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Organogenesis from cotyledon and hypocotyl-derived explants of japhara (*Bixa orellana* L.)

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A protocol for direct organogenesis in *Bixa orellana* (pink flowers variety) has been developed with significant organogenic response from rooted hypocotyls, hypocotyl segments, and cotyledonary leaf explants on Murashige and Skoog (MS) medium supplemented with 2.0 mg L^{-1} thidiazuron and 0.25% coconut water or 7.0 mg L^{-1} N^6-Benzyladenine and 0.1 mg L^{-1} α-naphthalene acetic acid. Thidiazuron in combination with coconut water promoted higher organogenic response in rooted hypocotyls. Similarly direct organogenesis was noticed from hypocotyls, cotyledonary leaf explants and shoot tip explants on half MS medium with 0.25 mg L^{-1} N^6-Benzyladenine (BA) and 0.5 mg L^{-1} indole-3-acetic acid (IAA). Best shoot elongation of shoot buds was achieved in the presence of 1.5 mg L^{-1} (BA) + 1.0 mg L^{-1}indole-3-butyric acid (IBA). The *in vitro* rooting of microshoots was good in the presence of 3.0 mg L^{-1} (IBA). Seventy percent of rooted plants survived after acclimatization.

Key words: organogenesis, axillary bud, *Bixa orellana*, hypocotyl, shoot, rooting

Introduction

Bixa orellana Linn. (Fam. Bixaceae), a native of tropical America, is widely cultivated in many other tropical countries, including southern India (SRIVASTAVA et al. 1999). The popular natural colourant of this plant 'annatto' is a rich source of the orange-red edible dye 'bixin' produced from the seed coat through a putative pathway from lycopene (JAKO et al. 2002). In purified form it is used as a natural food grade colorant, especially in colouring butter, cheese, ice creams, bakery products, edible oils, food stuffs and also for cosmetic and pharmaceutical applications (ANONYMOUS 1948, JONDIKO and PATTENDEN 1989). Being a safe, economical and easy-to-use product, among naturally occurring colorants, annatto ranks second in economic importance, with a current world consumption of 10,650 tons (SATYANARAYANA et al. 2003). Commercial plantations of this important species are raised through seedlings (APARNATHI et al. 1990). Since the bixin or annatto yield tends to vary from sample to sample, and from plant to plant, high yielding lines should be selected and vegetatively propagated for commercial plantations. *In vitro* regeneration of *Bixa orellana*

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may be used for the production of high-yielding and productive genotypes and could accelerate true-breeding programs. Studies on the regeneration of plantlets from axillary buds of seedlings (RAMAMURTHY et al. 1999, D'SOUZA and SHARON 2001) and through intervening seed callus (SHA et al. 2002), have been reported. In the present paper, a reliable regeneration system through organogenesis from cotyledons and hypocotyl explants of annatto is reported.

Materials and Methods

Plant material

Mature seeds from the red capsule (just before it turns brown) variety of *B. orellana* L. (pink flower-producing variety) were collected in the month of October 2002 from a 4 year plant of our Institute. The seeds were pre-treated with 0.5% bavistin solution (fungicide) for 2 hrs on a rotary shaker (New Brunswick, USA) at 90 rpm and thoroughly washed with sterile distilled water. This was followed by soaking the seeds in warm water (65 °C) for one and half hour and leaving it in the same water for 7 days at room temperature. During this period, the colour (carotenoids) of the seed coat leached out into the water. To get rid of any microbes (fungi or bacteria) that might have thrived during the soaking period, an initial treatment with 2–3 drops of Tween 20 (Himedia, Mumbai) and 1% NaOCl (v/v) (SRL, Mumbai), was given for 15 minutes, followed by 3 – 4 washes of 5 minutes each with sterile double distilled water. Later, 0.1% $HgCl_2$ (w/v) treatment was applied for 5 min, after which the seeds were thoroughly washed thrice with sterile water. The seeds were then inoculated on 40 ml aliquots of 0.7% agar (Himedia, Mumbai) or 0.35% phytagel (Sigma, USA) gelled MS medium and incubated in the dark for 15 days, after which they were transferred to a temperature of 25 ± 2 °C and light (45 µmol m^{-2} s^{-1}) for 16 h a day using fluorescent lights (Philips India Ltd) for 45 days.

Explants

In vitro grown seedlings with a length of 7–8 cm were used for the experiment. Cotyledonary leaf explants (10 x 10 mm size), hypocotyl segments (5–7 mm length) and rooted hypocotyls (50–65 mm length) were aseptically removed, and cultured either horizontally in sterile glass Petri dishes (Borosil) or vertically in test tubes (Borosil) 15 x 2.5 cm in size. In the case of rooted hypocotyls the hypocotyl tip was removed ~ 2 mm below the cotyledonary node.

Shoot bud induction medium

The rooted hypocotyl explants were placed upside down, on a shoot induction medium containing MS salts and vitamins (MURASHIGE and SKOOG 1962) supplemented with 0.25% coconut water (v/v) and thidiazuron at 0.5 to 3.0 mg L^{-1}. Similarly the hypocotyl segments and cotyledonary leaf explants were placed horizontally on the shoot induction medium and also on MS medium comprising the growth regulators naphthalene acetic acid (0.05 to 1.0 mg L^{-1}), and N^6-Benzyladenine (BA) (1–7 mg L^{-1}), which were tried individually and in combination to obtain the most suitable growth hormonal level for adventitious shoot

formation. Sucrose at 3% (w/v) was used as carbohydrate source. The pH was adjusted to 5.8 ± 0.2 before gelling with 0.7% (w/v) of agar (Hi media, India). Similarly, the shoot tip explants, hypocotyls and cotyledonary leaf explants were placed on ½ MS medium comprising 0.25 mg L^{-1} BA, 0.5 mg L^{-1} IAA for obtaining direct organogenesis. The cultures were kept at an incubation temperature of 25 ± 2 °C and in light (45 µmol m^{-2} s^{-1}) for 16 h per day using fluorescent lights (Philips India Ltd) for 60 days after an initial 48hr dark incubation.

Shoot bud elongation medium

Shoot buds derived from both rooted hypocotyls and hypocotyl segments were transferred to 150 ml Erlenmeyer conical flasks of 40 ml of MS medium containing 2.0 mg L^{-1} 2-isopentenyl adenine (2iP) or 1.5 mg L^{-1} BA with 1.0 mg L^{-1} IBA. Sucrose concentration was reduced to 2% (w/v) during this elongation stage.

In vitro rooting

The micro-shoots of size 4–5 cm length were excised from the original explant, and transferred to MS medium supplemented with IBA 3.0 mg L^{-1}. Rooted plantlets were removed from the medium, freed of agar by washing in running tap water and planted in a sand : compost mixture (1:2) at about 70–80% relative humidity under polyethylene hoods in the greenhouse. The plantlets were hardened for 30 days and then transplanted in the field.

Statistical analysis

For shoot bud induction 10 replicates of rooted hypocotyls, 30 explants each of cotyledonary leaf explants and hypocotyl segments (6 no. per plate), were used. Similarly, 10 replicates were used for shoot bud elongation and *in vitro* rooting. The experiment was repeated twice. Significant differences between the control and treatments were tested using the one way ANOVA test and significant values are presented in the tables. (AGRES 7.01 software).

Results

The inoculation of rooted hypocotyls upside down onto MS medium supplemented with 2.0 mg L^{-1} TDZ and 0.25% coconut water leads to the production of adventitious shoots from hypocotyl tip (80% response). A maximum of 6–8 and 20–22 shoots per explant, with a shoot length of 10–12 mm were obtained on media containing TDZ at 0.5 mg L^{-1} and 2.0 mg L^{-1}, respectively. In the absence of coconut water (0.25%) none of the TDZ concentrations tried were able to induce more than 3–4 shoots per explant (Tab.1). In the absence of hormones and coconut water shoot bud formation was absent and even callusing was not noticed. But rosette-like hard green clumps were observed and no further organogenesis from these clumps was noticed.

Organogenesis responses of *B. orellana* hypocotyl/ cotyledonary leaf segments varied from that of rooted hypocotyl explants (Tab.2). Adventitious roots were not induced in any

Tab. 1. Effect of TDZ and coconut water (CW) on shoot organogenesis in *Bixa orellana* L. *in vitro* (after 45 days).

Growth regulator (mg L^{-1})		Frequency of organogenesis (%)#		Number of shoots per responded explant		Shoot length range (mm)	
TDZ	+CW (%)	Rooted hypocotyls	Hypocotyl segments	Rooted hypocotyls	Hypocotyl segments	Rooted hypocotyls	Hypocotyl segments
0	0	20	2.0	–	–	–	–
0.5	0.25	40	50	8.0**	–	2–3	–
1	0.25	40	60	13**	1.5	4–6	4–6
2	0.25	70	60	22**	2.3*	10–16	3–7
3	0.25	30	40	2.25	–	2–3	–
0.5	0	40	60	RC	RC	RC	RC
1.0	0	45	60	RC	RC	RC	RC
2.0	0	60	70	RC	RC	RC	RC
3.0	0	40	30	RC	RC	RC	RC

* significant at $p < 0.05$; RC = Rosette-like clumps without any clear shoot bud formation; ** significant at $p < 0.01$;
# Thirty explants per treatment were used for the experiment.

Tab. 2. Effect of BA with NAA (0.1 mg L^{-1}) on shoot organogenesis in *Bixa orellana* L. *in vitro* after 45 d.

Growth regulator (mg L^{-1})	Frequency of organogenesis (%)#		Number of shoots per responded explant		Shoot length range (cm)	
N^6 benzyladenine	Cotyledonary leaf explant	Hypocotyl segments	Cotyledonary leaf explant	Hypocotyl segments	Cotyledonary leaf explant	Hypocotyl segments
1.0	20	20	1	1	2.1	1.8
3.0	40	30	1	1	2.3	2.5
5.0	40	50	1.2**	1.2**	3.2**	3.79**
7.0	60	70	1.92**	1.45**	4.44**	4.75**
9.0	20	40	1.42**	1.0	2.35*	2.6*
Control	0	0	0	0	0	0

* significant at $p < 0.05$; ** significant at $p < 0.01$; # Thirty explants per treatment were used for the experiment.

of the three types of explants on MS basal medium (control) devoid of growth regulators. Both cotyledonary leaf and hypocotyl explants initially showed little callusing from the cut surface of the explants on medium containing BA and NAA. Along with callusing, direct organogenesis in both cotyledonary and hypocotyl segments cultured in the presence of 0.1 mg L^{-1} NAA and 7.0 mg L^{-1}BA were mostly observed. A single prominent shoot formation was common from these explants. In the case of hypocotyl segment explants, callusing was more at the basal end and it eventually produced 2–3 shoots in 4–5 weeks of culturing. When the shoot tip, hypocotyls and cotyledonary leaf explants were cultured on ½ MS medium comprising 0.25 mg L^{-1}BA and 0.5 mg L^{-1} IAA direct organogenesis response was good (Tab.3). Shoot tip explants produced 10.5 shoot buds, while hypocotyl and cotyledon-

ary leaf explants produced a maximum of 30.5 and 3–4 shoot buds per explant respectively. Simultaneous callusing along with shoots was evident at the base of shoot tip explants and the same was absent in both hypocotyls and cotyledonary explants (Tab. 3).

Tab. 3. Direct organogenesis in *Bixa orellana* in the presence of BA (0.25 mg L^{-1}) and IAA (0.5 mg L^{-1}).

Explant	% of explants responded[#]	Number of shoot buds per responded explant	Number of shoot buds elongated	%Explants showed simultaneous callusing[#]
Shoot tip with a node	60	10.5**	6.2**	20
Hypocotyl	50	30.5**	5.4*	–
Cotyledonary leaf	40	3.2	2	–

* significant at $p< 0.05$; ** significant at $p< 0.01$. [#] Thirty explants per treatment were used for the experiment.

The scanning electron microscopic studies revealed shoot bud formation from cut surface of cotyledonary and hypocotyls explants. The buds or shoots formed during 8 weeks of culture were then transferred to the shoot elongation medium (Tab.4). The shoot buds formed in the primary induction medium with BA + NAA / TDZ+CW responded differently in the presence of 2.0 mg L^{-1} 2iP or 1.5 mg L^{-1} BA + 1.0 mg L^{-1} IBA. A maximum shoot length of 4.71 cm was noticed on the medium containing 1.5 mg L^{-1} BA + 1.0 mg L^{-1} IBA with 2–3 nodes (70% response), but in presence of 2.0 mg L^{-1} 2iP the number of nodes produced was 3–5 with a shoot length of 3.5 cm (50% response). Callus formation from the base of the explants was minimal in case of medium containing an optimum concentration of 2iP (2.0 mg L^{-1}).

Elongated shoots (>4.0 cm) were transferred to MS medium supplemented with IBA (3.0 mg L^{-1}) for rooting. It was observed that the shoots were rooted after 25 to 35 days with good root proliferation. IBA (3.0 mg L^{-1}) showed better rooting response by produc-

Tab. 4. Influence of 2iP or the combination of BA and IBA on shoot bud elongation of *Bixa orellana*

Growth regulator (mg L^{-1})			% of response[#]	Shoot length (cm)
2iP	BA	IBA		
1	0	0	30	2.33
2	0	0	60	3.4
3	0	0	40	2.71**
0	1	1	40	3**
0	1.5	1	70	4.71**
0	2.0	1	50	3.59**
0	0	0	30	1.8

** significant at $p< 0.01$. [#] Thirty explants per treatment were used for the experiment.

ing a maximum of 3.0 roots with a root length of 3.5 ± 0.52 cm and complete plantlets developed in 6 weeks in 90% of the shoots. The rooted plants were transplanted *ex vitro* and raised in pots under greenhouse conditions for one month, followed by field transfer. Approximately 70–80% of the plantlets survived.

Discussion

The significance of this study is the appearance of rapid direct organogenesis from hypocotyl and cotyledonary leaf explants and also from rooted hypocotyls. The rooted hypocotyl explants placed in an upside down position in medium as reported in *Capsicum annuum* require morpholino-ethylsulphonate (MES) for a response to be obtained (VALERA--MONTERO and OCHOA-ALEJO 1992, VINODKUMAR et al. 2005). But in the present study, without using MES, shoot bud formation from rooted hypocotyls was achieved in *B. orellana*. The nature of the hypocotyl explant was found to be important for organogenesis. The rooted hypocotyls showed better organogenesis than hypocotyl explants (Tab. 1). The distal ends of hypocotyl explants that were cultured in medium supplemented with TDZ + coconut water produced direct organogenesis, but callusing was noticed from the basal ends, which may be due to the auxin gradient (MUDAY and DELONG 2001). Coconut water addition to the medium in this study leads to organogenesis response in hypocotyl explants. This was due to its significant role in promoting callusing, embryogenesis as in case of *Coffea* sp. (GIRIDHAR et al. 2004) and shoot bud proliferation as in case of *Vanilla planifolia* (GIRIDHAR and RAVISHANKAR 2004). The organogenesis response from cotyledonary and hypocotyl explants in this study was further supported by a similar report by RAMAMURTHY et al. (1999). Even the anatomical studies revealed the initiation of calli from the cut surfaces of explants and the further commencement of differentiation was marked by the appearance of distinct cells with dense cytoplasm and prominent nuclei (data not shown) as reported earlier (RAMAMURTHY et al. 1999). Generally, these cells are located at a subepidermal level as well as in the center of the callus, as in *Viola tricolor* (BABBAR and SHARMA 1991). Cortical and epidermal cells formed an additional tissue to heal the wounded surface of the explant. In some regions of the explant the epidermis appeared to form a periderm--like structure, mainly in explants incubated on a TDZ + CW supplemented medium. The production of distorted leafy structures which do not produce normal shoots has been a major constraint in plant regeneration protocols, which was noticed in the presence of TDZ when it was used alone in Bixa (DE PAIVO NETO et al., 2003) and in other plants such as Lavender (TSURO et al. 1999); but not noticed in presence of CW (0.25%)+TDZ (2.0 mg L^{-1}). According to a recent report (de PAIVA NETO et al. 2003) though there was organogenesis from rooted hypocotyls in the presence of TDZ, impaired shoot elongation was encountered. But we overcome this problem in our experiment by using 1.5 mg L^{-1} of BA and 1.0 mg $L^{-1}$of IBA, in which good elongation of shoot buds was achieved. In the present report the optimum concentration of TDZ+CW produced organogenesis. Similarly, 2iP or BA+ IBA employed for elongation, promoted shoot elongation to a greater extent. This organogenesis method is useful for genetic transformation studies to improve this food-grade colour-producing plant.

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