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ORIGINAL ARTICLE

Effective Micropropagation method for *Bixa orellana* L.: A non-toxic dye-yielding tree

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ABSTRACT
This study presents the optimal protocol for the in vitro proliferation, ex-vitro rooting, and acclimation of Annatto (Bixa orellana L.) a plant valued for its invaluable annatto pigments. We developed a biotechnological strategy to increase culture efficiency since traditional propagation methods had drawbacks such as low seed germination and limited rooting success. The seeds were cultivated on MS medium enhanced with several cytokinins following surface sterilization. The maximum germination rate of 94.10±1.22% was attained at BAP (0.5 mg L^{-1}). Nodal shoot-tips and hypocotyl explants were inoculated with MS media containing BAP (2.0 mg L^{-1}) for shoot induction, yielding an average of 13.2±1.31 shoot buds per node. BAP (1.0 mg L^{-1}) and IAA (0.1 mg L^{-1}) were shown to be the most effective PGRs for shoot multiplication. This resulted in 23.2±0.91 shoots measuring 4.34±0.65 cm in length. Subculturing on MS medium supplemented with different concentrations of BAP, Kin, IAA, and 100 mg L^{-1} glutamine significantly promoted shoot proliferation, producing 29.7±2.11 shoots per culture vessel, each with an average length of 5.91±0.59 cm. 200 mg L^{-1} IBA was used to accomplish 90% ex vitro rooting using a mean of 6.3±0.67 roots per shoot. 70% of the plantlets in the black polybags survived as a result of the acclimatization process, which involved slow environmental changes in the greenhouse. This protocol offers a reliable technique for successful plant establishment and mass propagation, greatly increasing the efficiency of B. orellana production.
***Keyword**: In vitro propagation, annatto pigments, shoot multiplication, ex vitro rooting, acclimatization, synthetic dye.*

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INTRODUCTION

The small, evergreen perennial tree *Bixa orellana* L., have its place to the Bixaceae, is also known as Achiote or Annatto. Native to tropical South and Central America, it is widely cultivated in countries such as Peru, Mexico, Ecuador, Indonesia, Kenya, East Africa, and India, particularly in its southern regions [1]. 70–80% of the dried seed arils are made up of the carotenoid bixin, which is the main ingredient that contributes to annatto's use as a coloring agent in food, cosmetics, and textiles. [2, 3]. Also found in lower levels is the water-soluble norbixin (NBX). Bixin and norbixin, natural food colors, are utilized in cosmetic and medicinal purposes, as well as for a variety of food products [4]. These include antidiabetic, aphrodisiac, antipyretic, antimicrobial, antifungal, antidiarrheal, anti-inflammatory, insect repellent, and anticancer activities [5, 6, 7, 8]. Annatto pigments are preferred due to their affordability, safety, inert nature, and ease of handling. Because of the negative consequences of synthetic dyes, there is a greater need for natural colorants like annatto. The bixin content varies among plants due to its cross-pollinated nature [9]. With annatto being one of the 13 natural pigments approved by the US FDA for use as food coloring, market appeal for the product is predicted to rise [10]. In Ayurveda, the entire plant is utilized extensively and has considerable medicinal potential [1, 11]. The scarcity of enough plant material has hindered the commercial production of annatto, despite its significance in both medicine and the economy. There are drawbacks to traditional propagation techniques including seed and vegetative cuttings. Eira and Mello [12] observed that reproducing annatto by seed germination is challenging by variables factors such as "low viability (20%), low germination rates (5-7%), a long production cycle (~4 years), delayed germination (40 days), and seed dormancy".

In addition to good climate circumstances, the plant's growth depends on the soil's manganese content, which is necessary for effective germination. Recent research, however, by Bharti et al. [13], showed improvements in germination rates; seeds treated in water for 24 hours had the highest germination rate (52%) and mean daily germination (4.6). Furthermore, germination rates increased dramatically from 19% to 47% when mechanical scarification and 24-hour water soaking were combined. Due to strong phenolic substance seeping from the cut ends, which hinders roots, conventional proliferation through vegetative cuttings is likewise restricted [14].

Using different in vitro produced explants of *B. orellana*, mass propagation through tissue culture is an alternate strategy to overcome these propagation issues. Improving yield and reducing plant heterogeneity require the development of an easy-to-follow and effective regeneration process. There have been reports of *B. orellana* being micropropagated in vitro using various explants, although these techniques have often produced few shoot buds per explant, whereas remarkably effective shoot regeneration has been extensively documented for this species [14, 15, 16, 17, 18, 19, 20, 21, 22; 23]. Previous studies on the micropropagation of *B. orellana* have predominantly utilized seedling-derived explants. In vitro responses have been documented for explants such as hypocotyl segments [16, 24], shoot tips and nodal segments originating from immature seedlings [18, 19, 25]. Whereas two reports focus on leaf explants with a lower rate of multiplication [20, 25]. Tiwane et al. [26] optimized *B. orellana* propagation and achieved 3.2 shoots with BAP, Kin, charcoal, and IBA. Given this background, the current study aims to create an in vitro regeneration strategy for *B. orellana* using nodal shoot-tip explants obtained from in vitro germinating seedlings. Significant prospects exist for bulk commercial micropropagation using seedling nodal shoot-tip explants and for the genetic upgrading of this plant.

MATERIAL AND METHODS

Surface disinfection and germination of seeds

Fully grown seeds were gathered from completely mature from dried fruits of *B. orellana* that were growing by the side of the road in Hyderabad City and International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana (India). The seeds were thoroughly cleaned using tap water for two minutes, then treated for five minutes with 10% Tween 20 (Sigma Aldrich, USA), and then washed with sterilized water. Prior to being treated with 0.1% $HgCl_2$ for 4-5 minutes, the seeds were surface sterilized for one minute using 80% ethyl alcohol. Following sterilization, 4 to 5 times-worth of autoclaved water were used to rinse the seeds. In order to promote germination, the disinfected seeds were subsequently inoculated into MS or half-strength MS media that contained BAP and Kin. 30 ml of the medium was poured in 350 ml culture bottles (Borosil, Mumbai, India). Thirty grams of sucrose per liter was added to the baseline MS medium [27], and eight grams of agar (Hi-Media, Mumbai, India) was used to solidify it. The cultures were placed under a 16-hour photoperiod (40 µmol m^{-2} s^{-1}) of cool white fluorescent lighting at a temperature of (25±2 °C) after being kept in the dark for 5 to 8 days.

Shooting Induction Media and Cultural Environment

A range of explants, including shoot tips, nodal shoot-tips, nodal segments, and hypocotyls, were harvested from seedlings that were 2-3 weeks old. These explants were inoculated into MS medium that had been added with different conc. of BAP or Kin ranged from 0.0 to 4.0 mg L^{-1}; either by themselves or in combination with indole-3-acetic acid (IAA) of 0.1 mg L^{-1} or α-naphthaleneacetic acid (NAA) to initiate shoots. Additives such as ascorbic acid (50 mg L^{-1}), adenine sulfate, citric acid, and arginine (25.0 mg L^{-1} each) were also added to the MS medium, and HCl or NaOH was used to bring the pH down to 5.8. After pouring 15ml media to culture tubes measuring 25 x150 mm from Borosil, India, the culture media are sterilized for 15 minutes of standard condition (121°C; and 15-20 psi) by autoclave. The cultures were incubated and monitored under close observation in a growth room under the standard conditions listed above for light, temperature, and photoperiod.

Two techniques were used to further amplify shoots from in vitro induced shoots: (i) reculturing the mother explants (nodal shoot-tips) onto MS medium were fortified with BAP or Kin concentrations of 0.5, 1.0, 1.5, or 2.0 mg L^{-1}, either separately or in combination with 0.1 mg L^{-1} NAA or IAA, and the treatments were applied through three passages spaced four weeks apart, and (ii) Different combinations of BAP and Kin, or Kin with IAA and glutamine (Table 5), were tested by subculturing four to five clustered shoots in vitro on MS medium to evaluate their impact on growth. Using the second approach, shoot cultures grown in vitro were placed on new MS medium enriched optimum concentrations of L-glutamine (100 mg L^{-1}), Kin (0.2 mg L^{-1}, IAA (0.1 mg L^{-1}, and BAP (0.5 mg L^{-1}). This process was sustained over two years through subculturing every four to five weeks.

Ex Vitro Rooting and Acclimatization (EVRA) of Plantlets

Individual in vitro multiple shoot clumps were extracted, with selected microshoots ranging in length from 4 to 6 cm. Freshly made IBA or NOA solutions (ranges from 100 to 500 mg L^{-1}) were pulse-treated for five minutes on the basal ends of these microshoots. Following treatment, autoclaved bottles containing Soilrite® (Kel Perlite, Bangalore, India) were used to hold the microshoots that had received either IBA or NOA therapy plus the untreated controls. MS basal salts at one-fourth strength (10–12 ml per bottle) were used to hydrate the substrate every four to five days.

The bottles were sustained at a temperature (28±2°C) and high relative humidity (80–90%) by covering them with polycarbonate covers and placing them in a greenhouse close to the pad section. The polycarbonate caps were eventually removed after being progressively unscrewed over time of two to three weeks. The bottles were also relocated to the fan part of the greenhouse during this acclimation period, which had a higher temperature (34–36°C) and a lower relative humidity (45–50%).

Designing Experiments and Statistical Evaluation

Every culture tube holding a solitary shoot explant was handled as a single replicate. Each experiment was performed three times, with a total of twenty repetitions for every treatment employed in each experimental set. For every experiment, the mean's standard error was computed in order to evaluate variability. The statistical analysis included one-way ANOVA to uncover significant differences between treatments, followed by Duncan's Multiple Range Test (DMRT) for means comparison. The findings of these scientific experiments were analyzed using IBM® SPSS® Statistics software, version 27. A significance level of $p \leq 0.05$ was adopted, with $p \leq 0.01$ used for more precise differentiation. The precise interpretation of the experimental data was made feasible by this procedure, which ensured the reliability and validity of the statistical comparisons.

RESULT AND DISCUSSION

In Vitro Seed Germination and Surface Sterilization

Annatto seeds are known for their short lifespan and poor germination rates, which limit their propagation through conventional methods. In order to improve culture procedures, a biotechnological instrument for efficient in vitro propagation must be developed. In this study, seeds were disinfected using alcohol and a 0.1% $HgCl_2$ solution for 5 minutes to ensure 100% contamination-free seedlings. Using disinfected seeds supplemented with different cytokinins (BAP and Kin), the germination rate was measured by inoculating the seeds onto MS media. Within three to four weeks, the media with BAP (0.5 mg L^{-1}) showed a high germination rate of 94.10±1.22%, resulting in seedlings reaching a height of 3 to 4 cm (Table 1, Fig 1A). In contrast, the Kin-supplemented medium resulted in a lower germination rate compared to BAP. The best explant for creating an in vitro regeneration technique for shoot induction was the seedlings derived from the BAP-supplemented media. According to this, BAP is a better cytokinin to encourage seed germination and the growth of first shoots in annatto, which will help create reliable in vitro regeneration techniques.

Table 1: - Effect of various types of medium with/without PGRs on seed germination in *B. orellana*

Type of medium	Mean % of seed germination ±SE
MS+ HF	62.49±2.40^{c}
MS+ BAP (0.5 mg L^{-1})	94.10±1.22^{a}
MS+ BAP (1.0 mg L^{-1})	74.71±2.02^{b}
MS+ Kin (0.5 mg L^{-1})	66.66±2.40^{c}
MS+ Kin (1.0 mg L^{-1})	52.73±1.36^{d}

"Media: MS with additives ascorbic acid (50 mg L^{-1}), adenine sulfate, citric acid, and arginine (25.0 mg L^{-1} each). The superscript letters in each column correspond to the means, and DMRT at $P < 0.05$ signifies that there is no statistically significant difference between them"

Initiation of Culture and Multiple Shoot Production

For the micropropagation of *B. orellana*, out of various explants were tested, nodal and nodal shoot tip explants were able to propagate in vitro. 12.4±0.47 shoots on average were obtained with MS media having BAP (2.0 mg L^{-1}) and IAA (0.1 mg L^{-1}) and additives (Table 2, Fig 1B). In contrast to our findings, Vijayasekhar et al. [28] stated that TDZ (4.0 mg L^{-1}; Thidiazuron) was necessary to produce shoots (5.35 shoots per culture) from cotyledonary nodes. On the other hand, no shoots appeared from explants grown on greater concentrations of BAP or Kin in addition to NAA (Table 3, Fig 1C). Late subculturing (more than 4 weeks) resulted in callusing at the base of explants.

Table 2: - Response of several kinds of explants obtained from *B. orellana* seedlings that are just beginning to germinate

Explants Type	Response (%)	Means Shoots No. ±SE	Means shoots length (cm) ±SE
Shoot tip	62.49±2.40^{c}	5.20±0.17^{c}	3.35±0.16^{c}
Nodal shoot tip	94.11±1.22^{a}	12.4±0.47^{a}	4.28±0.13^{a}
Nodal segment	77.31±3.61^{b}	6.24±0.12^{b}	4.24±0.10^{a}
Hypocotyl	66.66±2.40^{c}	3.65±0.20^{d}	3.76±0.14^{b}

"Media: MS with additives ascorbic acid (50 mg L^{-1}), adenine sulfate, citric acid, and arginine (25.0 mg L^{-1} each). The superscript letters in each column correspond to the means, and DMRT at $P < 0.05$ signifies that there is no statistically significant difference between them"

Table 3: - Shows the impact of several cytokinin types or auxin (NAA) on bud break from *B. orellana* explants at the nodal shoot tips.

BAP conc. (mg L^{-1})	Kin conc. (mg L^{-1})	NAA conc. (mg L^{-1})	IAA conc. (mg L^{-1})	Response (%)	Mean Shoot No ± SE	Mean Shoot length (cm) ± SE
0.0	0.0	0.0		00±00^{i}	00±00^{j}	00±00^{h}
1.0	–	–	–	49.66±1.45^{g}	5.05±0.42^{fg}	3.64±0.21^{bcd}
2.0	–	–	–	86.33±1.45^{bc}	6.90±0.44^{cd}	4.52±0.22^{a}
3.0	–	–	–	80.00±1.15^{cd}	5.75±0.31^{ef}	3.69±0.15^{bc}
4.0	–	–	–	70.00±2.88^{e}	4.70±0.26^{fg}	3.27±0.18^{cdef}
–	1.0	–	–	40.00±2.88^{h}	3.60±0.23^{i}	3.19±0.14^{def}
–	2.0	–	–	80.00±2.88^{cd}	4.70±0.31^{fg}	3.91±0.09^{b}
–	3.0	–	–	69.00±2.30^{e}	4.95±0.26^{fg}	3.38±0.11^{cdef}
–	4.0	–	–	60.66±1.76^{f}	4.15±0.22^{gh}	3.27±0.09^{cdef}
1.0	–	0.1	–	55.66±1.20^{f}	6.60±0.21^{de}	3.28±0.13^{cdef}
2.0	–	0.1	–	95.66±2.33^{a}	12.4±0.47^{a}	3.63±0.09^{bcd}
3.0	–	0.1	–	85.33±2.90^{bc}	9.40±0.43^{b}	3.66±0.18^{bc}
4.0	–	0.1	–	75.00±1.73^{de}	7.80±0.23^{c}	3.16±0.16^{ef}
1.0	–	–	0.1	43.33±2.02^{h}	6.15±0.22^{de}	2.93±0.13fg
2.0	–	–	0.1	90.00±1.73^{ab}	9.75±0.79^{b}	3.45±0.06^{cde}
3.0	–	–	0.1	80.00±2.30^{cd}	10.00±0.27^{b}	3.12±0.09^{efg}
4.0	–	–	0.1	70.00±1.15^{e}	6.85±0.37^{cd}	2.73±0.09^{g}

"Media: MS with additives ascorbic acid (50 mg L^{-1}), adenine sulfate, citric acid, and arginine (25.0 mg L^{-1} each). The superscript letters in each column correspond to the means, and DMRT at $P < 0.05$ signifies that there is no statistically significant difference between them"

Table 4: - Effect of different combinations of auxin (IAA) and cytokinins on the repeated transfer of *B. orellana* cultures

BAP conc. (mg L^{-1})	Kin conc. (mg L^{-1})	NAA conc. (mg L^{-1})	IAA conc. (mg L^{-1})	Mean Shoot No ± SE	Mean Shoot length (cm) ± SE
0.0	0.0	0.0	0.0	0.0±0.0^{m}	0.0±0.0^{g}
0.5	–	–	–	12.05±0.18^{k}	3.93±0.14^{cd}
1.0	–	–	–	21.80±0.29^{c}	4.34±0.14^{bc}
1.5	–	–	–	20.1±0.21^{d}	3.99±0.32^{cd}
2.0	–	–	–	14.7±0.23^{gi}	3.41±0.21^{def}
–	0.5	–	–	9.80±0.34^{l}	3.93±0.15^{cd}
–	1.0	–	–	18.60±0.33^{f}	4.19±0.08^{bc}
–	1.5	–	–	17.10±0.32^{g}	3.95±0.14^{cd}
–	2.0	–	–	15.50±0.30^{h}	3.41±0.21^{def}
0.5	–	0.1	–	13.60±0.45^{kj}	3.85±0.20^{cd}
1.0	–	0.1	–	21.85±0.22^{b}	3.96±0.06^{cd}
1.5	–	0.1	–	19.90±0.41^{e}	3.44±0.10^{de}
2.0	–	0.1	–	15.30±0.28^{h}	2.98±0.25^{ef}
0.5	–	–	0.1	13.50±0.39^{j}	4.18±0.15^{bc}
1.0	–	–	0.1	23.20±0.21^{a}	4.58±0.22^{a}
1.5	–	–	0.1	18.60±0.22^{f}	3.86±0.18^{cd}
2.0	–	–	0.1	14.40±0.21^{i}	2.86±0.22^{f}

"Media: MS with additives ascorbic acid (50 mg L^{-1}), adenine sulfate, citric acid, and arginine (25.0 mg L^{-1} each). The superscript letters in each column correspond to the means, and DMRT at $P < 0.05$ signifies that there is no statistically significant difference between them"

During the multiplication phase, *B. orellana* mother explants were repeatedly transplanted to MS media that included IAA (0.1 mg L^{-1}) and BAP (1.0 mg L^{-1}). When this medium was compared to MS media that included either cytokinin alone or in conjunction with NAA, it was discovered to be the most successful. Table 4, Fig 1D shows that after 20–25 days and 2-3 subcultures, 23.2±0.21 shoots measuring 4.58±0.22 cm in length were produced on this medium. While BAP+IAA was more successful than BAP alone or in combination with Kin, utilizing BAP at a higher concentration (1.5 to 2.0 mg L^{-1}) in conjunction with NAA or IAA resulted in fewer shoots and more callusing at the base of the explants.

Shoot cultures obtained from both the methods (subculturing and re-culturing) were transferred to MS media fortified with L-glutamine (100 mg L^{-1}), Kin (0.2 mg L^{-1}, IAA (0.1 mg L^{-1}, and BAP (0.5 mg L^{-1}) in order to achieved shoot multiplication. L-glutamine is well-known for supporting and preserving cell activity. With this amino acid, in vitro shoots growth was accelerated and more shoots were produced, with an average of 29.7±2.11 shoots measuring 5.91±0.59 cm in length per culture vessel (Fig 1E). Glutamate is a vital component that facilitates the growth of shoots in a wide variety of plants. Studies have indicated that it is a useful source of nitrogen, as evidenced by the fact that it may be used in glutamine to greatly increase the growth of shoots in *Cucumis sativus* L. [29] and *Ficus religiosa* L. [30]. According to Borpuzari and Kachari [31], it also significantly promotes shoot multiplication in the biofuel plant *Jatropha curcas* L. and improves in vitro regeneration in *Aquilaria malaccensis* Lam. [32]. These investigations highlight the adaptable function of glutamine in plant tissue culture applications. To maintain ideal shoot multiplication, prevent medium browning, and prevent shoot tips from burning, regular subculturing every 22–28 days was necessary. In terms of shoot quantity and length, other cytokinin combinations examined did not produce results that were suitable (Table 5).

Table 5: The influence of auxin (IAA) in combination with different cytokinin types on the growth of *B. orellana* cultures

BAP conc. (mg L^{-1})	Kin conc. (mg L^{-1})	IAA conc. (mg L^{-1})	Glutamine (mg L^{-1})	Mean Shoot No ± SE	Mean Shoot length (cm) ± SE
0.0	0.0	0.0	0.0	0.0 ± 0.0^{l}	0.0 ± 0.0^{g}
0.1	0.2	–	–	14.65 ± 0.25^{i}	3.68 ± 0.22^{de}
0.25	0.2	–	–	23.15 ± 0.22^{e}	4.19 ± 0.21^{cd}
0.50	0.2	–	–	26.70 ± 0.26^{c}	5.42 ± 0.17^{a}
0.75	0.2	–	–	23.20 ± 0.39^{e}	3.63 ± 0.23^{de}
1.0	0.2	–	–	19.70 ± 0.38^{g}	2.97 ± 0.22^{f}
0.1	0.2	0.1	–	13.7 ± 0.20^{k}	3.66 ± 0.19^{de}
0.25	0.2	0.1	–	22.1 ± 0.40^{f}	4.68 ± 0.19^{bc}
0.50	0.2	0.1	–	28.60 ± 0.22^{b}	5.71 ± 0.19^{a}
0.75	0.2	0.1	–	21.40 ± 0.22^{f}	3.68 ± 0.24^{de}
1.0	0.2	0.1	–	18.35 ± 0.26^{h}	3.32 ± 0.22^{ef}
0.1	0.2	0.1	100	14.45 ± 0.43^{jk}	3.49 ± 0.21^{ef}
0.25	0.2	0.1	100	24.90 ± 0.31^{d}	4.78 ± 0.19^{bc}
0.50	0.2	0.1	100	32.95 ± 0.22^{a}	5.91 ± 0.13^{a}
0.75	0.2	0.1	100	23.35 ± 0.28^{e}	3.94 ± 0.21^{de}
1.0	0.2	0.1	100	21.65 ± 0.35^{f}	3.38 ± 0.20^{ef}

"Media: MS with additives ascorbic acid (50 mg L^{-1}), adenine sulfate, citric acid, and arginine (25.0 mg L^{-1} each). The superscript letters in each column correspond to the means, and DMRT at $P < 0.05$ signifies that there is no statistically significant difference between them"

Ex Vitro Rooting and Acclimatization (EVRA) of Plantlets

Rooting treatments were applied to excised in vitro-derived shoots. Following a 5-minute treatment with 200 mgL-1 of IBA, shoots exhibited the strongest rooting response—roughly 90% of them took root. Four weeks following treatment, an average of 6.3±0.67 roots (per shoot) with an average root length of 5.7±0.77 cm was formed (Table 6, Fig). When IBA or NOA concentrations were higher, the shoots turned brown and finally died, whereas lower IBA concentrations decreased the percentage of roots (Fig 1F). Initially, the rooted ex-vitro plantlets were kept in a greenhouse to aid in their acclimatization. To give the plantlets time to acclimate to reduced humidity and increased air circulation, they were progressively moved from the fan area to the greenhouse pad section. The plantlets that had hardened had successfully adapted when the bottle lids were gradually removed after twenty to twenty-five days.

Table 6: - Impact of different auxin doses on *B. orellana* shoot ex vitro root induction

IBA conc. (mg L^{-1})	NOA conc. (mg L^{-1})	Response (%)	Mean Root No ± SE	Mean Root length (cm) ± SE
0.0	0.0	00 ± 00^{f}	0.0 ± 0.0^{d}	0.0 ± 0.0^{e}
100	–	60.00 ± 2.88^{d}	4.20 ± 0.25^{c}	4.46 ± 0.18^{c}
200	–	90.00 ± 2.51^{a}	6.30 ± 0.14^{a}	5.68 ± 0.17^{a}
300	–	70.00 ± 1.73^{c}	5.20 ± 0.23^{b}	5.33 ± 0.16^{ab}
400	–	68.33 ± 2.40^{c}	4.90 ± 0.21^{b}	4.43 ± 0.19^{c}
500	–	60.00 ± 2.88^{d}	3.80 ± 0.18^{c}	4.43 ± 0.19^{c}
–	100	50.00 ± 2.88^{e}	3.60 ± 0.22^{c}	3.82 ± 0.12^{d}
–	200	80.33 ± 1.45^{b}	5.00 ± 0.22^{b}	4.83 ± 0.29^{bc}
–	300	69.66 ± 2.02^{c}	4.90 ± 0.25^{b}	5.11 ± 0.20^{ab}
–	400	60.66 ± 1.76^{d}	4.80 ± 0.17^{b}	4.82 ± 0.27^{bc}
–	500	55.00 ± 1.15^{de}	3.70 ± 0.19^{c}	3.50 ± 0.21^{d}

"The superscript letters in each column correspond to the means, and DMRT at $P < 0.05$ signifies that there is no statistically significant difference between them"

After hardening, the plantlets were put into black polybags. Following their placement in polybags, these plantlets underwent three stages of acclimation in the greenhouse, with transfers occurring every 15–18 days. Approximately 70% of the plantlets survived this procedure and adapted to the greenhouse's conditions. Previous studies on *B. orellana* tissue culture have emphasized similar rooting and acclimatization techniques [15, 18, 24, 33].

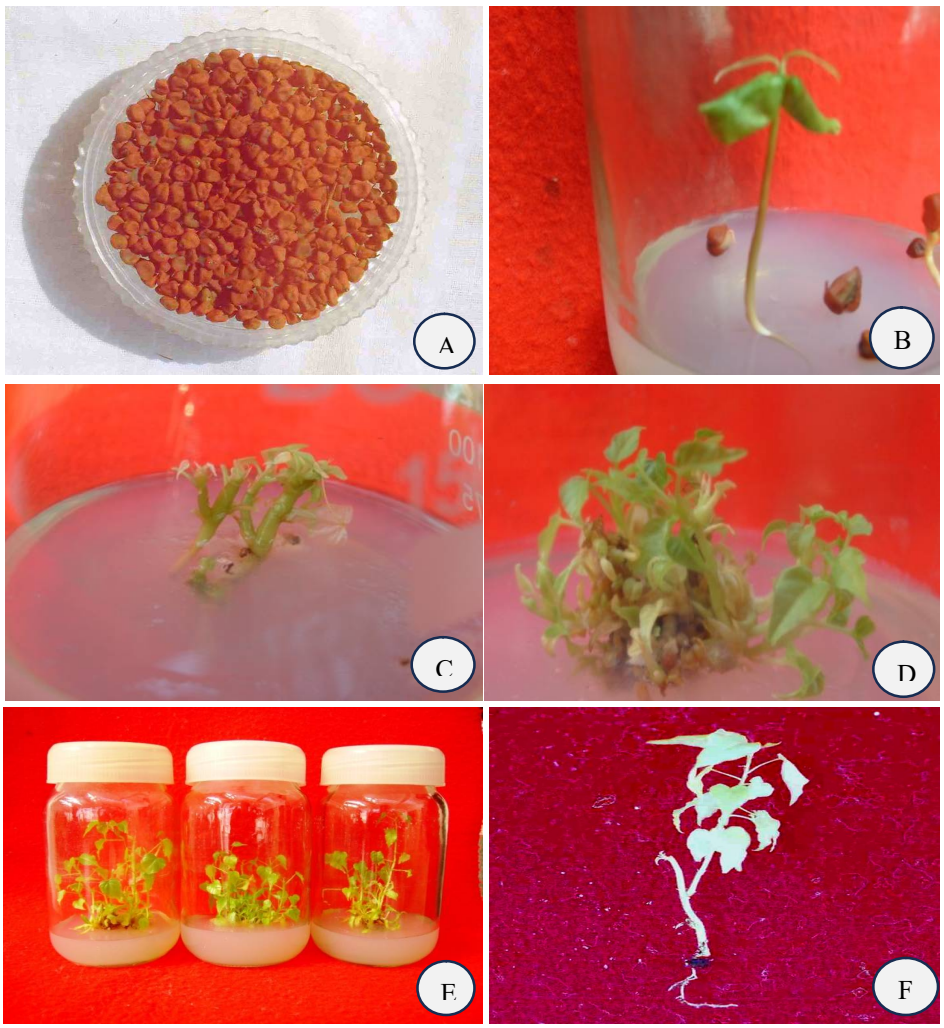

Fig. 1 Efficient clonal propagation of *Bixa Orellana* from seedling: - A. Seed of plants; **B**. In vitro seed germination; **C.** Bud breaking from explant of shoot tips of seedling on MS medium with 2.0 mg L^{-1} of BAP and 0.1 mg L^{-1} of IAA; **D.** Repeated transfer of explants on 1.0 mg L^{-1} of BAP and 0.1 mg L^{-1} of IAA; **E.** multiplication of in vitro raised shoots on 0.5 mg L^{-1} of BAP, 0.2 mg L^{-1} of Kin, 0.1 mg L^{-1} of IAA, and 100 mgL^{-1} of glutamine; **F.** Ex vitro rooted plantlets of in vitro raised shoots on 200 mgL^{-1} of IBA for 5 minutes.

CONCLUSION

This study presents an enhanced in vitro protocol for *Bixa orellana* that solves the drawbacks of traditional propagation techniques, including the issues of low seed germination and restricted rooting success. We improved the mass propagation efficiency greatly by achieving high rates of shoot induction, multiplication, and roots by the refinement of culture conditions, which included the application of particular amounts of auxins and cytokinins. With 70% of the plantlets surviving, the robustness of this approach is further validated by the effective ex vitro rooting and adaptation. Large-scale *B. orellana* cultivation is made feasible by this biotechnological method, which also helps to broaden the commercialization of this valuable species and produce annatto pigments in a sustainable manner.

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