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RESEARCH ARTICLE

An efficient in vitro propagation methodology for Annatto (*Bixa orellana* L.)

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Abstract Efficient methods were developed for both in vitro seed germination and micropropagation of an economically important dye yielding multipurpose tree, *Bixa orellana* L. Mature seeds were inoculated onto Murashige and Skoog (MS) medium supplemented with different concentrations of gibberellic acid (GA_3). Highest frequency of germination (93.3 %) was recorded on medium supplemented with 3 μM GA_3 against 13.33 % in control. Nodal explants cultured on MS medium fortified with 5 μM isopentanyl adenine (2-iP) produced maximum explants response (93.3 %) and highest number of shoots (35.71). Addition of relatively higher concentration (15 μM) of benzyl adenine (BA) resulted in the production of significantly ($P<0.05$) reduced number of shoots (12.66). Sucrose at 87.6 mM was found to be the best carbohydrate source for multiple shoot induction compared to glucose and fructose. Regenerated shoots (3–4 cm) were rooted (95.5 %) on agar gelled MS medium supplemented with 10 μM indole-3-butyric acid (IBA). In vitro developed plantlets with well-developed roots were potted and acclimatized initially in the growth chamber and then moved to a green house with 83.3 % survival. The present protocol avoids the use of auxins in shoot multiplication medium, which will lower the cost, avoid callus formation and thus reduces the possibility of somaclonal variation in the regenerated plants. The method is efficient to produce over 32,000 hardened plants within a 10-month culture period starting from a single nodal explant.

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Introduction

Annatto (*Bixa orellana* L.) is a small sized tree cultivated in the tropical and subtropical regions of the world. The plant is chiefly valued for its pigmented seeds which are the source of orange red natural colorant. In commerce, annatto ranks second among the natural colorants with a world consumption of 10,650 tons/year (Satyanarayana et al. 2003). This natural pigment has an ever-increasing demand from food and cosmetic industries, as the natural color is safe for consumption and for skin applications. It is one of the 13 basic pigments derived from natural sources that are currently permitted by the US-FDA and also specified as permitted color in European Union. In India, it is accepted under Prevention of Food Adulteration Act. Hence there is an ever-growing market for annatto (Carvalho 1999; Collins and Hughes 1991). The whole plant is medicinally important and is used in various systems of medicine.

Traditionally the plant is propagated by cuttings and seeds. Vegetative propagation by cuttings has serious drawbacks such as the difficult nature of cuttings to root due to leaching of gummy substances (Sharon and D'Souza 2000). Currently commercial plantations of this species are being raised through seeds (Aparnathi et al. 2003). Commercial cultivation of this crop is not very attractive due to the lack of quality planting materials. Therefore in vitro propagation of annatto could be an alternative to produce large number of superior planting materials. Moreover, development of a reliable in vitro regeneration system is a pre requisite for genetic transformation system and generating polyploids aimed at increasing the levels of carotenoids in the seed coat.

To initiate in vitro cultures, seeds were preferred as starting material (Benson et al. 2000), as they are most capable, fully organized structures to respond in an in vitro environment. In vitro germination of seeds yields large number of aseptic plants that can be inoculated for initiating tissue culture (Mercier and Kerbauy 1997). In vitro protocols using seedling explants have been successfully employed for rapid propagation and manipulation of several woody perennials e.g., *Pterocarpus marsupium* (Husain et al. 2007).

Previous reports on micropropagation of *B. orellana* are also based on seedling-derived explants. In vitro response of explants like hypocotyl segments (Neto et al. 2003; Parimalan et al. 2007), nodal segments and shoot tips derived from young seedling (D'Souza and Sharon 2001; Nassar et al. 2001; Parimalan et al. 2008, 2009, 2010; Ramamurthy et al. 1999) and leaf segments from seedling (Almeida et al. 1996) are available. Role of ethylene in in vitro rooting of micropropagated shoots are also available (Neto et al. 2009). In the previous reports however, raising in vitro seedling was a tedious process coupled with a reduced rate of multiplication. An efficient micropropagation protocol is still lacking in *B. orellana* using nodal explants. The present study sought to develop an effective protocol for micropropagation using low levels of 2-iP or BA without an intervention of auxin in the multiplication medium.

Materials and methods

In vitro seed germination Due to severe infection, seeds from fully mature dehiscent fruits were avoided, instead 7-week after fruit set, mature, indehiscent fruits collected from a 4-year-old tree growing in the Botanic garden of University of Kerala, Kariavattom was used as seed source.

The collected fruits were processed by removing bristles like outgrowth by using a fine razor blade. The intact fruits were then treated with diluted (1 % v/v) solution of polysorbitol detergent (Labolene, Mfg. Qualigen's Fine Chemicals, Mumbai, India) for 30 min, followed by several rinses in water and then submerged in 0.1 % (w/v) carbondazim fungicide (Bavistin, Mfg. BASF, Mumbai, India) for 1 h and washed. Subsequently the fruits were treated with mercuric chloride (0.1 %) solution containing few drops of surfactant (Tween 20) for 10 min followed by several rinses with sterile distilled water in the laminar air flow chamber and finally the fruits were dipped in alcohol and flamed. Seeds were aseptically excised from the fruits and used for inoculation on to germination medium (15 ml) prepared in culture tubes (25×150 mm) plugged with non-absorbent cotton. Single seed per culture tube was inoculated.

Effect of GA_3 on germination The seeds were cultured in vitro, on MS medium supplemented with GA_3 (0.0, 1.5, 3.0, 4.5 and 6.0 μM) and 3 % sucrose. The pH of the medium was adjusted to 5.8 and solidified with 0.7 % Difco Bactoagar (Qualigen's, Mumbai, India) before autoclaving at 121 °C and 108 kPa for 20 min. A set of seeds inoculated on hormone free MS medium served as the control.

The number of germinated seeds was recorded from the fifth day onwards at 5 days interval for 45 d. Mean time taken for germination (MTG) was calculated (Hartman and Kester 1983) using the equation $MTG = \sum (nxd)/N$, where n = number of seeds germinated, d = period of incubation in days, N = total number of germinated seeds at the end of the test.

If stated otherwise, all cultures were incubated at 25± 2 °C in a culture room with 50-$\mu molm^{-2}$ s^{-1} irradiance provided by cool white fluorescent tubes (40 W; Philips, India) and were exposed to a photoperiod of 16-h and 55± 5 % of relative humidity.

In vitro shoot multiplication Nodal segments excised from 20-d-old axenic seedlings were used for in vitro shoot multiplication. The nodal segments (1.5–2.0 cm) were excised and cultured on MS medium supplemented with different concentrations (0.5, 1.0, 2.5, 5.0, 10.0 and 15.0 μM) of BA or 2-iP. When most of the axillary shoots attained a length of 3–4 cm, usually within 4 weeks were excised and segmented into 1–1.5 cm size having a single node were subcultured on to MS medium supplemented with 5 μM 2-iP for further multiplication. The multiplication through this way was continued for the six subcultures without adversely affecting rate of multiplication and undesirable callus formation. Also in vitro-raised shoots (>3 cm) from 3rd subculture product onwards was used for rooting experiments.

To study the influence of carbohydrate sources on multiple shoot proliferation, nodal segments were cultured on MS medium supplemented with different carbohydrates viz. sucrose, glucose or fructose at different concentrations.

In vitro rooting In vitro rooting of shoots were conducted using excised shoots (>3 cm). The MS medium supplemented with auxins viz., IBA and NAA at varying concentrations (2.5, 5.0, 10.0 and 15.0 μM) along with 3 % sucrose and 0.7 % agar was tested. Cultures for rooting were kept at 16-h light conditions.

To identify suitable length of shoots for rooting, shoots were classified into five groups (< 1.0 cm, 1.1–

2.0 cm, 2.1–3.0 cm, 3.1–4.0 cm, 4.1–5.0 cm). These shoots were planted on agar gelled MS medium containing 10 μM IBA.

After root initiation (20 d), the plantlets were transferred to half strength MS liquid medium containing 2 % sucrose for root elongation.

Acclimatization Plantlets with well-developed shoot and roots thus obtained were deflasked. They were washed gently under running tap water and transplanted into plastic pots containing soil: vermiculite (1:1). Potted plants were incubated in a growth chamber at 25±1 °C and 16-h photoperiod with a light intensity of 50-μmolm^{-2} s^{-1}. Initially, for the first 2 weeks, plants were covered with micro holed transparent polyethylene bags to maintain humidity. They were watered on 5 days intervals with one fourth-strength MS salt solution. When the plants were completely weaned (4 weeks) they were moved to shade house. Three-month-old plants were planted in the field and their performance is being observed.

Experimental design and statistical analysis All experiments were conducted using a completely randomized block design. Every treatment composed of three replications and each replication block represented by ten 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). The shoot number, maximum shoot length and the percentage of explants producing shoots were recorded 4 weeks after inoculation. The mean number and length of roots developed from the regenerated shoots and the percentage of shoots producing roots were also determined after four-week incubation on rooting medium.

Results and discussion

In vitro seed germination Method adopted for fruit sterilization, excision of seeds from fruits and subsequent culture resulted in the establishment of cent percent aseptic cultures. In the previous works on tissue culture of annatto, sterilization of seeds reported as tedious and lengthy process including one-week agitation in gyratory shaker and warm water treatment to break the dormancy (Parimalan et al. 2007, 2008; 2009; Sharon and D'Souza 2000). The present sterilization and seed culture establishment is therefore rapid and efficient to provide axenic seedlings for further in vitro culture of annatto.

Effect of GA_3 on germination The effect of different concentrations of GA_3 on in vitro seed germination of *B. orellana* was examined (Table 1). The addition of GA_3 in MS basal medium significantly ($P<0.05$) enhanced in vitro germination. Highest germination (93.3 %) was observed in 3 μM GA_3 supplemented medium. In the control, least germination (13.3 %) was observed. The seeds inoculated on to 3 μM GA_3 supplemented medium showed emergence of radicle within a week and recorded least MTG (15.16). Concentration of GA_3, beyond 3 μM did not show any significant ($P<0.05$) difference (Table 1) in MTG. In all the concentrations of GA_3 tested, significant ($P<0.05$) decrease in the time taken for germination was noted. The seedlings become 4-leaf stage within a period of 20 d (Fig. 1a). In the previous reports on in vitro multiplication of annatto (Parimalan et al. 2009), the seed germination and seedling growth together took 2 months. Thus by using GA_3 in the germination medium the time can be considerably reduced. Addition of GA_3 (3 μM) in the germination medium did not cause any morphological abnormality to in vitro raised seedlings. In *B. orellana,* seeds were sensitive to exogenous application of GA_3 and found to enhance seed germination (Joseph et al. 2010).

In vitro shoot multiplication Nodal segments from 20 d-old in vitro seedlings were used for shoot multiplication. Enlargement of shoot buds at the nodal region was observed in 7 d-old cultures. Development of adventitious shoot buds was noticed in 20 d after culture from the lower cut end of nodal explants. Nodal segments cultured on various hormonal concentrations showed significant ($P<0.001$) response. Cultures growing in 2-iP (5 μM) supplemented medium showed emergence of axillary shoots (Fig. 1b) and development of adventitious shoot buds

Table 1 Effect of different concentrations of GA_3 supplemented MS medium on in vitro germination of *B. orellana* seeds

GA_3 Conc. (μM)	% of germination[a]	MTG[a]
0.0	13.33^{d}	29.13^{a}
1.5	56.66^{b}	21.33^{b}
3.0	93.33^{a}	15.16^{c}
4.5	53.33^{b}	15.36^{c}
6.0	26.66^{c}	15.60^{c}
Main Effect F Df (n-1) 4	85.80***	110.83**

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

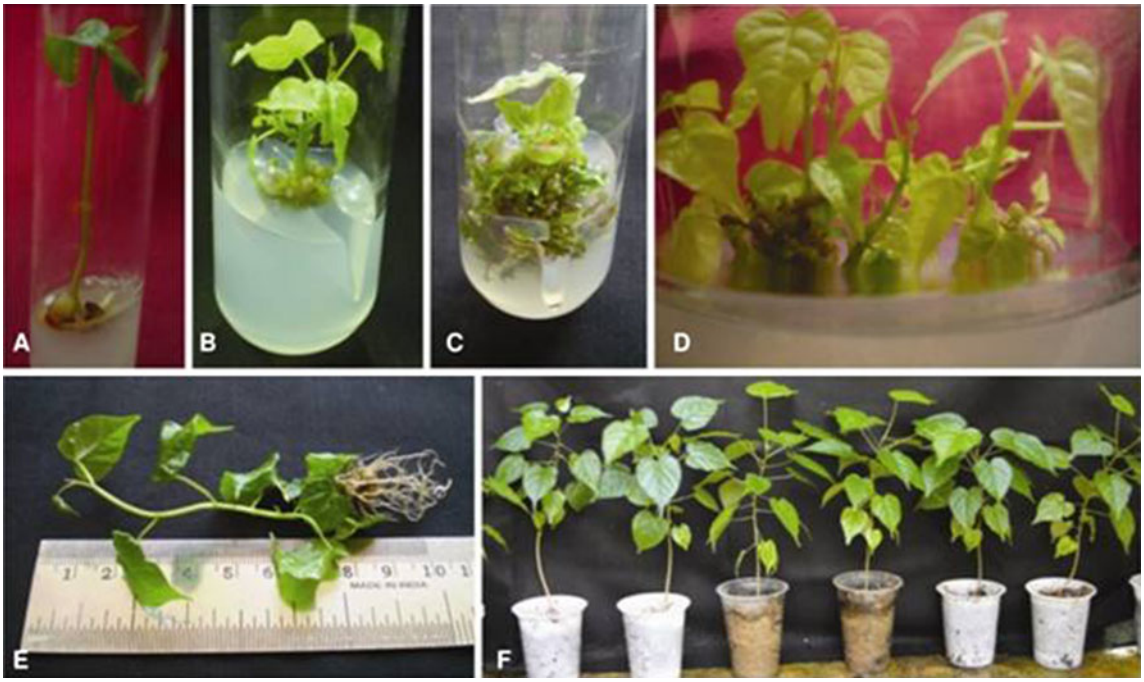

Fig. 1 In vitro propagation of *B. orellana* using in vitro raised seedling explant **a** In vitro seed germination on MS medium supplemented with 3 μM GA_3, **b** Axillary bud elongation and initiation of multiple shoots from nodal segments cultured on MS medium containing 5 μM 2-iP, **c** Adventitious shoot bud proliferation on MS medium with 5 μM 2-iP, **d** Shoot multiplication and subsequent elongation of shoots in MS + 5 μM 2-iP **e** 4-week-old well rooted in vitro raised plantlet before potting, **f** Acclimatized plants growing in green house (2-months after potting)

(Fig. 1c). Axillary shoot development could not be induced on growth regulator free medium. ANOVA revealed significant ($P<0.001$) interaction between cytokinin type and concentration in evoking both explants response and shoot bud development (Table 2). 2-iP (5 μM) supplemented MS medium thus standardized as the best medium for multiple shoot induction (35.7 shoots per explant) from nodal segments of seedlings with 93.3 % response. Shoots developed in this hormonal concentration sustained an active growth and therefore elongated in to 5.96 cm size on fourth week of culture (Fig. 1d). Subculturing of nodal segments excised from 5 μM 2-iP supplemented medium was continued for six multiplication cycles without any decline in the rate of multiplication and morphological abnormality of nascent shoots.

The type and concentration of carbohydrate significantly influenced shoot proliferation (Table 3). Explants cultured on medium containing 87.6 mM sucrose as carbohydrate produced the maximum response (93.3 %). The number of shoots (35.71) and shoot length (5.96) was also found to be the highest at this concentration of sucrose. Callusing was observed at higher concentrations (131.2 mM) of sucrose and lower concentrations of glucose (43.3 and 87.6 mM). No callus formation was observed in all the three concentrations of fructose. The lowest culture response (43.3 %) was observed in 43.3 mM fructose supplemented medium.

In the previous reports on *B. orellana* (Table 6), the number of shoots obtained was only 3–5 per explant (D'Souza and Sharon 2001) in B5 medium supplemented with very high concentration of triacontanol along with 2-iP. However, in the present study 2-iP was found to be the best cytokinin for nodal explants to produce 35.7 shoots. In contrast to present findings that superiority of 2-iP over BA, Ramamurthy et al. (1999) has reported effectiveness of BA in multiple shoot induction from nodal explants. Earlier reports on organogenesis in *B. orellana* (Table 6) indicate rate of multiplication limited to around 10 shoots per explant (Parimalan et al. 2008, 2009). In the previous report (Parimalan et al. 2008) both auxin, (phenyl acetic acid, 14.7 μM) and cytokinin (BA 31.1 μM) were used. Excessive concentrations of auxins and/or cytokinins in the shoot multiplication medium, however, can result in the development of morphologically variant plants (George 1996), callus development and subsequent shoot regeneration. Shoots regenerated through callus can often variant from its source material. The appearance of somaclonal variation (Larkin and Scowcroft 1981) in the in vitro regenerated plants can be greatly reduced by using lower levels of 2-iP (Debnath and McRae 2001; Marcotrigiano and McGlew 1991). In another report using nodal shoot tip explants (Parimalan et al. 2010), 12–13 elongated shoots were produced by the incorporation of putrescine in the medium, still then callus formation was noticed. In the present work use of auxin in shoot

Table 2 Effect of BA and 2-iP on shoot multiplication using nodal explants of *B. orellana*

Cytokinin	Conc. (μM)	Explant response[a] (%)	No. of shoots/explant[a]	Mean shoot length[a] (cm)
Control	0.0	0.00^{g}	0.00^{i}	0.00^{i}
2-iP	0.5	4.5^{f}	1.43^{hi}	1.50^{cde}
	1.0	43.2^{de}	4.58^{fg}	2.25^{bc}
	2.5	63.5^{bc}	20.11^{c}	5.83^{a}
	5.0	93.3^{a}	35.71^{a}	5.96^{a}
	10.0	73.5^{bc}	3.53^{gh}	1.47^{cde}
	15.0	33.3^{e}	0.94^{i}	1.33^{cde}
BA	0.5	1.2^{fg}	1.72^{hi}	1.05^{de}
	1.0	33.3^{e}	5.99^{ef}	2.65^{b}
	2.5	43.3^{de}	7.75^{e}	1.96^{bcd}
	5.0	56.7^{cd}	10.66^{d}	1.42^{cde}
	10.0	76.8^{b}	23.26^{b}	0.92^{e}
	15.0	63.5^{c}	12.66^{d}	0.70^{ef}
Treatment Df (n-1)	12	37.43***	220.60***	37.22***
Auxin type(T) Df (n-1)	1	5.20	2.69	80.391***
Auxin conc.(C) Df (n-1)	5	50.60***	206.374***	34.780***
T X C Df (n-1)	11	26.79***	204.923***	33.074***

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

multiplication medium was avoided. This omission reduces the cost as well as chance of somaclonal variation in the regenerated plants.

In vitro rooting Shoots were rooted after 10–15 d of culture on rooting medium. In vitro rooting was accompanied with elongation of shoots. Rooting response of micro shoots cultured in different auxin (NAA or IBA) supplemented medium varied significantly (Table 4). The maximum rooting response (95.5 %) and number of roots per shoot (20.23) was recorded in medium supplemented with 10 μM IBA. Choice of auxin and concentration had significant ($P<0.001$) effect on rooting. Increasing concentration of auxins caused development of callus at the basal portion of the micro shoots. The number of roots increased with increasing concentration from 2.5 to 10 μM and decreases thereafter. With respect to rooting percentage and number of roots, there was significant ($P<0.001$) interaction between auxin type and concentration. D'Souza and Sharon (2001) found that the average root length of annatto was affected

Table 3 Effect of different carbohydrate sources and its concentrations on multiple shoot induction using nodal explants of *B. orellana* cultured in 5 μM 2-iP supplemented MS medium

Carbon source	Conc. (mM)	% response	No. of shoots	Shoot length	Callus development
Glucose	43.3	73.5^{cd}	10.76^{e}	2.14^{d}	+
	87.6	67.7^{cde}	8.16^{f}	2.56^{c}	++
	131.2	60.0^{de}	4.30^{i}	3.50^{b}	−
Fructose	43.3	43.3^{f}	5.23^{hi}	2.66^{c}	−
	87.6	53.3^{ef}	6.20^{g}	1.63^{e}	−
	131.2	70.4^{cd}	12.30^{d}	1.10^{f}	−
Sucrose	43.3	76.8^{bc}	14.66^{c}	2.96^{c}	−
	87.6	93.3^{a}	35.71^{a}	5.96^{a}	−
	131.2	87.0^{b}	19.40^{b}	3.56^{b}	+
Treatment Df (n-1)	8	9.55***	537.59***	106.78***	
Carbon source (S) Df (n-1)	2	27.04***	1329.05***	230.34***	
Carbon conc.(C) Df (n-1)	2	2.24	187.11***	29.77***	
S X C. Df (n-1)	8	9.55***	537.59***	106.78***	

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

Table 4 Effect of auxins on rooting of *B. orellana* micro shoots in vitro

Auxin	Conc. (μM)	% of response	Root number	Root length(cm)
Control	0.0	0.0[f]	0.00[h]	0.00[e]
IBA	2.5	50.0[c]	11.15[d]	3.13[a]
	5.0	73.5[b]	16.86[b]	2.61[b]
	10.0	95.5[a]	20.23[a]	1.70[c]
	15.0	33.3[d]	12.83[c]	0.63[d]
NAA	2.5	53.4[c]	5.31[f]	3.06[a]
	5.0	60.1[bc]	6.92[e]	1.90[c]
	10.0	46.6[cd]	2.90[g]	1.78[c]
	15.0	16.4[e]	2.46[g]	0.77[d]
TreatmentDf(n-1)	8	59.10***	227.535***	106.015***
Auxin type (T)Df(n-1)	1	37.61**	991.74***	3.22
Auxin conc.(C)Df(n-1)	3	40.11 ***	40.81***	156.66***
T X C Df(n-1)	7	28.21 ***	179.57***	70.15***

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

by increased concentrations of NAA (0.05–4.03 μM) in the rooting medium.

Rooting response of different sized shoots varied significantly ($P<0.05$). Shoots length ranging 3.1–4.0 cm showed 95.5 % rooting. Lowest rooting response (4.5 %) was observed in shoots of <1.0 cm (Table 5). The efficient rooting of regenerated shoots and the survival of plantlets in the soil are the important steps in the successful micropropagation. In this study both IBA and NAA produced roots. Addition of IBA is found to be more effective to induce roots in *B. orellana.* The superior effect of IBA has been previously documented in other tree species (Siril and Dhar 1997; Tiwari et al. 2002). De Klerk et al. (1997) reported the strong inhibition of root growth with NAA (4 mM) in apple rootstock Jork 9, whereas IBA produced longer roots (11 mM) at the optimal concentrations. The superior effect of IBA on the root development in these plant species including *B. orellana* might be due to preferential uptake, transport and stability over NAA and subsequent gene activation (Ludwig-Muller 2000). Shoots planted in hormone free medium (control) failed to produce roots. Time taken for rooting in the present protocol is less compared to previous report (Parimalan et al. 2008) with the same type of growth hormone (Table 6). This attributes to the effect of explants type or due to the genotypic difference. Due to the lack of rooting in hormone free medium and dramatic increase of rooting due to exogenous auxin supplementation, annatto, in accordance to Marks and Simpson (2000) can be considered as a difficult-to-root category of woody plants.

Acclimatization Four-week-old rooted plantlets (Fig. 1e) were transferred to plastic pots containing soil: vermiculite mixture (1:1) were survived (83.3 %). Potted plants showed emergence of new leaves within 3-weeks of planting. In vitro raised plants showed uniform morphological features (Fig. 1f) and active growth in ambient conditions. Three-month-old, well-acclimatized plants were planted in the field.

The above study on in vitro multiplication demonstrates a possible step towards efficient in vitro propagation using MS medium containing 5 μM 2-iP for multiple shoot induction and 10 μM IBA for rooting. Direct shoot proliferation from nodal segments seems to be suitable for the rapid propagation of annatto. Shoot multiplication can be enhanced with repeated subculture under a mass propagation or production strategy. These results will be helpful to future research on in vitro propagation of adult trees. Using present method it is estimated that a single nodal segment can produce over 32,000 hardened plants within a 10-month culture period. This simple and rapid propagation protocol is reproducible and can be used as a

Table 5 Effect of shoot size on in vitro rooting response of *B. orellana* shoots

Shoot size (cm)	% of response	Root number	Root length
<1.0	4.5[d]	0.66[e]	3.66[a]
1.1–2.0	46.6[c]	10.53[d]	2.83[ab]
2.1–3.0	83.6[ab]	16.33[b]	1.76[ab]
3.1–4.0	95.5[a]	20.23[a]	1.70[ab]
4.1–5.0	63.5[bc]	5.80[c]	0.53[c]
Main Effect F Df (n-1) 4	34.66***	101.41***	2.07

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

Table 6 Summary of tissue culture work on *Bixa orellana*

Reference	Explant and mode of regeneration	Treatment/Medium	Result	Remarks
Ramamurthy et al. 1999	Node (Indirect)	B5 + BA 1.5 mgl^{-1} + IBA 1 mgl^{-1} for shoots and rooting in IBA 1 mgl^{-1}	4–6 shoots along with callus	Low frequency of multiplication, requires 5–6 months to get plantlets with 30–60% response
Nassar et al. 2001	Coty. node, node (Direct)	WPM + 1 mgl^{-1} lBA/Kin + 0.2 mgl^{-1} NAA + 100 mg Tyrosine + 40 mgl^{-1} AS for shoot and rooting on WPM/1/2 MS + 3 mgl^{-1} NAA	Few shoots	low frequency of multiplication
D'Souza and Sharon 2001	Shoot apex, node (Direct)	B5 + 2iP 4.9 μM + TRIA for shoot and rooting in 0.05 μM/2.7 μM NAA	9 shoots per shoot apex and 5 shoots per node explants	low frequency of multiplication, high conc. of TRIA
Khan et al. 2002	Seed (Indirect)	MS + 10 μM BA + 5 μM NAA for shoot and 1/2MS + 5 μM IBA for rooting	6–7shoots per callus	Indirect regeneration, low frequency of multiplication
Neto et al. 2003	Hypocotyl (HY), rooted HY (Direct)	MS + 4.56 μM Zeatin for shoot and rooting in 5.0 μM IBA	3–4 shoots	Mechanical scarification of seeds may cause contamination and damage to embryo
Parimalan et al. 2007	HY, rooted HY, coty. leaf, nodal shoot tips (Direct)	MS + TDZ + CW/BA + NAA for shoots. and IBA 3 mgl^{-1} for rooting	rooted hypocotyls give 22 shoots per explant	Seed dormancy breaking and sterilization tedious, chances for contamination of seeds, shoot length is only 10–16 mm
Parimalan et al. 2008	Nodal shoot tip explants (Direct)	MS + 31.1 μM BA + 14.7 μM PAA for shoot and 4.9 μM IBA for rooting	6–13 primary shoots	Seed dormancy breaking and sterilization tedious process, shoot length is less than 1.5 cm, callusing, high conc. of hormones
Parimalan et al. 2009	Nodal shoot tip (Direct)	MS + 8.87 μM BA + 0.05 μM IAA + 11.2 μM TRIA for shoot and rooting in 4.9 μM IBA	6–18 primary shoots	Seed dormancy breaking and sterilization tedious process, shoot ength is less than 1.5 cm, callusing
Parimalan et al. 2010	Nodal shoot tip (Direct)	MS + 6.6 μM BA + 4.9 IBA + Putrescine 800 μM for shoot and rooting in 4.9 μM IBA	12–13 shoots with maximum shoot length of 7.3 cm	Seed dormancy breaking and sterilization tedious process, callusing,
Present study	Node (Direct)	MS + 5 μM 2-iP for shoot and rooting in 10 μM IBA	35.71 shoots per explant	Sterilization protocol simple, avoids contamination chances and seed germination percentage significantly high with the use of GA_3 in germination medium. High frequency regeneration without auxin in multiplication medium, avoid callusing, chances for variation and reduce cost of production. Time taken for rooting is less.

**HY* hypocotyl, *AS* Adenine sulphate, *CW* Coconut water

supporting system in the improvement program of *B. orellana*.

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