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Multiplication of Annatto (*Bixa orellana* L.) using cotyledons and leaf explants

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Annatto (*Bixa orellana* L.) is an important natural dye yielding plant. To improve the regeneration capacity of annatto, the effects of explants size, plant growth regulators, light regime and leaf position were evaluated using cotyledons and leaf explants derived from seedlings. Explants having entire lamina with petiole showed maximum response. The cotyledonary explants produced highest regeneration frequency (78.1%) and maximum number of shoots (12.4) on Murashige and Skoog medium supplemented with 20 µM benzyl adenine (BA). Leaf explants on MS medium containing 20 µM BA gave 67% regeneration frequency and 11.3 new shoots per explant. A photoperiod of 16/8 h (light/dark) was found to be superior to continuous dark or light. Position of leaf on the seedling had significant role in organogenic response and is found decreasing from the base to shoot apex. The regenerated shoots were rooted on MS medium supplemented with 10 µM IBA. The rooted plantlets were transferred initially in the growth chamber and then moved to a green house. The present protocol can be utilized for the mass multiplication and also for effective genetic transformation aimed at the improvement of annatto.

Key words: *Bixa orellana*, cotyledon, leaf explants, plant regeneration

Restrictions and concern over the use of synthetic colourants have led to an increase in the demand for natural colours. Annatto is a carotenoid dye derived from the pulpy seed coat. It is synthesized by a single terrestrial plant *Bixa orellana* L. (family Bixaceae). It is ranked as the world's second most important natural colourant yielding yellow to red colour (Satyanarayana *et al*., 2003). Annatto is widely used to colour cheese, butter and other dairy products. It has a special interest among food processors and cosmetic companies because the natural colour is safe for consumption and skin applications. The annatto dye is Generally Recommended as Safe (GRAS) for human consumption in food products. 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 colour in European Union. In India, it is accepted under Prevention of Food Adulteration Act (PFA Act 1954), which is aimed at adulteration and misbranding of foods. Annatto dye is used in the treatment of diabetics, as haemostatic, anti-dysenteric, diuretic, laxative, febrifuge, measles, gonorrhea, digestive agent, diarrhea, kidney and skin diseases (Srivastava *et al*., 1999). Due to use in the food and cosmetic industries, there is an ever increasing demand for annatto. Efficient *in vitro* regeneration protocols will be helpful for the multiplication of elite *B. orellana* plants and

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also in the genetic transformation experiments.

The availability of an *in vitro* regeneration system is a prerequisite for effective genetic transformation. Adventitious shoot regeneration is most preferred if *Agrobacterium*-mediated gene transfer is to be achieved and leaf explants are best suited for both adventitious shoot formation and *Agrobacterium*-mediated gene transfer experiments (Kantia and Kothari, 2002). Previous reports on micropropagation of *B. orellana* are 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; Joseph *et al.*, 2011; Parimalan *et al.*, 2010) are available. Based on leaf explants two reports are available (Almeida, 1996; Parimalan *et al.*, 2007) with a reduced rate of multiplication. This underline the need for developing an efficient regeneration protocol using leaf derived explants. The present study was conducted with an objective to standardize a reproducible protocol which can be used at a commercial scale for the clonal multiplication and also to facilitate genetic transformation of *B. orellana,* using cotyledons and leaf explants derived from *in vitro* germinated seeds.

Materials and Methods

Mature, fruits prior to dehiscence collected from red variant growing in the Botanic garden of University of Kerala, Kariavattom was used as seed source. The collected fruits were sterilized and subjected *in vitro* seed germination as reported earlier (Joseph *et al.*, 2011). Cotyledons and leaves from *in vitro* raised seedlings were used as explants for *in vitro* multiplication.

Murashige and Skoog (1962) medium containing 3% (w/v) sucrose and 0.7% agar (Sisco Research Laboratories, Mumbai, India) was used for the *in vitro* seed germination. In the regeneration medium plant growth regulators (Sigma–Aldrich, Bangalore, India) at different concentrations were incorporated into the basal media. The pH of the medium was adjusted to 5.8 before autoclaving at 121^{o}C and 103 kPa for 15 min. Fully expanded leaf and cotyledons of about 1-2.5 cm length were excised from *in vitro* developed seedlings and cultured in 25 x 150 mm culture tube containing 15 ml of sterilized semi solid medium for initiation of cultures. Cotyledonary leaves and leaves (1.5-2.0 cm in length) with petiole were selected for the study. The petiole of leaves were placed into the medium and the lamina of leaves are placed horizontally on the surface of medium in such a way that the abaxial side of lamina are in contact with the medium. Cultures were incubated at 25±2^{o}C, irradiance 50 $\mu molm^{-2}s^{-2}$ provided by cool fluorescent tubes (40 W; Philips, India) and were exposed to a photoperiod regime of 16 h and 55±5% of relative humidity (RH).

To study the role of lamina and petiolar region of the leaf explant, different portions of the lamina were removed so as to have explants with full, one half, one quarter or no lamina with the petiolar cut end were inoculated into agar gelled MS medium supplemented with 3% sucrose and 20 µM BA.

Cotyledons and leaf explants were cultured on MS medium supplemented with isopentanyl adenine (2-iP), benzyl adenine (BA) or kinetin at varying concentrations (2.5, 5, 10, 20 or 40 µM) on the regeneration of multiple shoots was studied.

To investigate the effect of light regime on shoot regeneration, cotyledons and leaf explants were cultured on MS medium supplemented with 20 µM BA. Various photoperiodic conditions used in the study are; 16/8 h (light/dark), 0/24 h or 24/0 h.

To investigate the effect of leaf position on multiple shoot induction, cotyledons and four leaves I, II, III and IV

leaves counting from the next leaf of cotyledons were inoculated on MS medium supplemented with 20 µM BA. Leaves including portions of petiole but without any axillary buds were excised from *in vitro* raised seedlings. Cotyledons and four leaves were collected from 2-month-old seedlings raised *in vitro* was used for the experiment.

Microshoots obtained from fourth subculture onwards were used for *in vitro* rooting. Experiments on *in vitro* rooting of shoots were conducted with shoots measuring >2.5 cm in length. The MS medium supplemented with IBA at 10 µM along with 3% sucrose and 0.7% agar was used for *in vitro* rooting (Joseph *et al.,* 2011). Cultures for rooting were kept at 16 h photoperiod.

Plantlets with well-developed shoot and roots 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^{o}C and under 16 h photoperiod with a light intensity of 50 µmolm^{-2}s^{-1}. Initially, for the first two weeks, plants were covered with micro holed transparent polyethylene bags to maintain humidity. They were watered on five days intervals with one fourth-strength MS salt solution. When the plants were completely weaned (4 weeks) they were moved to a green house.

All experiments were conducted using a completely randomized block design. Each treatment composed of three replications and each replication block was represented by twelve culture tubes. Data on various parameters such as percentage of explants producing shoots, shoot number, maximum shoot length were recorded six weeks after inoculation and was subjected to 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).

Results and Discussion

Leaf explants with intact lamina showed maximum regeneration response (66.8%) and the response decreases with the reduction in the lamina tissue (Table 1). Petiolar explants devoid of lamina failed to produce organogenic response, instead callus response was noticed. The number of shoots significantly decreases from 11.23 per explant in leaf explants with full lamina to zero in explants without lamina or lamina alone (without petiole). Formation of adventitious shoot buds directly from the petiole of the explants was noticed. At the initial stage of shoot formation, no callus formation was observed (Fig. 1a). However, at a later stage callus formation occurred mainly from the laminar portion of *in vitro* cultured leaf segment. These results suggest the promotive effect of lamina through the establishment of a gradient of diffusible factors, including endogenous phyto-hormones. Similar results were reported in plants like *Paulownia fortune* (Kumar *et al.,* 1998).

After two weeks of inoculation of cotyledons and leaf explants, enlargement and swelling of petiolar cut end occurred. Initiation of shoot buds from the swollen tissues and also the adjoining portions of petiole occur after 4 weeks of culture. A transverse section through the basal swollen portion of the petiole showed initiation of meristematic zones characterized by darkly stained nuclei (Fig. 1f). Explants cultured on growth regulator free medium failed to respond *in vitro*.

Cotyledons cultured on MS medium fortified with BA (20 µM) resulted in 78.1% response and production of 12.4 new shoots (Table 2, Fig. 1b). Leaf explants cultured on MS medium containing BA (20 µM) showed

significantly (P<0.05) high response (67%) and maximum (11.3) number of shoots (Fig. 1c). Addition of BA in the medium resulted in the development of multiple shoots directly from the petiolar region. Callus development from the laminar portion was noticed at a later stage (Fig. 1e). In 2-iP supplemented medium, shoot buds were originated from the leaf margins and from the midrib of adaxial side (Fig. 1d). Low frequency shoot development was noticed in leaf explants cultured in kinetin (20 or 40 µM) supplemented medium.

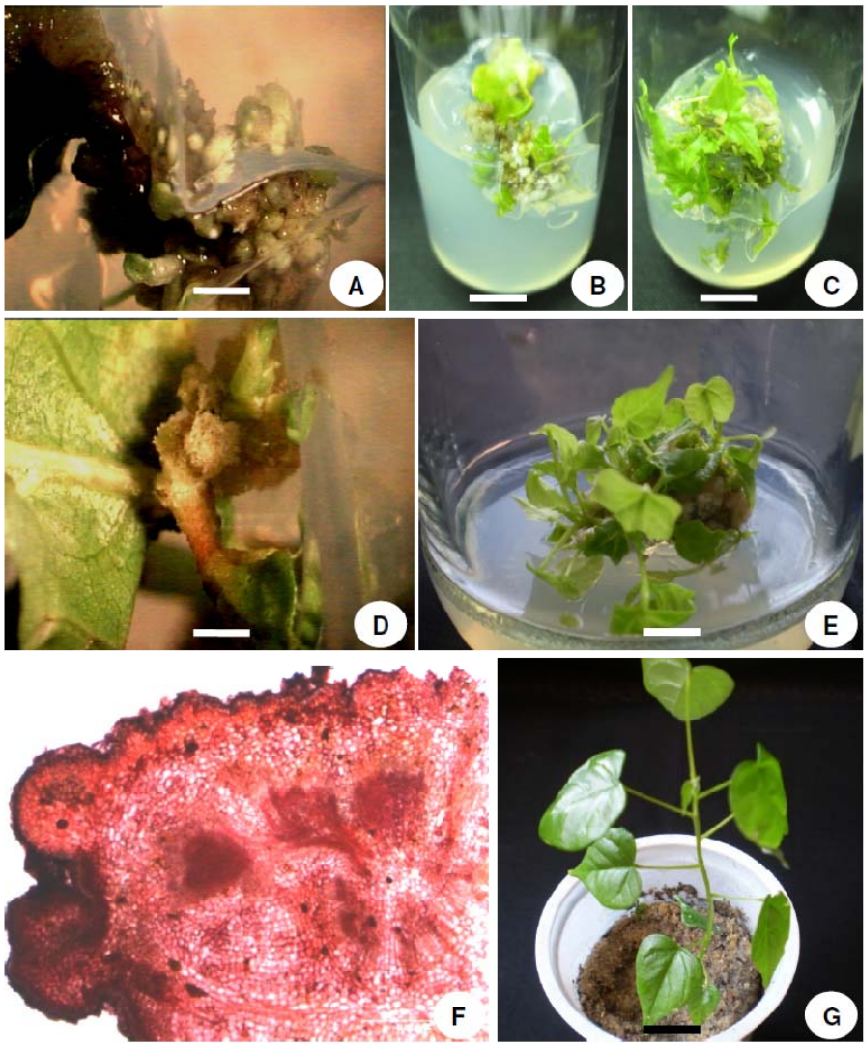

Fig. 1. Adventitious shoot development from Cotyledons and leaf explants of *Bixa orellana*. A. Shoot bud initiation from petiolar region of cotyledonary leaf explants in MS medium supplemented with 20 µM BA supplemented medium. B. Multiple shoot induction of cotyledon after 4 weeks of culture MS+BA 20 µM (bar = 2.4 cm). C. Adventitious shoot formation of leaf explants in MS medium supplemented with 20 µM BA (bar = 2.3 cm. D. Shoot bud initiation from the lower portion of midrib in 2-iP supplemented medium. E. Shoot elongation and callus formation in MS medium supplemented with MS+BA 20 µM after 5 weeks (bar = 1.2 cm). F. Transverse section of petiolar cut end showing the initiation of meristematic zones in MS+BA 20 µM. G. Rooted plant transferred to plastic cups.

Table 1: Effect of the size of lamina and presence of petiole on adventitious shoot induction in leaf explants of *B. orellana* in MS medium supplemented with 20 µM BA.

Explant	% response	No. of shoots	Callus formation
Full lamina	66.8±2.95 a	11.23±0.12 a	-
One half lamina	36.0±1.64 b	3.47±0.20 b	+
one quarter lamina	10.8±4.21 c	0.83±0.44 c	++
Without lamia	0.0±0.00 d	0.0±0.00 c	+++
Lamina alone	0.0±0.00 d	0.0±0.00 c	+++
Main Effect F Df (n-1) 4	163.776***	453.216***	

[a]Means with in a column followed by same letters are not significantly different as determined by DNMRT test (P<0.05). + indicates the intensity of callus formation. ***significant at P<0.001 level.

Table 2: Effect of different cytokinins on adventitious shoot induction from cotyledons and leaf explants of *B. orellana.*

PGR	Conc. µM	% of shoot regeneration		Mean no. of shoots per explant	
		Cotyledon	Leaf	Cotyledon	Leaf
2-iP	2.50	0.0±0.00 f	0.0±0.00 g	0.0±0.00 i	0.0±0.00 h
	5.00	19.4±2.13 d	24.8±3.24 cd	0.7±0.15 h	1.7±0.14 d
	10.00	55.6±1.60 b	38.8±1.66 b	1.8±0.11 f	1.2±0.29 de
	20.00	16.2±3.95 de	8.3±0.00 ef	1.0±0.28 gh	0.6±0.33 fg
	40.00	0.0±0.00 f	0.0±0.00 g	0.0±0.00 i	0.0±0.00 h
BA	2.50	0.0±0.00 f	0.0±0.00 g	0.0±0.00 i	0.0±0.00 h
	5.00	36.0±1.66 c	16.2±3.95 de	2.3±0.14 e	1.4±0.23 de
	10.00	61.1±1.63 b	30.6±1.60 bc	4.5±0.14 c	2.9±0.20 c
	20.00	78.1±1.83 a	67.0±3.03 a	12.4±0.26 a	11.3±0.20 a
	40.00	38.8±1.66 c	24.8±3.24 cd	5.9±0.17 b	4.1±0.23 b
Kin	2.50	10.8±2.43 e	0.0±0.00 g	0.1±0.05 i	0.0±0.00 h
	5.00	22.1±1.98 d	3.8±5.58 f	0.9±0.10 gh	0.3±0.15 gh
	10.00	38.8±1.64 c	16.0±3.83 de	1.2±0.11 g	0.7±0.06 fg
	20.00	55.6±1.59 b	30.5±1.74 bc	2.6±0.20 e	1.7±0.11 d
	40.00	52.8±1.59 b	27.7±1.74 bc	3.5±0.15 d	1.1±0.15 ef
Control	0.0	0.0±0.00 f	0.0±0.00 g	0.0±0.00 h	0.0±0.00 h
Main Effect F Df (n-1)	15	131.048***	45.233***	465.471***	269.794***

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

Table 3: Effect of different photoperiodic conditions on adventitious shoot formation on MS medium supplemented with 20 μM BA.

Light/ Dark	% of shoot regeneration		Mean no. of shoots per explants	
	Cot leaf	Leaf	Cot. Leaf	Leaf
16/8	75.5±0.00 a	69.6±1.86 a	12.13±0.18 a	11.16±0.12 a
0/24	13.5±2.43 c	10.8±2.43 c	1.2±0.11 c	0.83±0.16 b
24/0	30.6±1.60 b	33.3±2.82 b	2.76±0.14 b	1.4±0.18 b
Main Effect F Df (n-1) 2	139.013***	60.748***	1522.210***	1316.580***

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

Table 4: Response of leaves excised from *in vitro* seedling cultured on MS medium containing 20 μM BA.

Leaf position	% of response	Mean no of shoots per explants	Mean length of shoots (cm)
Cotyledon	72.3±1.76 a	12.13±0.14 a	1.60±0.11 a
I	67.0±3.03 a	11.46±0.12 b	1.20±0.11 b
II	38.8±1.66 b	3.56±0.20 c	0.76±0.08 c
III	19.4±2.13 c	1.63±0.12 d	0.53±0.06 c
IV	0.0±0.00 d	0.00±0.00 e	0.00±0.00 d
Main Effect F Df (n-1) 4	143.225***	1758.884***	48.543***

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

In *B. orellana*, previous reports on (Almeida, 1996, Parimalan *et al.,* 2007) on direct organogenesis from cotyledons and leaf explants, shoot production was less efficient (3.2 shoots/explants) or non responsive (Neto *et al.,* 2003). The result of the present investigation shows that the type and concentration of cytokinin is an important factor in the shoot induction in *B. orellana*.

Both cotyledon and leaf explants exposed to different photoperiodic conditions showed varying response. The 16 h photoperiod significantly increased the shoot regeneration frequency, compared to the 24 h light or dark treatments (Table 3). Cultures incubated at continuous dark resulted in the development of callus. The number of shoots per explants was also found to be less in continuous light. The shoot length was found to be high in dark and the shoots showed symptoms of etiolation. In continuous light no initial callus formation was observed. Even though normal shoots were obtained in continuous light the number of shoots was found to be less.

Amoo *et al.* (2011) reported an enhanced production of adventitious shoots in cultures maintained under a 16 h photoperiod than those under continuous light in *Barleria greenii*. Possibly the dark periods (following the 16 h photoperiod)

might promote the synthesis or accumulation of hormones and / or metabolites which stimulate growth during the ensuing 16 h light periods (Amoo *et al.,* 2011; Economou and Read, 1986). Light plays significant role in plant growth either by its direct effect on photosynthesis or by influencing the function of growth regulators (Neumann *et al.,* 2009). Furthermore, a high accumulation of CO_2 in culture vessels has been reported during the dark period of a light/dark cycle (Fujiwara *et al.,* 1987, Serret *et al.,* 1997). Availability of high CO_2 could result in an increased rate of photosynthesis and reduced photo-respiration, at least during the early phase of the subsequent light period in cultures maintained under a 16 h photoperiod. The increased rate of photosynthesis coupled with reduced photorespiration will result in improved growth rate in cultures maintained in 16 h light. The 16/8 h photoperiod reduces the energy consumption and thus can significantly reduce the cost of *in vitro* propagation.

The reduced number of shoots and growth of shoots in continuous light may be due to photo-inhibition caused by over exposure to light (Daniel, 1997) or by a limitation of CO_2 supply under high light intensity (Williams *et al.,* 1992). It may also be due to the photo-oxidation of endogenous IAA, due to over exposure to light (Economou and Read, 1986). The present observations on *B. orellana* are in agreement with *Dierama erectum* (Koetle *et al.,* 2010) showing that the control of photoperiod can enhance shoot production.

In vitro response of leaf explants derived from different position on source plant and cotyledon showed differential response. Cotyledon and I leaf exhibited significantly high (P<0.05) response compared to leaf explants derived from tip portion (Table 4). Cotyledon explants produced response (72.3%) and new shoot buds (12.13). The percentage response and the number of shoots per explants found decreasing in accordance to increasing order of leaf position from the base to the apex (Table 4). The leaf most proximal to apical dome failed to respond. Similar results were reported in *Solanum laciniatum*, where callus growth and shoot development was more rapid on explants cut from mature leaves which had just completed full expansion (George, 2008). The oldest leaves ontogenetically had the greatest capacity for adventitious shoot initiation and can be correlated with the gradient of endogenous phytohormones that exist in the seedling (Vasil and Vasil, 1999). The leaves near the apex will have highest endogenous auxin levels and those near the base the least. The oldest leaves from the seedlings were more responsive to BA for shoot induction, due to the reduced auxin level that counteracts the shoot initiating effect of cytokinin. Similar results were observed in plants like *Mentha spicata* (Li *et al.,* 1999). The variation in the organogenic response depending on leaf position may also attribute to physiological gradients along the stem which may determine the interaction site of growth regulators, carbohydrates or metabolites and the specific nodal position at which leaves are developed (Brown and Thorpe, 1986).

In vitro developed shoots measuring >2.5 cm in length were rooted in MS medium supplemented with IBA at 10 µM along with 3% sucrose and 0.7% agar. Four-week-old rooted plantlets (Fig.1g) were transferred to plastic pots containing soil: vermiculite mixture (1:1) was survived (80.7%). Potted plants showed emergence of new leaves within 3-weeks of planting. Well-acclimatized plants were transferred to field.

An efficient protocol for *in vitro* propagation of *B. orellana* using cotyledon and leaf explants were developed. The developed procedure is simple, efficient and reproducible. In the present study micro-propagation technique of annatto was further

optimized by the manipulation of size of explants, light regime and position of explants on the seedling explants. Cent percent of shoot buds developed in MS medium supplemented with 20 µM BA were elongated to shoots and thus the system can be utilized for the effective genetic transformation aimed at the improvement of annatto by increasing pigment production.

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