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RESEARCH ARTICLE

Micropropagation of annatto (*Bixa orellana* L.) from mature tree and assessment of genetic fidelity of micropropagated plants with RAPD markers

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Abstract An in vitro propagation technique based on axillary bud proliferation was developed for the first time to mature annatto (*Bixa orellana* L.) tree. Nodal segments cultured on Murashige and Skoog (MS) medium supplemented with 1.0 μM benzyl adenine (BA) and tender coconut water (10 %) showed significantly high ($P<0.05$) explant response (67.0 %), development of elongated shoots (3.36), shoot buds (8.9) and shoot elongation (3.53 cm). Cytokinins like zeatin, isopentenyl adenine (2-iP), kinetin, or thidiazuron (TDZ) were inferior to BA to induce multiple shoots. Seasonal variations significantly affected the in vitro response of nodal explants. In vitro rooting experiments have showed 55.6 % rooting on MS medium containing 15 μM indole-3-butyric acid (IBA). Alternatively, in vitro raised shoots were rooted (61.1 %) ex vitro, by 10 mM indole-3-butyric acid (IBA) for 30 s. The results of the RAPD marker system revealed the genetic stability among the micropropagated plants. The present protocol in brief, can be used for the clonal propagation of the superior genotype and preservation of germplasm.

Keywords Benzyl adenine · Ex vitro rooting · Multiple shoots · Nodal segment · Tender coconut water

Introduction

Bixa orellana L. (Family Bixaceae) is a small tree widely cultivated in tropical and sub tropical regions of the world for the extraction of annatto dye from the seeds. Among the naturally occurring colorants, annatto ranks second in its economic importance. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) raised the Acceptable Daily Intake (ADI) for bixin to 0–12 mg/kg body weight (FAO/WHO 2007), leading to substantial increase in market demands of annatto dye. Plantations of *B. orellana* were traditionally raised through seedlings (Joseph et al. 2010). Since the plant is cross pollinated one, uniform yield cannot be expected from such plantations. Vegetative propagation by cuttings is possible (Joseph et al. 2011a) however, trails because of low rooting coupled with unavailability of enough branch cuttings for mass production of superior planting materials. For raising commercial plantations large numbers of vegetatively propagated plantlets obtained from high yielding plants are required. In lieu of this, development of an efficient in vitro propagation methodology for yield proven plus trees will be helpful in raising superior planting materials and thus increase the productivity substantially. However, all the earlier attempts to micropropagate *B. orellana* were based on seedling derived explants (D'Souza and Sharon 2001; Khan et al. 2002; Parimalan et al. 2008, 2009, 2011; Joseph et al. 2011b). In the present communication, we report a protocol for the micropropagation of 6-year-old mature *B. orellana* tree using nodal explants. Morphological and RAPD analysis of the field transferred hardened plants were also carried out to test the genetic fidelity of micropropagated plants.

Materials and methods

Plant material Extensive germplasm exploration survey was carried out in different parts of Kerala state to find out high yielding elite plants of *B. orellana*. After preliminary screening, 6-year-old adult tree, growing in Medicinal plant garden, Department of Botany, University of Kerala, Kariavattom (8º33′03.86″ N; 76º52′38.64″ E; 18 m asl)

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was used as explant source. The yield traits of the plant *viz*., seed output (2.93 kg per plant per year) and total bixin yield per year (24.3 g) were high in the selected plant compared to an average yielder (1.5 kg per year and 7.0 g bixin per year).

The donor plant was pruned, so as to induce fresh shoots. Healthy shoots of 5–6 cm length were collected from current season's growth. Explants were segmented into 2–2.5 cm sized segments having a single node with an axillary bud. They were first wiped with alcohol soaked cotton followed

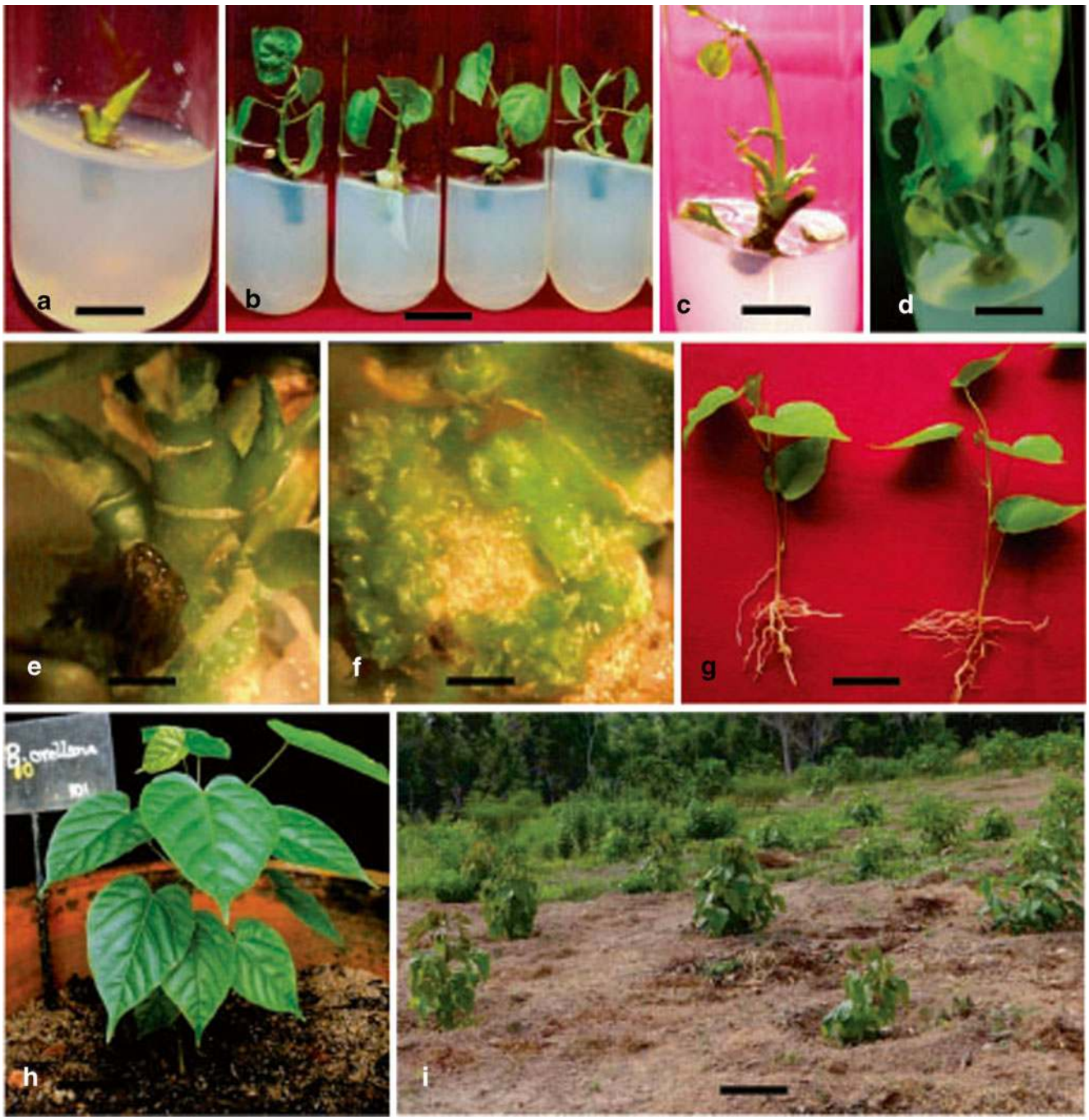

Fig. 1 In vitro propagation of mature *Bixa orellana* using nodal segments collected from 6-year old tree, **a** Elongation of axillary bud (bar=2 cm), **b** Shoot elongation and initiation of multiple shoots in MS medium containing 1 μM BA along with 10 % tender coconut water (bar=0.65 cm), **c** Multiple shoot development (bar=1 cm) on MS+1 μM BA and 10 % coconut water (4 weeks after culture), **d** Shoot multiplication in MS+BA (1 μM) BA+10 % coconut water (bar=0.8 cm), **e**–**f** Stereo microscopic view of multiple shoots from axillary bud and adventitious shoot buds (bar=5.5 cm, 6.2 cm), **g** Ex vitro rooted plants raised through 10 mM IBA treatment for 30 s (bar=1.5 cm), **h** 2-month-old-plants transferred to pots (bar=0.55 cm), **i** Micropropagated plants 3-months after field transfer (bar=0.075 cm)

Table 1 Effect of different concentrations of BA on multiple shoot induction in *B. orellana* nodal segments

Benzyl adenine (μM)	% response	No. of shoot buds	No. of shoots	Shoot length (cm)
Control	0.0±0.00 d	0.00±0.00 e	0.00±0.00 c	0.00±0.00 d
0.5	3.7±5.56 c	0.66±0.33 d	0.33±0.33 c	0.20±0.20 d
1.0	41.7±0.00 a	4.33±0.17 a	2.16±0.16 a	2.83±0.16 a
1.5	30.6±1.60 ab	3.06±0.06 b	1.00±0.00 b	1.26±0.13 b
2.0	19.4±2.13 b	2.20±0.11 c	1.00±0.00 b	0.70±0.05 c
2.5	8.3±0.00 c	0.00±0.00 e	0.00±0.00 c	0.00±0.00 d
Main effect F Df (n-1) 5	34.96***	119.24***	29.64***	62.26***

Means±SE within a column followed by same letters are not significantly ($P<0.05$) different as determined by Duncan's New Multiple Range Test (DNMRT). ***significant at $P<0.001$ level

by thorough washing in running tap water for 30 min, and then treated with 1 % polysorbitol detergent (Labolene, Mfg. Fischer Scientific Chemicals, Mumbai, India) for 20 min and subsequently immersed in 0.1 % (*w*/*v*) carbondazim fungicide (Bavistin, Mfg. BASF, Mumbai, India) for 1 h. The explants were then surface disinfected with 85 % ethanol for 30 s followed by treatment with mercuric chloride solution (0.05 %) for 5 min and 4–5 times washing in sterile distilled water. The surface sterilized explants were trimmed into 1–1.5 cm long segments and were cultured in vitro for shoot proliferation.

Culture media and conditions Murashige and Skoog (1962) medium containing 3 % (*w*/*v*) sucrose and 0.7 % agar (Sisco Research Laboratories, Mumbai, India) were used. Plant growth regulators (Sigma–Aldrich, St. Louis, US) at different concentrations were incorporated into the basal media. The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C and 108 kPa pressures for 15 min. Single explant was cultured in 25×150 mm culture tube containing 15 ml of sterilized medium for initiation of cultures. Cultures were incubated at 25±2 °C in a culture room with 40 $\mu mol m^{-2} s^{-1}$ irradiance provided by cool fluorescent tubes (40 W; Philips, India) and were exposed to a photoperiod of 16 h and 55±5 % of relative humidity.

Multiple shoot induction Single nodal segments were used as explants for in vitro shoot multiplication experiments. Experiments were conducted with different concentrations of benzyl adenine (BA) at 0.5, 1.0, 1.5, 2.0 or 2.5 μM. Growth regulator free media was served as control.

In vitro response of explants on medium supplemented with 1 μM BA in combination with different concentrations (5, 10, 15 or 20 %) of filter sterilized (0.22 μm pore size) tender coconut water was studied. Medium without coconut water but containing 1 μM BA was used as control to compare the effect of different concentrations of coconut water on multiple shoot induction.

To compare the efficiency of BA with other cytokinins on multiple shoot induction, fresh explants were cultured on medium containing 10 % tender coconut water along with 1.0 μM isopentenyl adenine (2-iP), kinetin, thidiazuron (TDZ) or filter sterilized (0.22 μm pore size) zeatin. When the axillary shoots attained a length of 3–4 cm, they were excised, segmented and further multiplied on agar gelled MS medium containing 1.0 μM BA and 10 % tender coconut water.

Effect of explant collection month Under natural conditions in the state Kerala (the southernmost part of peninsular India), fresh sprouts of *B. orellana* appear during early summer (March–April). Active vegetative growth phase continues till July. Flowering begins in July and extends till October. To determine the most favorable season for culture establishment, explants were collected every month from January to December and cultured on MS medium containing 1.0 μM BA and 10 % tender coconut water.

Table 2 Effect of different concentrations of tender coconut water on multiple shoot induction in *B. orellana* nodal segments in MS medium supplemented with 1 μM BA

Tender coconut water (%)	% response	No. of shoot buds	No. of shoots	Shoot length (cm)
0.0	41.7±0.00 b	4.23±0.12 c	2.06±0.06 b	2.90±0.05 b
5	47.2±1.60 b	4.03±0.08 c	2.23±0.14 b	1.73±0.06 c
10	67.0±3.03 a	8.90±0.26 a	3.36±0.17 a	3.53±0.14 a
15	38.8±1.60 b	4.93±0.14 b	2.23±0.03 b	1.73±0.06 c
20	19.4±2.13 c	2.76±0.14 d	1.50±0.00 c	1.23±0.03 d
Main Effect F Df (n-1) 4	27.95***	201.95***	39.79***	131.89***

Means±SE with in a column followed by same letters are not significantly ($P<0.05$) different as determined by DNMRT. ***significant at $P<0.001$ level

Table 3 Effect of MS medium supplemented with 1.0 μM various cytokinins along with 10 % tender coconut water on multiple shoot induction from nodal explants of *B. orellana*

Cytokinin (1.0 μM)	% response	No. of shoot buds	No. of shoots	Shoot length (cm)
Control	0.0±0.00 d	0.00±0.00 d	0.00±0.00 d	0.00±0.00 e
Zeatin	44.4±1.60 e	0.00±0.00 d	1.10±0.10 c	0.90±0.17 d
Isopentenyl adenine	41.5±2.82 c	1.23±0.14 c	1.60±0.11 b	1.40±0.11 c
Kinetin	61.1±1.60 ab	0.00±0.00 d	1.86±0.18 b	2.50±0.15 b
Benzyl adenine	66.6±0.00 a	8.80±0.26 b	3.50±0.11 a	3.63±0.13 a
Thidiazuron	55.6±1.60 b	12.00±0.57 a	0.00±0.00 d	0.00±0.00 e
Main Effect F Df (n-1) 5	153.11***	401.60***	146.12***	147.32***

Means±SE with in a column followed by same letters are not significantly ($P<0.05$) different as determined by DNMRT. ***significant at $P<0.001$ level

In vitro rhizogenesis and hardening Micro shoots (> 2.5 cm) obtained from fourth subculture onwards were used for in vitro rooting. Agar gelled (0.7 %) MS medium containing 2 % sucrose, and auxins [α-Naphthalene acetic acid (NAA) or IBA] at varying concentrations (5, 10, 15 or 20 μM) was tested. The cultures were incubated in light at16-h photoperiod. After 4 weeks, in vitro rooted plantlets were washed in running tap water to remove traces of agar and the plantlets were transplanted into polyethylene cups (diameter = 7 cm and height = 8 cm) containing a mixture of autoclaved soil and vermiculite. They were covered with polyethylene bags for 4 weeks to maintain high relative humidity. Potted plantlets were maintained initially in growth chamber at 25±2 °C, 55±5 % RH, under 16 h of photoperiod with a light intensity of 40 $\mu mol m^{-2} s^{-1}$. Polyethylene bags were removed upon emergence of new leaves (usually within 4 weeks) and acclimatized plantlets were transferred to green-house.

Ex vitro rooting Microshoots (>2.5 cm in length) obtained from the fourth subculture onwards were used for ex vitro rooting experiments. Shoots were washed thoroughly in running tap water and the excess water from the cut ends were removed with a blotting paper. The shoots were pulse treated by dipping in freshly prepared auxin solutions (IBA or NAA) in 50 % ethanol at varying concentrations (2.5, 5, 10 or 20 mM) for 30 s. Microshoots dipped in 50 % ethanol were used as control. They were then planted in polyethylene cups (diameter = 7 cm and height = 8 cm) containing a soil: vermiculite (1:1) mixture enriched with ¼MS salt solution and placed in a hardening chamber (28±2 °C, RH 90 %; 40 $\mu mol m^{-2} s^{-1}$ irradiance). Initially, plants were covered with micro-holed polyethylene bags. When plants were completely weaned (4 weeks), they were moved to a shade-house. Three month-old-plants were planted in the field.

Field planting and morphological evaluation Three month-old-plants were used for field transfer. To transfer the in vitro plants, 50 m×50 m plot was selected. Plants were planted at 2 m×2 m spacing by preparing small (15×15×15 cm) pits. As an initial trial, 24 plants were transferred and were watered regularly. The plantation was raised in three replication blocks and each block represented by eight plants. The plantation subjected to intermittent weeding and other field maintenance activities regularly. The field transferred plants were used to record various metric and non metric growth characteristics such as collar diameter, leaf number, leaf size, leaf color, leaf vein color, petiole length, petiole color, stem color and number of branches. The data was scored on the basis of three replication blocks. Both metric and non metric characters were tabulated to determine level of morphological uniformity of the in vitro regenerants.

Genomic DNA extraction and RAPD analysis of regenerants Three months after field transfer, fresh, young leaves were collected from ten randomly selected micropropagated plants and also from the mother plant. DNA extraction was made with 100 mg of young leaves, using modified CTAB procedure (Dellaporta et al. 1983). DNA concentration and purity was determined by using Biophotometer (Eppendorf, Germany).

RAPD amplification was performed using a thermal cycler (Biorad Master Cycler). The reaction mixture composed of 1.5 μl 10x buffer (Taq buffer A with Tris-pH 9.0, KCl, 15 mM

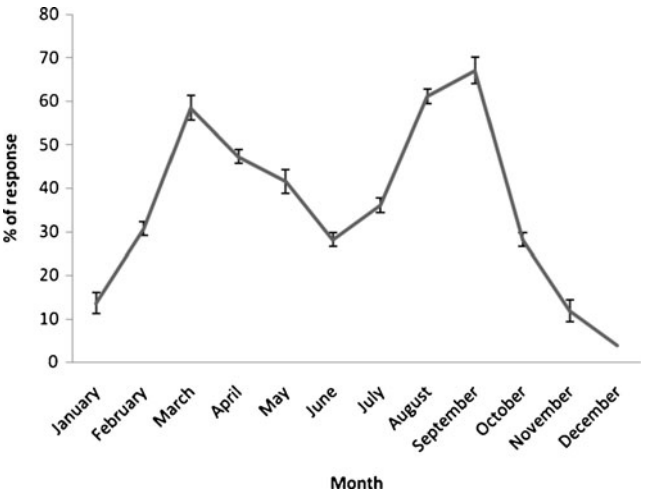

Fig. 2 Effect of season on the response of nodal explants in *Bixa orellana*

Table 4 Effect of auxin type and concentration on in vitro rooting of *Bixa orellana*

Auxin*	Conc (μM)	% of response	No. of roots	Root length (cm)
Control	0.0	0.0±0.00 f	0.00±0.00 g	0.00±0.00 f
NAA	5.0	3.7±5.56 e	0.56±0.28 f	0.76±0.38 e
	10	22.4±2.13 c	1.50±0.05 d	1.83±0.03 c
	15	38.8±1.66 b	2.63±0.08 b	2.56±0.12 b
	20	28. 0±1.60 bc	2.03±0.08 c	1.23±0.08 de
IBA	5.0	10.7±2.43 d	1.10±0.10 e	0.76±0.08 e
	10	36.0±1.66 b	2.56±0.08 b	2.43±0.08 b
	15	55.6±1.60 a	4.06±0.08 a	3.66±0.08 a
	20	19.4±2.13 cd	1.76±0.08 cd	1.6±0.08 3 cd
Treatment Df (n-1)	8	34.71***	99.70***	55.69***
Auxin type(T) Df (n-1)	1	38.30***	57.40***	21.80***
Auxin conc.(C) Df (n-1)	3	7.34**	127.65***	79.94***
T X C Df (n-1)	7	19.09***	69.91***	39.15***

MS medium containing 2 % sucrose. Means±SE with in a column followed by same letters are not significantly ($P<0.05$) different as determined by DNMRT. **significant at $P<0.01$ level; ***significant at $P<0.001$ level

$MgCl_2$ and gelatin), 1.2 μl 10 mM dNTP mix, 0.15 μl of Taq polymerase (Genei, Bangalore, India) and 1.2 μl primer (Eurofins mgw/Operon, Bangalore, India), 50 ng DNA template were made upto 15 μl with autoclaved distilled water. The amplification program was as follows; initial denaturation for 4 min at 95 °C followed by 40 cycles of denaturation for 30 s at 94 °C, annealing for 1 min at 37 °C, extension for 2 min at 72 °C and finally ending with one cycle for 5 min at 72 °C. After amplification, PCR products were stored at −20 °C till performing electrophoresis. Amplified products were mixed with 2 μl of bromophenol blue dye solution before loading. A total of 20 random 10-mer primers (Eurofins mgw/Operon, Bangalore, India) were screened. Of these 9 primers, that could generate discernible and reproducible bands were selected for formal amplification are OPI 01, OPI 02, OPI 04, OPI 06, OPI 07, OPI 09, OPI 14, OPI 18 and OPI 20.

Amplified products were separated by electrophoresis on an agarose (1.8 %*w*/*v*; SRL, Mumbai) gel, mixed with ethidium bromide and submerged in 1x Tris-Acetic acid-EDTA (TAE) buffer (pH 8.0) at a constant voltage (100 V). The profiles were documented by UV trans-illuminator equipped gel documentation system (JH BIO, Alpha Innotech, USA). The molecular size of amplified fragments was compared with 1 kb DNA ladder (Genei, Bangalore, India).

Experimental design and statistical analysis All the experiments were conducted using a completely randomized block design. Every treatment was composed of three replications and each replication block was represented by 12 culture tubes. Data on various parameters were evaluated by analysis of variance (ANOVA) and mean values were compared with Duncan's New Multiple Range Test (DNMRT). Data scored in percentages were subjected to arcsine transformation before analysis, and then converted back to percentages for presentation in the tables (Snedecor and Cochran 1962).

Table 5 Effect of auxin type and concentration on ex vitro rooting of *B. orellana*

Auxin	Conc. (mM)	% of response	No. of roots	Root length (cm)
Control	0.0	0.0±0.00 f	0.00±0.00 d	0.00±0.00 e
IBA	2.5	13.5±2.43 cd	1.23±0.08 cd	0.60±0.11 cd
	5	30.6±1.60 bc	5.46±0.67 b	1.23±0.20 bc
	10	61.1±1.63 a	11.60±1.70 a	3.00±0.10 a
	20	3.7±5.56 e	1.26±0.64 cd	2.03±1.02 ab
NAA	2.5	22.4±2.13 b	1.03±0.12 cd	0.43±0.03 cd
	5	50.0±2.77 a	2.46±0.18 c	2.06±0.12 ab
	10	10.8±2.43 d	1.73±0.12 cd	1.43±0.08 bc
	20	0.0±0.00 f	0.00±0.00 d	0.00±0.00 e
Treatment Df (n-1)	8	49.39***	32.10***	8.53***
Auxin type(T) Df (n-1)	1	9.39**	47.28***	6.72*
Auxin conc.(C) Df (n-1)	3	49.03***	28.88***	7.19**
T X C Df (n-1)	7	31.88***	27.25***	5.99*

Means±SE with in a column followed by same letters are not significantly ($P<0.05$) different as determined by DNMRT. *significant at $P<0.05$ level; **significant at $P<0.01$ level; ***significant at $P<0.001$ level

Table 6 Growth performance of tissue cultured plants of *B. orellana* in the field

Plantation blocks	Plant height (cm)	No. of leaves	Petiole length (cm)	Leaf length (cm)	Leaf width (cm)	Collar diameter (mm)	Petiole thickness (mm)
1	15.0	12.8	3.6	41.0	40.8	5.0	2.2
2	14.7	11.5	3.6	40.6	37.0	4.7	2.0
3	17.3	12.5	3.6	40.0	36.0	4.7	2.0
Main effect F Df (n-1)=2	6.98*	5.33 ns	0.196 ns	0.175 ns	5.46*	1.09 ns	5.66*

*Significant at $P<0.05$ level; NS-non significant F value

The percentage of explants producing shoots, mean number of shoot buds (<0.5 cm) per explant, mean number of elongated shoots per explant, maximum shoot length were recorded 4 weeks after inoculation. In ex vitro rooting experiments, the mean number and length of roots developed from shoots and the percentage of shoots producing roots were also determined after 4-week incubation.

Results and discussion

Multiple shoot induction Initial response of explants cultured on media containing various levels of growth regulators was the elongation of axillary bud (Fig. 1a). Among the different concentrations of BA, 1 μM BA gave 41.7 % response and shoot buds (Table 1). Maximum explants response (67 %) was noticed when BA (1 μM) was used in conjunction with 10 % tender coconut water (Fig. 1b). The number of shoot buds (8.9), shoots longer than 0.5 cm (3.36) and the shoot length (3.53 cm) was significantly ($P<0.05$) high in this concentration (Table 2; Fig. 1c, d). In addition to axillary bud proliferation, adventitious shoot buds were also developed (Fig. 1e, f). These shoot buds were elongated after the removal of elongated axillary shoots. Explants cultured on growth regulator free medium failed to respond.

The explant response to various cytokinins (zeatin, 2-iP, kinetin, BA or TDZ) on shoot production was varied significantly ($P<0.001$). The percentage response and the number of shoots produced per explant on medium supplemented with BA was significantly ($P<0.05$) higher than other cytokinins (Table 3). The addition of TDZ in the medium produced highest number of shoot buds (12.0). However, further growth of these buds was arrested. This observation is in conformity with other reports on in vitro propagation of trees like *Pterocarpus marsupium* (Husain et al. 2007) and *Quercus rubra* (Vengadesan and Pijut 2008). Inhibition of shoot elongation and shoot fasciations were recorded. This may possibly due to over activity of TDZ or due to the activity of phenyl groups in TDZ (Huetteman and Preece 1993). Previous report on the use of TDZ in tissue culture of *B. orellana* seedling explants have shown that TDZ alone in multiplication medium resulted in the production of distorted leafy structures with impaired shoot elongation (Neto et al. 2003). Present findings with mature *B. orellana* were also in tune with this report. Parimalan et al. (2007) have reported higher organogenic response with 20–22 shoots per explants from rooted hypocotyl explants in medium supplemented with 2.0 mg/l TDZ and 0.25 % coconut water. In the absence of coconut water, as per the report (Parimalan et al. 2007) there was a drastic reduction in shoot bud development. In the present study, it was evident that even in the presence of coconut water, mature tissues of *B. orellana* failed to elongate in 1 μM TDZ supplemented medium. In the present work, coconut water along with low concentration of BA was found to be effective to induce new shoot buds from nodal segments of mature plant.

BA is reported to be among the most effective and affordable cytokinins used in micropropagation techniques (Amoo et al. 2011). The effectiveness of BA over other cytokinins on development of shoots possibly attributes to the ability of plant tissues to metabolize natural hormones more readily than the synthetic growth regulators. Further, BA could induce production of natural hormones such as zeatin within tissues (Zaerr and Mapes 1982). It is also possible that the amount of BA conjugated to medium was less compared to other hormones. As a result, large amount of BA exist as free or severable forms, which are readily

Table 7 Qualitative morphological features of tissue cultured *B. orellana* plants growing in the field

[a]Color as per Wilson (1938, 1941)

Plantation blocks	Leaf color[a]	Leaf vein color[a]	Petiole color[a]	Stem color[a]
1	Spinach green	Nasturium orange	Purple madder	Peach
2	Spinach green	Nasturium orange	Purple madder	Peach
3	Spinach green	Nasturium orange	Purple madder	Peach

Table 8 Primer sequences, number of bands per primer and the band size in RAPD profile of in vitro raised plantlets of *B. orellana*

Primer	Primer sequence (5′–3′)	Total number of amplified bands	Band size (range in bp)
OPI 01	ACCTGGACAC	4	142–1,650
OPI 02	GGAGGAGAGG	11	81–2,750
OPI 04	CCGCCTAGTC	12	240–2,597
OPI 06	AAGGCGGCAG	8	122–1,306
OPI 07	CAGCGACAAG	6	89–1,717
OPI 09	TGGAGAGCAG	8	43–1,767
OPI 14	TGACGGCGGT	4	11,247–4,366
OPI 18	TGCCCAGCCT	5	258–2,136
OPI 20	AAAGTGCGGG	7	109–2,390

available to plant tissues from the medium (Buah et al. 2010).

In woody perennials, juvenility is to be restored for making an adult plant amenable to clonal propagation. Usually transition from juvenility to maturity limits clonal propagation from adult plant (Boerjan 2005). The present findings in *B. orellana* had shown that the combination of BA and coconut water can induce in vitro development of both axillary buds and adventitious shoot buds from mature tissues. Beneficial effect of BA with other additives in multiple shoot induction in single node shoot tips of seedling explants in *B. orellana* was reported (Parimalan et al. 2008, 2011). Seedling derived nodal explants gave maximum response (93.3 %) and number of shoots (35.71) in 2-iP (5 μM) supplemented MS medium (Joseph et al. 2011b). But in the case of nodal explants of mature tree, 2-iP was found to be inferior to BA at 1 μM concentration (Table 3).

Effect of explant collection month The plant material collected during September gave the maximum (67 %) response. The least explant response (Fig. 2) was recorded in the months of December and January, may be due to the cessation of growth during this period and consequent bud dormancy. The influence of phenophase and physiological conditions of source tree in the establishment of cultures observed in the present study is in agreement with other reports (Preece 2008; Goyal et al. 2012).

In vitro rhizogenesis and hardening Rooting coupled with elongation of shoot was occurred in rooting medium. Rooting response of micro shoots cultured on different auxin (NAA or IBA) supplemented medium varied significantly with the type and concentration (Table 4). Addition of IBA (15 μM) in the rooting medium significantly ($P<0.001$) improved rooting (55.6 %), root number (4.06) and root length (3.66 cm). The control shoots planted in growth regulator free medium failed to root. In the previous works, IBA (5 μM and 4.9 μM) was reported to induce maximum rooting in micro shoots derived from various seedling explants (Neto et al. 2003; Parimalan et al. 2008; Joseph et al. 2011b). However, another report (D'Souza and Sharon 2001) suggest the effectiveness of varying concentrations of NAA to induce adventitious roots. Various concentrations of NAA tested in the present study were inferior to IBA (5 μM). The present findings had shown that even though both IBA and NAA were able to induce roots, IBA was preferred as it can induce maximum response and number of roots.

Ex vitro rooting Auxin type and concentration had significant ($P<0.001$) effects on rooting (Table 5). Of the two auxins (IBA and NAA) used for ex vitro rooting, micro

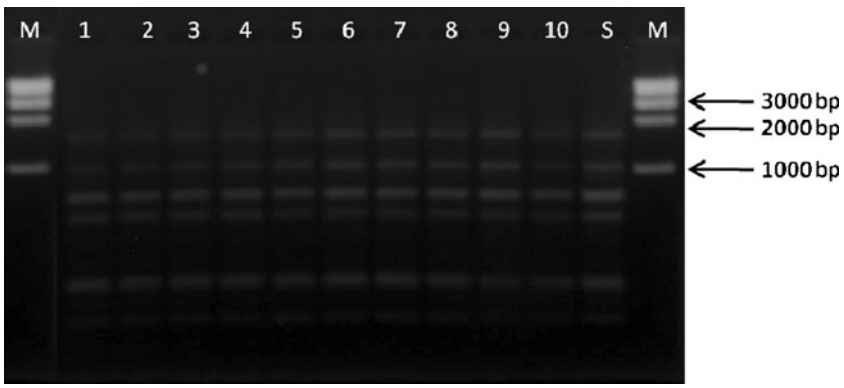

Fig. 3 RAPD profile of micropropagated plants and the mother plant of *Bixa orellana* generated with OPI 07. Lane M-1 kb DNA ladder, lane 1–10 in vitro raised plants, lane S-source (mother) plant

shoots treated with a 10 mM IBA solution for 30 s resulted in maximum rooting (61.1 %), number of roots (11.60) and root length (3.0 cm; Fig. 1g). Control shoots planted after 50 % ethanol treatment (30 s) failed to root (Table 5). Ex vitro rooting is an attractive method to micropropagators as it synchronizes rooting and hardening of plants. Rhizogenesis through ex vitro offers the opportunity to improve the biological as well as economical efficiency of micropropagation; to save time, labour and resources (Borkowska 2001). The successful use of pulse treatment for ex vitro rooting of micro shoots was previously reported in plants like *Tectona grandis* (Tiwari et al. 2002) and *Embelia ribes* (Dhavala and Rathore 2010).

Four-week-old rooted plants were transferred to pots containing soil: vermiculite mixture (1:1). Potted plants (Fig. 1h) showed emergence of new leaves within 3-weeks of planting. Micropropagated plants showed uniform morphological features and active growth at ambient conditions.

Field trial and morphological evaluation Field planted plants survived (87.5 %) and resumed active growth within 4 weeks of transfer. The young plants within 3 months were well established (Fig. 1i). Majority of morphological parameters showed non-significant differences between replication blocks (Table 6), in turn, indicating high level of uniformity among regenerants. Plant height of 3 month old plants ranged 14.77 to 17.3 cm (Table 6). At this growth phase all plants showed nearly 11–12 fully expanded spinach green leaves. Each leaf lamina has 40–41 mm length and 36–41 mm diameter. Records on number of leaves per plant, petiole length, leaf length and collar diameter showed insignificant differences among replication blocks. Whereas plant height, leaf width and petiole length showed significant ($P<0.05$) F value. Qualitative traits such as color of stem, petiole, and leaf vein were similar in all the plants growing in the field (Table 7). Thus evident that micropropagted plants are maintaining a high degree of uniformity.

Assessment of genetic fidelity of regenerants using RAPD analysis A total of 65 RAPD bands were generated with nine OPI primers (Table 8). Out of these 65 bands, cent percent showed monomorphic bands. Bands ranged in size from 81 bp to 4,366 bp. The number of bands produced per primer ranged between 4 and 12. On an average 7.2 bands per primer was observed. Maximum number of bands was produced by the primer OPI 04 and minimum by OPI 14.

RAPD analysis of in vitro raised and the mother plant did not show any polymorphism (Fig. 3) indicating genetic stability of the plants. Since RAPD profiles in sampled plants are similar, high degree of clonal uniformity among regenerants can be presumed. Thus, in vitro raised plants evolved by adopting present protocol were true-to-type and could be due to regeneration of the plants from pre-existing meristem without any intermittent callus phase.

The protocol described above is reproducible. Using this procedure it is estimated that a single explant can produce over 200 true-to-type hardened plants within a 10-month culture period. It can be used for the clonal propagation of the superior genotype and preservation of *B. orellana* germplasm.

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