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BRIEF COMMUNICATION

Micropropagation of *Bixa orellana* using phytohormones and triacontanol

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Abstract

An efficient micropropagation protocol for annatto (*Bixa orellana* L.) was achieved using nodal shoot tip explants. Shoot buds were obtained on the Murashige and Skoog (MS) medium supplemented with various concentrations and combinations of indole-3-acetic acid (IAA), N^6-benzyladenine (BA) and triacontanol (TRIA). Maximum of 213 shoot buds along with 18 primary shoots were produced on MS medium containing 0.05 μM IAA, 8.87 μM BA, and 11.2 μM TRIA. The primary shoots elongated best on MS medium containing 6.66 μM BA and 2.45 μM indole-3-butyric acid (IBA). The regenerated shoots rooted best on MS medium supplemented with 4.9 μM IBA. The *in vitro* rooted plantlets were hardened and establishment rate under field conditions was 70 to 80 %.

Additional key words: annatto, callus, *ex vitro* transfer, multiple shoots, organogenesis.

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Bixa orellana L. (*Bixaceae*) an evergreen shrub or tree native in Brazil, has been widely cultivated in many tropical countries for the orange-red edible dye bixin produced over the aril portion of the seeds (Jondiko and Pattenden 1989). Though *in vitro* shoot multiplication from cotyledon and nodal explants was reported earlier (Ramamurthy *et al*. 1999, D'Souza and Sharaon 2001, De Paiva Neto *et al*. 2003), the number of shoots formed was only 3 to 5 per explant.

Triacontanol [$CH_3(CH_2)_{28}CH_2OH$] - a naturally occurring plant growth promoter (Ries *et al*. 1977) - is a component of the epicuticular waxes of *Medicago sativa* (Chibnall *et al*. 1933). Effect of triacontanol on micropropagation (Reddy *et al*. 2002), somatic embryogenesis (Giridhar *et al*. 2004) and production of the secondary metabolites (Giridhar *et al*. 2005) was described. The present study utilizes triacontanol to produce a commercially viable micropropagation protocol from the nodal shoot tip explants and further evaluates bixin yield after field establishment.

Red capsules of *Bixa orellana* L. were harvested during October 2002 from Ramakrishna Ashram, Mysore, India. Seeds were collected, initially washed with 70 % ethanol, then treated with 0.5 % *Bavistin* solution for 2 h on a gyratory shaker at 90 rpm, washed thoroughly with running tap water and transferred to a flask containing warm water (65 °C) and kept at ambient temperature for a week to break the dormancy. Imbibed seeds were treated with 1 % NaOCl (v/v), for 15 min, rinsed with sterile distilled water, treated with 0.1 % $HgCl_2$ for 5 min and rinsed with sterile distilled water. Hence surface sterilized seeds were inoculated on to the Murashige and Skoog (1962; MS) medium supplemented with 3 % sucrose and 555 μM of myo-inositol, 0.7 % agar (*Hi-media*, Mumbai, India) or 0.35 % *Phytagel* (*Sigma-Aldrich*, St. Louis, USA) (pH was adjusted to 5.7 ± 0.1). Initially, cultures were maintained in dark for two weeks (till radicle emergence) and then were transferred to 16-h photoperiod with irradiance of 45 μmol m^{-2} s^{-1} and temperature of 25 ± 2 °C. From the one-month-old seedlings, shoot tip, nodal and nodal shoot tip explants were dissected and used for further experiment.

The explants were inoculated on to the MS medium supplemented with either of the two cytokinins benzyladenine (BA; 4.44 - 17.74 μM) or 2-isopentenyladenine

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Abbreviations: 2iP - 2-isopentenyl adenine; BA - N^6-benzyladenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog; NAA - naphthalene acetic acid; TRIA - triacontanol.

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(2iP; 4.92 - 14.76 µM) along with an auxin indole-3-acetic acid (IAA 0.05 - 5.7 µM) and triacontanol (TRIA; 5.56 - 45.6 µM). All hormones were obtained from *Sigma*.

The primary shoots (1 - 2 cm long) and callusing stump containing shoot bud primordia (25 - 30 shoot buds) that are obtained from the shoot induction medium have been inoculated on to MS medium supplemented with BA (4.44 - 8.87 µM) or 2iP (4.92 - 14.76 µM) along with IBA (2.45 - 9.8 µM) for shoot elongation. Elongated shoots of 4.0 - 5.0 cm length were inoculated on to MS medium supplemented with IAA (2.75 - 11.4 µM), IBA (2.4 - 9.8 µM), α-naphthaleneacetic acid (NAA; 2.75 - 10.8µM) or TRIA (5.56 - 45.6 µM) for rooting. *In vitro* rooted plantlets were removed from the medium, washed in running tap water and planted in sand:compost mixture (1:2) and are maintained in greenhouse for a month and then transplanted to field. Various morphological parameters, *e.g.*, fruit length, fruit width, fruit mass, number of seeds per fruit, days to maturity and pigment content were evaluated.

For anatomical studies, callus obtained from explants at the time of differentiation were fixed in 35 % paraformaldehyde : glacial acetic acid : 70 % ethanol at the ratio of 1:1:9 (v/v/v) for 3 h, dehydrated in an ethanolic graded series after which, free hand sections were made, stained with safranin and observed under microscope.

In vitro study followed a randomized block design with ten replicates and the observations were recorded for each replicate. One-way *ANOVA* was applied to test their significance and the means were separated using *t*-test at $P < 0.05$.

Though shoot tip explants, nodal explants and single node shoot tip explants were used, only nodal shoot tip explants responded. Nodal shoot tip explants produced a maximum of 213.6 ± 12.5 shoot buds and 18 ± 1.4 primary shoots (Table 1, Fig. 1*A,B*) when inoculated on to the MS medium containing 11.2 µM TRIA, 8.87 µM BA, and 0.05 µM IAA with 80 % response. Irrespective of explants used, shoot differentiation did not occur, when cytokinins concentration was below 4.4 µM BA or 4.92 µM 2iP. Callusing from the base of the nodal shoot tip explants could be seen in a week after inoculation and bud formation from this callus started after two weeks. After further two weeks, shoots were 1 - 2 cm long. Histological studies revealed the initiation of calli from the base of explants and further commencement of differentiation to shoot buds was visible by the appearance of distinct cells with dense cytoplasm and prominent nuclei (Fig. 1*C*). Scanning electron microscopy revealed that the meristemoids differentiated to for shoot buds (Fig. 1*D*) which in turn elongated to produce shoots later.

Elongated shoots (~ 4.0 cm) when inoculated on to MS medium supplemented with 4.9 µM IBA gave a maximum root length of 4.8 cm with an average of 4.11 roots per plantlet (Table 3, Fig. 1*F*). 70 - 80 % of the plantlets survived upon hardening in greenhouse

Table 1. Influence of growth regulators on shoot multiplication in *B orellana* L. Means ± SE, $n = 10$; ** - $P < 0.01$ ([a] -callusing from the base of explant is common in all hormonal combinations except MS basal medium; [b] - 22.4 and 45.6 µM of TRIA produced deformed leaf like structures).

BA [µM]	2iP [µM]	IAA [µM]	TRIA [µM]	Response [%]	Number of shoot buds [explant^{-1}][a]	Number of shoots
0	0	0	0			
4.44	0	0	0	-	-	1.2 ± 0.2
8.87	0	0	0	40	-	1.7 ± 0.25
17.74	0	0	0	30	-	1.3 ± 0.4
0	4.92	0	0	-	-	1.1 ± 0.4
0	9.84	0	0	10	-	1.2 ± 0.51
0	14.76	0	0	25	-	1.2 ± 0.48
0	0	0	5.56	-	-	-
0	0	0	11.2	30	8.9 ± 0.16*	2.1 ± 0.38
0	0	0	22.4[b]	-	-	-
0	0	0	45.6[b]	-	-	-
8.87	0	0.05	0	60	8.5 ± 1.2	2.5 ± 0.6
8.87	0	0.57	0	40	8.2 ± 1.5	2.3 ± 0.5
8.87	0	5.70	0	30	8.4 ± 0.9	2.5 ± 0.9
8.87	0	0.05	5.56	50	65.5 ± 4.8**	8.2 ± 1.1**
8.87	0	0.05	11.2	80	213.6 ± 12.5**	18.0 ± 1.4**
8.87	0	0.05	22.4	70	120.5 ± 10.4**	10.0 ± 0.8*
8.87	0	0.05	45.6	50	37.6 ± 4.5*	3.2 ± 0.2*

Shoot buds and primary shoots that are produced from nodal shoot tip explants when inoculated on to the MS medium containing 6.66 µM BA and 4.9 µM IBA for elongation; a maximum of 18 ± 1.5 shoots with a shoot length of 4.5 cm were produced (Table. 2; Fig. 1*E*). Using 2iP in place of BA did not give much response and produced only one or two shoots.

Table 2. Development of shoots from *in vitro* shoot buds of *Bixa orellana* under the influence of BA and IBA combination. The callus stump consists of approximately 25 - 30 shoot buds. Means ± SE, $n = 10$; ** - $P < 0.01$.

2iP [µM]	BA [µM]	IBA [µM]	Response [%]	Shoot number [callus^{-1}]	Shoot length [mm]
4.92	0	0	40	1.8 ± 0.40	8 ± 1.50
9.84	0	0	40	2.3 ± 0.12	9 ± 1.20
14.76	0	0	60	2.0 ± 0.18	8 ± 1.00
9.84	0	2.45	50	2.4 ± 0.25	10 ± 2.00
9.84	0	4.9	40	2.6 ± 0.14	10 ± 0.90
9.84	0	9.8	40	2.4 ± 0.60	7 ± 0.20
0	4.44	0	40	3.0 ± 0.24	12 ± 0.50
0	6.66	0	40	4.2 ± 0.50	16 ± 0.90
0	8.87	0	40	3.5 ± 0.70	14 ± 0.60
0	6.66	2.45	60	14.0 ± 1.20**	32 ± 1.60**
0	6.66	4.9	80	18.0 ± 1.50**	45 ± 2.20**
0	6.66	9.8	50	11.0 ± 0.90**	26 ± 2.00**

Fig. 1. *A* - Callus mediated shoot buds formation from the base of nodal shoot tip explant on medium containing 8.87 μM BA, 0.05 μM IAA and 11.2 μM TRIA (*bar* = 4 cm). *B* - Magnified dorsal view of this shoot bud. *C* - Histology of shoot regeneration from callus (*bar* = 200 μm). *D* - Electron microscopic studies showing the initiation of the shoot buds (*bar* = 300 μm). *E* - Shoot bud elongation into plantlets on the medium containing 6.66 μM BA and 4.9 μM IBA (*bar* = 4 cm). *F* - *In vitro* rooting of shoots on medium containing 4.9 μM IBA (*bar* = 4 cm). *G* - Potted plant after five months (*bar* = 10 cm). *H* - Established tree at fruiting stage after 24 months (*bar* = 15 cm; inset - mature fruit bunch).

Table. 3. *In vitro* rooting of *B. corellana* micro-shoots. Means ± SE, $n = 10$; * - $P < 0.05$, ** - $P < 0.01$. IAA at concentrations 8.45 and 11.4 μM or TRIA 5.56 - 45.6 μM did not induce rooting.

IAA [μM]	IBA [μM]	NAA [μM]	Response [%]	Root number [shoot^{-1}]	Root length [cm]
2.75	-	-	40	1.0 ± 0.75	0.8 ± 0.11
5.70	-	-	50	1.0 ± 0.47	1.2 ± 0.12
-	2.45	-	30	6.0 ± 1.41**	3.2 ± 0.17*
-	4.90	-	90	4.1 ± 0.75 *	4.8 ± 0.52**
-	7.35	-	70	3.5 ± 0.65	3.6 ± 0.26 *
-	9.80	-	50	1.4 ± 0.51	2.0 ± 0.14
-	-	2.75	50	3.2 ± 0.42	2.2 ± 0.17
-	-	5.40	70	3.3 ± 0.46	2.4 ± 0.20
-	-	8.15	20	2.0 ± 0.16	2.0 ± 0.32
-	-	10.80	20	1.5 ± 0.57	1.6 ± 0.19

(Fig. 1*G*) and they were transplanted to field (Fig. 1*H*). The height of 24-month-old plants transplanted from the greenhouse was 200 - 250 cm and they have 14 - 15 branches. They were very similar to seedling-based field grown plants of the same age (Fig. 1*H*). The other morphological parameters such as fruit size, fruit mass and days required for dehiscence of fruit, along with the pigment content were also compared between the tissue culture-based and seedling-based plants of same age and found to be better in micropropagated plants (Table 4).

The present report describes an efficient protocol for the production of plantlets through tissue culture using nodal shoot tip explants of *B. orellana* under the

Table. 4. Comparison of field grown tissue culture-based and seedling-based plants (most parameters were measured 70 d after anthesis). Means ± SE, $n = 10$.

Parameter	Tissue culture derived plant	Seedling derived plant
Seed dye content [% d.m.]	2.56 ± 0.52	2.15 ± 0.36
Fruit mass [g]	12.56 ± 0.24	10.76 ± 0.24
Fruit length [cm]	4.80 ± 0.26	4.00 ± 0.15
Fruit width [cm]	4.00 ± 0.18	3.50 ± 0.21
Number of seeds [fruit^{-1}]	59.60 ± 3.9	58.10 ± 2.3
Time to fruit dehiscence [d]	88 - 94	68 - 72

influence of TRIA (maximum of 213.6 shoot buds and 18 shoots per explant). TRIA has a synergistic effect in shoot multiplication when combined with BA and IAA (Table 1), which is not the case when supplemented alone. MS medium supplemented with TRIA alone gave only 2 - 3 shoots along with fluffy callus. Effect of triacontanol as a growth regulator had been reported already by Ries *et al* (1977). Though some reports of its use in apple (Ma *et al*. 1990) or *Capsicum* (Reddy *et al*. 2002) cultures have been reported earlier, this is the first report in annatto on identifying its synergistic and consistent effect on increased pigment content after transplanting them to field conditions. Improvement in pigment content in tissue culture based plants may be attributed to TRIA, as TRIA is known to enhance secondary metabolites (Yaseen and Tajuddin 1998, Fraternale *et al*. 2003, Giridhar *et al*. 2005).

Some attempts were made earlier for direct and indirect regeneration as well as for somatic embryogenesis of *Bixa* by using leaf, cotyledonary node and hypocotyls as explants with various hormonal combinations and concentrations. Ramamurthy *et al.* (1999) reported 4 - 6 shoots per nodal explant with simultaneous callusing by using B_5 medium supplemented with different cytokinins in combination with IBA. Moreover, it requires 5 - 6 months to get plantlets with 30 - 60 % response from explants. D'Souza and Sharaon (2001) used very high concentration of TRIA (up to 300 μM) along with 2iP which resulted in minor response. Though callusing from seed surface followed by organogenesis was achieved (Sha Valli Khan *et al.* 2002), reproducibility of the protocol was very low. A report on direct organogenesis by De Paiva Neto *et al.* (2003) described 3 - 4 shoots by using thidiazuron. From our study, it is evident that selection of a suitable explant together with phytohormone combination is a crucial factor for *in vitro* shoot multiplication in *B. orellana* as in case of *Capsicum* spp. (Sanatomibi and Sharma 2008) and *Piper methysticum* (Zhang *et al*. 2008). Apart from obtaining high frequency of *in vitro* shoots, this study shows the increased bixin content in the seeds of annatto under field conditions, which is essential for export. Thus, we conclude that TRIA can be used efficiently for micropropagation of *B. orellana.*

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