp amplicon of partial *gus* gene, Lane M 3-kb DNA ladder. **c** Southern blot analysis of transgenic *Bixa orellana*. Genomic DNA (approximately 40 µg) was digested with *EcoRI*, which recognizes a single site within pCAMBIA 1305.2, and hybridized with the *gus* probe. *i* Control (untransformed plants). *ii*, *iii* PCR-positive transformed plants



1999; Song and Sink 2005). After 2 months, stable expression studies for co-cultivated explants were analyzed using 117 explants and the frequency of stable transformation was found to be 2.56%. However, all of those somatic embryos that were produced from the transformed somatic embryo expressed blue color upon histochemical GUS analysis (Fig. 8) and are further confirmed by PCR analysis using partial *gus* and *hptII* primers (Fig. 9a, b).

Somatic embryos that are obtained from selection medium were maintained in RBANGT liquid medium with reduced concentration of antibiotics (hygromycin 5 mg l⁻¹ and cefotaxime 125 mg l⁻¹) for further development of somatic embryos. In the third sub-culture, antibiotics were completely removed and maintained for further production of secondary somatic embryos. For Southern blot analysis, genomic DNA isolated from transformants was digested with *EcoRI* and stable integration of transgene into genome was confirmed (Fig. 9c). No hybridization signal was detected in the controls (untransformed). A transformation protocol in annatto was attempted previously, but it was only a transient expression study involving mannose as a transformation marker (de Pavia Neto et al. 2003b). However, in this study, *Agrobacterium*-mediated transformation of *B. orellana* was achieved using somatic embryos.

In conclusion, we have developed an efficient method for direct and callus-mediated somatic embryogenesis for *B. orellana*. Particularly in callus-mediated somatic embryogenesis, vigorous production of somatic embryos in liquid medium was the best part of the protocol. Moreover, for the first time, a highly competent somatic embryo-based *A. tumefaciens*-mediated stable transformation was standardized that has a wide range of applications in improving annatto pigment through genetic engineering.

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