Development of Molecular Techniques for Studying the Metabolism of Carotenoids in *Bixa orellana* L.

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Abstract. The tropical plant *Bixa orellana* L. (annatto) is the sole source of bixin, the most frequently employed natural pigment in the food industry. Little is known about the physiology, biochemistry, and molecular genetics of this crop. Our purpose was to establish a set of analytical tools that could be applied in the genetic improvement of *B. orellana*, particularly for the screening of characteristics such as bixin content and resistance to diseases or pests. Some preliminary results on the study of carotenoid synthesis are presented. In vitro cultures from several *B. orellana* tissues were established and DNA, RNA, and proteins were extracted from them and analyzed. Similarly, bixin and total carotenoids were quantified.

Bixa orellana L. is a perennial tree, native to the tropical Americas, which may have originated in the Amazon basin (Arce, 1999). The seeds of this plant, commonly known as "annatto" ("achiote" in Mexico) have a high content of an orange-red pigment, which consists mainly of the apocarotenoid bixin and has been used for many years as a bread dye and in vegetable oils and drinks (Dendy et al., 1965). Demand for bixin declined considerably when the synthetic Congo Red colorant was introduced. However, as the demand for natural dyes continues to increase, bixin is again being traded in large quantities on the international market. Its economic importance warrants further study of *B. orellana* to obtain crops with greater pigment production, higher disease resistance, and improved agronomic characteristics that will optimize field management. Bixin, the first cis-carotenoid to be isolated from natural sources, is the only one found in *B. orellana*. This 24-carbon compound belongs to the apocarotenoid family, whose members are formed via oxidative degrada-

tion of 40-carbon carotenes. It represents >80% of the total carotenoids in the seedcoat and 2% to 3% of the total seed weight (Mercadante et al., 1996). The chemical nature of the pigment, as well as that of other isoprenoids present, such as all-E-geranylgeraniol, farnesylacetone, geranylgeranyl octadecanoate, geranylgeranyl foramate, and α -tocotrienol, is well known from studies of seed extracts (Jondiko and Pattend 1989; Mercadante et al., 1996). However, little is known about the physiology, biochemistry and molecular biology involved in pigment production. In vitro culture techniques have become a powerful tool for studying basic and applied problems of plant biology (Zenk, 1978). In the last 10 years, these techniques have been utilized for studying gene expression and other biochemical and molecular processes. The objective of our work was to develop several methods for studying gene expression, enzyme regulation, and plant genome organization related to carotenoid accumulation in *B. orellana* in order to facilitate future study of the carotenoid biosynthesis pathway in this plant.

Materials and Methods

Explant preparation. *Bixa orellana* cv. India was used as the source of explants for in vitro cultures. The tested explants came from leaf and stem segments of plantlets germinated in vitro, as well as from cross-sections of immature fruits. Fruit were collected during October and November, which is the fruit production season for *B. orellana*. Seeds were harvested between January and March, stored in darkness at low relative humidity to assure good germination potential, and disinfected by soaking in 70% ethanol for 15 min, followed by a 3-min rinse in sterile distilled water. They were then washed in a 50% dilu-

tion of commercial bleach (Cloralex™ at 6%; Allen Co., Puebla, Mexico), followed by three rinses in sterile water. An incision was then made in the seed coat to facilitate germination, and seeds were disinfected again for 7 min in Cloralex at 6%. All washes were done using constant agitation under sterile conditions. To stimulate germination after disinfection, seeds were placed in sterile distilled water for 72 h. Finally, they were placed in magenta boxes on solid MS (Murashige and Skoog, 1962) medium, and kept under constant light at 25 to 27°C. To produce explants, immature fruit were harvested and their external spines removed with a scalpel. The fruit sections were then washed for 3 min with 70% ethanol, rinsed with sterile distilled water, and submerged for 3 min in the same disinfectant solution used for the seeds. Finally, they were rinsed four times with sterile distilled water and placed in magenta boxes on MS medium.

Callus induction and maintenance. Explants from the leaves, stem segments and cotyledons of plantlets germinated in vitro were used in callus induction. In all cases, PC basal medium (Phillips and Collins, 1979) was used, with different ratios of 1-naphthalene-acetic acid : 6-benzylaminopurine (NAA : BAP) for each explant type. Explants from leaves and cotyledons were cultivated on 0.54 μ M NAA plus 8.88 μ M BAP. Those from stems were cultivated on 2.68 μ M NAA plus 4.44 μ M BAP, and those from immature fruit on 13.43 μ M NAA plus 11.09 μ M BAP. Once callus was formed, it was maintained on medium containing NAA : BAP at a ratio (μ M : μ M) of 0.54 : 8.88 (leaves), 2.68 : 4.44 (stems), or 8.05 : 11.09 (immature fruit). Calli were subcultured every 30 d.

Genomic DNA isolation. The quality of the DNA isolated is critical in molecular genetic studies. The DNA in *B. orellana* tissues was isolated as described by Rogers and Benedich (1985) and quantified using a DNA Quantification Kit and a VersaFluor Multiple-Wavelength Fluorometer (Bio-Rad Corp., Hercules, Calif.). To verify integrity, samples of nondigested DNA and of DNA digested with EcoRI, EcoRV, and HindIII restriction enzymes were run through electrophoresis in a 1% agarose gel and stained with ethidium bromide (ETB) to verify integrity.

Isolation of total RNA. Total RNA was extracted using the hot phenol method according to De Vries (1992), and modified by including selective precipitation with urea and LiCl as proposed by Schuler and Zielinski (1985). Tissue (1 g) was crushed in liquid nitrogen and subsequently resuspended in 20 mL of a buffer containing 50 mM Tris-HCl at pH 8.5, 10 mM EDTA, 200 mM NaCl, and 20 mL of 50 phenol : 49 chloroform : 1 isoamyl alcohol. The mix was incubated for 10 min at 80 °C under constant agitation and cooled immediately on ice. The mix was then centrifuged at 8000 g, in a J2-21 centrifuge (Beckman Corp., Palo Alto, Calif.) and the supernatant collected and mixed with one volume of 50 phenol : 49 chloroform : 1 isoamyl alcohol at room temperature. After separating the organic phase by centrifugation, the supernatant

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was collected, one volume of isopropanol was added, and the mixture was precipitated at -20°C for 30 min, then centrifuged at 8000 g_n for 30 min. The pellet was resuspended in 5 mL TE buffer (Tris-EDTA: 10 mM Tris HCl, pH 7.4, 1 mM EDTA, pH 8), and a second precipitation was performed with two volumes of cold absolute ethanol and 0.1 volume of 5 M NaCl at -20°C for 30 min, to eliminate the excess phenol. The suspension was centrifuged at 8000 g_n for 20 min and the pellet was resuspended in 0.5 mL of TE buffer. To remove the DNA, 0.25 volumes of both 8 M urea and 10 M LiCl were added, the suspension was left for 12 h at -20°C , and then centrifuged at $15,000\text{ g}_n$ for 30 min at 4°C in an 5417 R Eppendorf centrifuge (Eppendorf Corp., New York, N.Y.). The pellet was then resuspended in 1 mL of 20% ethanol and washed with 1 mL of 49 chloroform : 1 isoamyl alcohol for 5 min at 3000 g_n to eliminate polysaccharide traces. The supernatant was incubated with two volumes of cold absolute ethanol and 0.1 volume of 3 M potassium acetate for 20 min at 20°C , and then centrifuged again at $15,000\text{ g}_n$ for 20 min at 4°C . The recovered RNA was resuspended in 0.3 mL of TE buffer and stored at -80°C for later use. The RNA was quantified using a spectrophotometer at 260 nm and the integrity was verified by non-denaturing 1% agarose gel electrophoresis.

Protein determination and HMGR assay. For protein extraction and assay of enzyme activity, samples of callus tissue were collected after 30 d of culture. Following the methodology proposed by Chappell et al. (1991), 1 g of the tissue was macerated in liquid nitrogen and homogenized in a buffer with 50 mM KCl, 5 mM MgCl₂, 250 mM sucrose, 15 mM EDTA, 100 mM KPO₃ pH 7.5, 5 mM dithiothreitol (DTT), 1 mM phenyl methyl sulphonyl fluorure (PMSF) and 7.5 mg·mL⁻¹ leupeptine. To recover the microsomal proteins, the extract was centrifuged at $15,000\text{ g}_n$ for 10 min, and the supernatant centrifuged at $100,000\text{ g}_n$ for 45 min. The pellet was then resuspended in a solution containing 100 mM potassium phosphate buffer, pH 7.5, 20% glycerol (v/v), 5 mM EDTA, 100 mM KPO₃ pH 7.5, 50 mM DTT, 1 mM PMSF, and 7.5 mg·mL⁻¹ leupeptine. Proteins were quantified according to Peterson (1977).

The HMGR activity was quantified by formation of [3-¹⁴C]-mevalonate from [3-¹⁴C]-hydroxy-methylglutaryl CoA in a reaction mixture containing 3 mM NADPH, 5 mM DTT, 700 pmol [3-¹⁴C]-hydroxy-methylglutaryl CoA (0.04 μCi) (CFA732; Amersham Corp., Buckinghamshire, England), and microsomal protein (10–60 μg) in a final volume of 26 mL. The reaction mixture was incubated for 1 h at 18 to 20°C and the mevalonate produced was separated by lactonization using 5 mL of mevalonate lactate as carrier. The mix was incubated for 15 min at 20°C , and then extracted with 300 mL of ethyl acetate and 125 mL of saturated phosphate buffer pH 7.5. The organic phase, containing the radioactive mevalonate, was recovered and counted by scintillation. Three replicate samples were analyzed for each type of callus.

Fig. 1. Callus from *B. orellana* fruit and plantlet explants obtained using the protocol described in the methods section. (A) Three types of callus produced after 30 d of culture: (a) white callus from stems; (b) white/brown callus from leaves; (c) brown callus from fruits. (B) Growth curves for the three types of callus tissues.

Carotenoid and bixin quantification. Pigments were extracted and quantified using methodologies standardized for seeds (Brandl, 1988; Lancaster and Lawrence, 1995, 1996; Luf and Brandl, 1988; Rouseff, 1988; Scotter et al., 1994). Vegetative tissues (2 g) from in vitro cultures were ground to a fine powder and mixed with 2 mL of 75 acetonitrile : 10 methanol : 15 isopropanol in a Polytron (model T25; Labortechnik, Seelbach, Germany). The resulting slurry was agitated constantly for 45 min in the dark at low temperature, and in a nitrogen atmosphere to eliminate atmospheric oxygen, thereby avoiding carotenoid oxidation and degradation. The suspension was then centrifuged twice at 3000 g_n for 20 min. The supernatant was recovered in a fresh tube, dried with nitrogen, and dissolved in 40 μL of 75 acetonitrile : 10 methanol : 15 isopropanol. Total carotenoids and bixin were quantified using a DU-65 spectrophotometer (Beckman Corp., Palo Alto, Calif.) with absorbance at 500 and 404 nm, according to McKeown and Mark (1962).

High performance liquid chromatography (HPLC). Callus extracts were subjected to HPLC analysis. After testing several eluants, a solvent mix similar to that used during the

extraction was applied at a flow rate of 1.5 mL·min⁻¹. Samples were injected in a C-18 reverse phase column (25 cm long $\times$ 4.6 mm i.d.) packed with lichrosobond (model 9679; Alltech, Deerfield, Ill.), and the absorbency was recorded at 493 nm, as described by Biacs and Daoud (1994). A bixin standard, purified from annatto seeds based on its R_f (0.72) by thin-layer chromatography (TLC) and using the same extraction solvent mix, was used as a control.

Results and Discussion

Callus culture establishment. Four callus cultures were established from aseptically germinated plantlets or from fruit. Callus formation occurred on several types of explants and tissues. Explants from plantlets exhibited tones from green to light yellow, whereas those from fruit showed a marked tendency to turn rapidly from creamy white to intense brown. Regardless of the explant source, all calli were friable, suggesting that they are suitable for the induction of cell suspension cultures (Fig. 1A). Figure 1B shows the growth dynamic for three more representative lines of callus culture. Cultures were classified into

Table 1. Quantification of DNA, total RNA, and proteins in different in vitro cultures of *B. orellana*.

Tissues	Total RNA ($\mu\text{g}\cdot\text{mL}^{-1}$)	DNA ($\mu\text{g}\cdot\text{mL}^{-1}$)	Proteins ($\mu\text{g}\cdot\text{mL}^{-1}$)
Roots	300 ± 75^z	294^y	6.01^y
Stems	246 ± 89	107^y	6.05^y
Leaves	181 ± 42	107^y	7.26^y
White callus	121 ± 1	81 ± 97	1.19 ± 0.03
White/brown callus	124 ± 24	35 ± 38	2.81 ± 0.41
Brown callus	151 ± 13	529 ± 371	1.78 ± 0.060

^zStandard deviation, n = 3

^yOne experiment only.

Table 2. Quantification of total carotenoids and bixin in different in vitro cultures of *B. orellana*. Leaves, stems, and roots were obtained from 3-week-old plantlets, and callus cultures were collected at day 30 (i.e., during exponential growth).

Tissues	Total carotenoids ($\text{mg}\cdot\text{kg}^{-1}$)	Bixin ($\text{mg}\cdot\text{kg}^{-1}$)
Roots	449 ± 30.4^z	266 ± 43.2
Stems	$6,567 \pm 186$	$4,530 \pm 162.4$
Leaves	$10,526 \pm 251$	$4,600 \pm 188.9$
White callus	142 ± 21.6	9 ± 25.1
White/brown callus	300 ± 48.2	141 ± 30.4
Brown callus	635 ± 54.5	400 ± 46.7

^zStandard deviation, n = 3

three types according to phenotype: white callus from stems (Fig. 1Aa), white/brown callus from leaves (Fig. 1Aa), and brown callus from immature fruit (Fig. 1Ac).

The latent period lasted 10 d for callus obtained from stems, leaves, and fruit. The exponential growth phase, manifested by a considerable increase in the mass of callus, was evident from day 10 onward (Fig. 1B). This phase extended beyond 35 d of culture, with growth continuing even when media were not changed for long periods (3–4 months). However, the coloration changed after 30 d of culture. This change was used as a visual marker to select cultures for molecular studies of carotenoid biosynthesis.

The methodologies proposed for the extraction of nucleic acids, proteins, and bixin were optimized for both plantlets and callus cultures. Good yields of nucleic acids were obtained, especially in roots (Table 1). However, the wide variations observed in the plantlet tissues were due to interference by their high polysaccharide contents. Despite this, the RNA isolated from these tissues was suitable to carry out expression studies with different genes, and the DNA isolated was adequate to carry out PCR amplifications as well as for performing Southern blots (Narváez et al., in preparation). Protein yield was lowest in callus tissues. However, the protein obtained were adequate to carry out HMGR enzymatic activity, as shown below. The callus cultures also varied in their capacity to accumulate bixin and other carotenoids (Table 2). Such a trait makes them suitable for the study of genes involved in the carotenoid pathway.

DNA isolation. Total DNA was obtained from seedlings and calluses. Few difficulties were encountered during DNA extraction with these types of cultures, although the DNA isolated from the seedlings and brown callus

tissue was partially contaminated with polysaccharides. Even when the yields were low (Table 1), the extracted DNA was of good quality, and suitable for molecular studies (data not shown).

RNA isolation. Several RNA isolation protocols have been reported using various compounds in the extraction buffer, such as guanidinium thiocyanate (Chomczynski and Sacchi, 1987), hot phenol (De Vreis et al., 1992), and Tris-Borate (López-Gómez and Gomez-Lim, 1992); others, such as that of Schuler and Zielinski (1985), in which urea and LiCl are used, are based on selective precipitation. However, none of these methods produced *B. orellana* RNA of sufficient quality or quantity. Therefore, we had to develop a better extraction method.

By using the method proposed in this work, high yields (Table 1) of good quality RNA were recovered (Fig. 2). This protocol allows the separation of polysaccharides and DNA from the extracted RNA. The isolated RNA conserved its integrity, shown in Fig. 2. Spectrometry at 260 nm was used for quantification (Table 1). The high yield (up to $300 \mu\text{g}\cdot\text{mL}^{-1}$), and quality of the RNA obtained with this methodology are appropriate for expression studies. The RNA recovered using this method has been used with good results in dot blot, and northern blot tests and RT-PCR (data not shown). Having a reliable method for RNA extraction from *B. orellana* tissues is the first step toward the construction of cDNA libraries.

Protein determination and HMGR assay.

Fig. 2. Electrophoresis of *B. orellana* RNA in 1% agarose gel stained with ethidium bromide. The RNA was extracted from *B. orellana* tissues cultured in vitro using the technique described in the methods section. The 18 S and 28 S ribosomal bands, characteristic of intact RNA, are visible (arrow). Lane 1, leaves; Lane 2, white/brown callus from leaves; Lane 3, white callus from stems; Lane 4, brown callus from fruit.

Protein extraction is the key to the study of the enzymes involved in isoprenoid biosynthesis in in vitro cultures of *B. orellana*. A method proposed by Chappell et al., (1991) for *Nicotiana tabacum* L. was used in the present study for the initial protein extraction from different tissue cultures in vitro, and resulted in high protein yields ($1.2\text{--}6 \text{ mg}\cdot\text{mL}^{-1}$) (Table 1). Because of the high pigment and polysaccharide content of *B. orellana*, centrifugation procedures had to be modified. The protein integrity was verified using an acrylamide native gel, with good results.

To determine whether the isolated proteins were appropriate for enzyme assay, the enzyme HMGR was chosen to begin the biochemical study of *B. orellana* tissues cultured in vitro, since this enzyme is important in isoprenoid synthesis (Bach, 1995; Chappell 1995). The enzymatic activity method was adapted from the technique proposed by Chappell (1991). The HMGR enzyme analysis was done using 30-d cultures, since cultures at this age were at the exponential growth phase, and exhibited color changes (Fig. 1). As a control for this methodology, activity was assayed using leaves, since this tissue has high HMGR activity (Learned and Connolly, 1997; Moore and Oishi, 1993). The activity observed was within the range of 0.75 to $2.7 \text{ pKat}\cdot\text{mg}^{-1} \text{ Prot}$ in all the tissues. However, no significant differences were found. The leaves had $0.75 \text{ pKat}\cdot\text{mg}^{-1} \text{ Prot}$ and, as it was lower than that observed in callus cultures, this appears to be an efficient method for measuring HMGR activity. However, HGMR activity in

all tissues remained low, even after 1 h of incubation. Only small differences were found among callus cultures, despite differences in color, which indicated that these tissues were adequate for analysis of enzymatic activity.

Bixin determination. Different protocols for carotenoid extraction were tested in *in vitro* cultures. Most of the methods available for bixin quantification have been designed for annatto extracts or for foods colored with this pigment (Lancaster and Lawrence 1995, 1996; Luf and Brandl, 1988; Rouseff, 1988; Scotter et al., 1994). Thus, a protocol for carotenoid extraction from different tissues was developed for the present study by modifying that proposed by Biacs and Daood (1994). Table 2 shows spectrophotometric representations of bixin and carotenoid accumulation according to McKeown and Mark (1962). The level of bixin was good in all the tissues except in white callus from stems. The highest levels of carotenoid and bixin were observed in the plantlet leaves and brown callus from fruit (Table 2).

HPLC analysis in callus culture. Since the spectrophotometric quantification was carried out on organic extracts from different *B. orellana* tissues, this assay could be subject to interference from other compounds with similar spectral properties. To improve such results, a HPLC protocol was developed for bixin separation from the other pigments present in our callus. After testing various solvent mixtures, the system described by Biacs and Daood (1994) was chosen. Application of this technique to mature seeds allowed the clear resolution of a major peak at 5.95 min, which exactly matched the retention time of the bixin purified by TLC (control). The intensity of such a peak strongly suggests that it corresponds to bixin, since it represents >80% of the seed carotenoids, as previously noted (Jodinko and Pattenden, 1989; Lancaster and Lawrence, 1996; Scotter et al., 1994). The HPLC analysis revealed important differences in carotenoid profiles among all the tissues analyzed (Fig. 3). No major peak at the 5- to 7-min interval was observed in extracts from callus culture, suggesting the absence of bixin in these tissues. These results confirmed those obtained by spectrophotometer (Table 2). In fact, chromatograms from white calli exhibited barely any peak between 5 and 7 min. This is the first use of HPLC in the separation of carotenoids from non-seed *B. orellana* tissues. In the future, the use of HPLC will facilitate the study of bixin accumulation, as well as the analysis of other carotenoids in the tissues.

Conclusions

In the present study, we developed and adapted a set of methodologies to facilitate research on the pigment-rich plant *B. orellana*, and on bixin, its main pigment. We were able to extract bixin, DNA, RNA, and proteins from various organs of the plantlets and callus produced. In fact, different kinds of callus cultures exhibited different pigment contents, allowing their use in the study of gene and enzyme regulation of carotene production.

Fig. 3. HPLC chromatograms of carotenoids in *B. orellana* callus tissues. The carotenoids were extracted and the samples analyzed by HPLC as described in the methods section. Samples were collected during the exponential growth stage (day 30): (A) white callus; (B) white/brown callus; (C) brown callus; and (D) seed extracts. Bixin purified for TLC (inside replots) was used as a control for retention time of bixin. Arrow indicates bixin peak. The legend "Time" indicates the exact retention time.

The *in vitro* cultures provide the additional advantage of ensuring the availability of plant material, regardless of phenological and environmental conditions. Some of the tissues provided good subjects for protein or nucleic acid extractions, although none contained high amounts of bixin. However, according to our results, only leaf tissue had all the necessary characteristics for the study of bixin biosynthesis, since it contains high quantities of bixin.

This set of methodologies can be useful for multiple genetic, molecular genetic, and chemical analyses, and biochemical studies that eventually could lead to a better understanding of the synthesis of these secondary carotenoids, and how these traits, linked to production, are inherited. The protocols should permit the application of any marker in the selection of high bixin-producing plants.

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