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In Vitro Culture studies of *Bixa orellana* L: II- Bixin accumulation in root and hypocotyl derived callus

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ABSTRACT

Root and hypocotyl derived calli of Bixa orellana L., cultured on MS medium supplemented with 2 mg/l 2-iP and 0.05 mg/l NAA showed many localized area having orange pigmentation. The pigments were confirmed to be bixin after spectrophotometric analysis. After 2 passages of subculture the pigment accumulation decreased. No further continuation of bixin biosynthesis could be induced even after incorporation of various carotenoid precursor viz. Geranylgeranylpyrophosphate (50-200 ugl/l GGPP), Mevalonic Acid (50-200 mg/l MA), Gibberellic Acid (0.1- 1 mg/l GA) or carotenoid biosynthesis cofactor Manganese (10-40 mg/l Mn) into the callusing medium. However, GGPP and MA did enhance the accumulated bixin content in the first month.

Key words: *Bixa orellana*, Bixin, Callus, Carotene, Geranylgeranylpyrophosphate, Mevalonic acid, Gibberellic acid, 2-isopentenyl adenine (2-iP).

INTRODUCTION

Bixa orellana L. (annatto) is a unique plant having massive storage of bixin, a carotenoid, in the vacuoles of aril layer of seeds. In mature dried seeds, the stored pigments can be extracted as crude powder of carotenoids. Carotenoids represent one of the largest group of pigments found in nature, are widespread in plants [1 & 2]. Bixin present in annatto is an orange dye used as food colorant mainly in dairy products and also in textile. Current trend shows that it is being increasingly cultivated for use in body care preparations (creams, lotions and shampoos). Schules [3] has shown that it has UV protection as well as antioxidant and liver protective properties. This health related characteristics have given renewed impetus to carotenoid research.

In vitro productions of carotenoids in callus derived from different organs of various plants have been reported [4]. Occurrence of colored pigments in calli derived from root and hypocotyl explants of *Bixa orellana*, led to a study of possibility of using callus culture as a system to produce bixin.

MATERIALS AND METHODS

Callus Culture - Seeds were surface sterilized with 2% sodium hypochlorite having few drops of Tween-20 for 10 min. Treated seeds were washed thrice with autoclaved double distilled water and then dipped in 0.2% mercuric chloride for 5 minutes and again washed thrice with autoclaved double distilled water, each time for 5 minutes. Surface sterilized seeds were taken in distilled water and various parts viz. cotyledon; embryo, seed coat etc were dissected.

Since seeds are organs of storage of bixin; from mature seeds of Bixa *orellana* zygotic embryos, cotyledon, seed coat with tegmen and whole seeds were taken as explant for callus production.

Moreover, shoot apex (5 mm), node (5mm), hypocotyls (5mm) and root (20mm) explants from 1 month old, *in vitro* germinated seedlings were also used for generating calli.

Basal medium used for callus formation was Murashige and Skoog's [5] MS medium supplemented with 1 and 2 mg/l 2- iso pentenyl adenine (2-iP) and 0.05, 0.5 and 1 mg/l Naphthalene acetic acid (NAA).

To enhance bixin contents in callus carotene precursor 50-200 µg/l Geranylgeranylpyrophosphate (GGPP), 0.05 – 1 mg/l Mevalonic acid (MA), 0.1-1 mg/l Gibberellic acid (GA) and 10-40 mg/l Manganese a co-factor for caroteoid biosynthesis, was also added to the callusing medium separately.

Bixin Extraction and Estimation - Bixin was extracted from 1, 2 and 3 months old calli derived from root and hypocotyl explants. Calli older than that did not show pigmentation.

For bixin extraction, 1 g macerated callus was extracted with 10 ml chloroform in a capped vial and kept for 48 h at 37^{o}C. It was centrifuged at 1000 rpm for 10 min. Supernatant was decanted and dried in vacuum fume hood. It was further dried to powder in an oven at 105^{0}C. 20 mg of dried powder was dissolved in 50 ml chloroform and optical density was measured at 467 nm and bixin was estimated [6].

RESULTS AND DISCUSSION

Callusing did not occur from any of the seed derived explant. All the explants taken from seedlings produced callus on MS medium containing 1 or 2 mg/l 2-iP. Callus derived from only root and hypocotyl explants showed presence of pigmented spots. Hence the bixin assay and study were done only for the callus from these explants.

Addition of 0.05 mg/l NAA to both 1.0 and 2.0 mg/l 2 iP containing medium enhanced the growth of root and hypocotyl derived calli. Growth was measured by recording increase in fresh weight after 1, 2 and 3 months after inoculation (Table-1). Other concentrations of NAA were inhibitory. The trend of growth response of both root and hypocotyl derived calli to both NAA and 2 iP were the same.

One month after inoculation the calli derived from both the explants showed spotted orange pigment accumulation (Fig-1a) only in those calli which were growing on medium containing 2 mg/l 2 iP along with 0.05 and 0.5 mg/l of NAA.

Table 1 - Fresh weight of callus derived from 5 mm long hypocotyl and root explants from In vitro germinated *Bixa orellana* seedlings

Values are mean of 5 determinants

MS medium with PGRs (mg/l)		**Fresh weight (g) of callus derived from Hypocotyl and Root explants**					
2 iP	**NAA**	1 month after inoculation		2 month after inoculation		3 month after inoculation	
		Hypocotyl	Root	Hypocotyl	Root	Hypocotyl	Root
1.0	0.00	0.24 ± 0.02	0.22 ± 0.02	0.27 ± 0.03	0.24 ±0.04	0.39 ± 0.04	0.36 ± 0.01
1.0	0.05	0.38 ± 0.01	0.35 ± 0.01	0.48 ± 0.03	0.46 ± 0.46	0.54 ± 0.03	0.53 ± 0.03
1.0	0.5	0.23 ± 0.03	0.21 ± 0.03	0.28 ± 0.02	0.26 ± 0.26	0.35 ± 0.01	0.33 ± 0.01
1.0	1.0	0.21 ± 0.04	0.17 ± 0.04	0.24 ± 0.04	0.21 ± 0.21	0.28 ± 0.02	0.25 ± 0.04
2.0	0.00	0.28 ± 0.01	0.26 ± 0.01	0.36 ± 0.02	0.34 ± 0.34	0.40 ± 0.02	0.40 ± 0.03
2.0	0.05	**0.42 ± 0.02**	**0.39 ± 0.02**	**0.62 ±0.01**	**0.60 ± 0.60**	**0.74 ± 0.03**	**0.72 ± 0.04**
2.0	0.5	0.24 ± 0.01	0.23 ± 0.01	0.29 ± 0.03	0.28 ± 0.28	0.38 ± 0.03	0.36 ± 0.02
2.0	1,0	0.23 ± 0.03	0.21 ± 0.01	0.30 ±0.04	0.30 ± 0.30	0.34 ± 0.02	0.34 ± 0.02

In the 2nd month in general pigmentation increased in all the cultures (Fig. 1b), but it started to decline in the 3rd month (Table-2) onwards on further subculture. Callus derived from root explant had more bixin than callus derived from hypocotyl explant. Though some cultures showed visible orange coloration; e.g. culture growing on medium containing GA_3 or Mn. they could not be extracted in measurable amounts in the entire replica. Moreover they were not reproducible in all the determinants. For such readings a ± symbol is shown in the table 2.

Carotenoid formation in roots of carrot has been done [4] by cytokinin (kinetin) and auxin (2,4-D). In the present work also bixin could be synthesized in root as well as hypocotyls derived callus, in the presence of cytokinin (2-iP) and auxin (NAA). In carrot, roots are the organ having carotenoids. But in *Bixa orellana* seeds are the organs for storage of carotenoids, hence accumulation of carotenoids in callus cells derived from root and hypocotyls are an interesting observation.

Table 2 - Bixin content in callus derived from root and hypocotyl explants of *Bixa orellana*

Values are mean of three determinants (+ = Visible color but not measurable amount & - = Absence of bixin)

MS medium augmented with		% Bixin content in callus derived from Hypocotyl and root explants					
2 iP	NAA	1 month		2 month		3 month	
		Hypocotyl	Root	Hypocotyl	Root	Hypocotyl	Root
1.0	0.00	+	+	_	+	-	_
1.0	0.05	+	+	-	0.8	-	+
1.0	0.5	+	+	-	+	-	_
1.0	1.0	+	+	-	+	-	_
2.0	0.00	+	+	_	+	_	+
2.0	0.05	1.5	3.0	3.5	5.0	1.0	3.5
2.0	0.5	0.5	0.5	1.0	2.5	0.3	1.0
2.0	1,0	+	0.5	+	0.5	_	+

It has been suggested [7] suggested that MA is a precursor for carotene biosynthesis. Addition of 0.05 mg/L MA to callusing medium showed measurable amount of bixin in the callus during the first month though it was less than in the control, in calli derived from both root and hypocotyls explants, which declined after 2nd month (Table-3).

Table 3. Effect of Geranygeranylpyrophosphate (GGPP), Mevalonic Acid (MA), Gibberellic Acid (GA_3) and Manganese (Mn) on bixin content in callus (cultured on callusing medium MS + 2 mg/l 2 iP and 0.05 mg/l NAA) derived from hypocotyl and root of *Bixa orellana*

Values are mean of three determinants.(+ =Visible color but not recordable amount. - = Absence of bixin)

Callusing medium + Test	% Bixin content in callus derived from Hypocotyl & root explants					
compounds	1 month		2 month		3 month	
	Hypocot	Root	Hypocot	Root	Hypocot	Root
50 µl / l GGPP	+	+	+	+	_	_
100 µl / l GGPP	0.4	0.5	+	+	_	_
200 µl / l GGPP	+	+	+	+	_	+
0.05 mg/l MA	0.05	0.07	+	+	_	+
0.5 mg/l MA	+	+	+	+	_	_
1.0 mg/l MA	+	+	+	+	_	_
0.1 mg/l GA_3	+	+	_	_	-	-
0.5 mg/l GA_3	+	+	_	_	-	-
1.0 mg/l GA_3	+	+	_	_	-	-
10 mg/l Mn	_	_	_	_	_	_
30 mg/l Mn	_	_	_	_	_	_
40 mg/l Mn	_	_	_	_	_	_

GGPP (100 µg/L) an intermediary compound of the carotenoid biosynthesis pathway showed almost 10 times more bixin accumulation that in the callus treated with MA only in the first month; but not as much as control. However bixin content declined and disappeared from the callus by the end of 2nd month.

There has been report that numerous plants were able to form phytoene a carotenoid using Mn^{2+} as phytoene synthase cofactor [8, 9 & 10]. Taking that as a clue, Mn was incorporated into the medium as cofactor. Though higher concentration of Mn (10-40 mg/L); which is usually incorporated in MS medium at 22.3 mg/L; did not adversely affect the growth of callus derived from both the explants, but it distinctly stopped appearance of bixin spots in the callus.

GA, one of the known phyto-hormone related to carotenoid biosynthesis, inhibited both growth and bixin content in the callus.

The results suggested that calli derived from root and hypocotyl explants can be further developed to be a source of bixin production. Since bixin is stored in vacuoles, cell culture of root and hypocotyls can also be used for *in vitro* production of bixin and their easy expulsion into the culture media. Further work is necessary to understand the role of various factors in carotenoid biosynthetic pathway, type and age of explants (though the aril layer of *Bixa orellana* accumulates bixin, the organ and cells that synthesizes bixin is not known.) and growth regulators to achieve *in vitro* bixin production

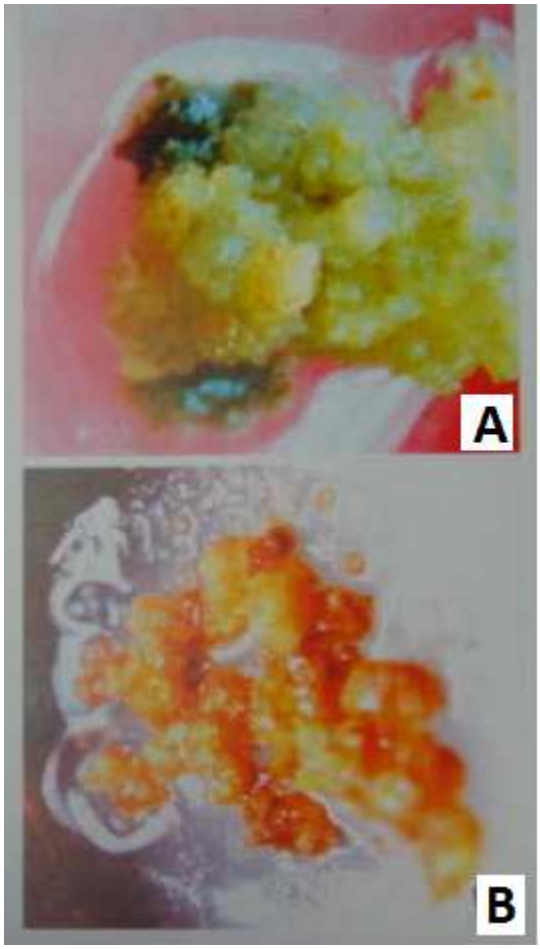

Fig. I. Callus of *Bixa orellana* cultured on MS medium supplemented with 2 mg/l 2 iP + 0.05 mg/l NAA. (A) One month old callus showing spots of bixin (B) Two months old callus showing red bixin content.

From the observations it can be concluded that with aging bixin production capacity of callus declines and bixin can be produced in young growing callus. Moreover hypocotyl and root, both non-chlorophyllous tissues seems to be encoded for bixin production But how and where it is synthesized *in vivo* and accumulated in aril layer of seeds could be another area of investigation.

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