DANIELE VIDAL FARIA

BIXIN BIOSYNTHESIS IN ANNATTO (*Bixa orellana* L.) LEAVES CULTURED *IN VITRO*: INFLUENCE OF IRRADIANCE, SPECTRAL LIGHT QUALITY AND THE METHYLTRANSFERASE INHIBITOR 5-AZACYTIDINE

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do programa de Pós-Graduação em Botânica, para obtenção do título de *Doctor Scientiae*.

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(Coorientador)

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(Coorientador)

Wagner Campos Otoni
(Orientador)

Ao meu pai José Virgílio de Faria
À minha mãe Marilda de Sousa Vidal de Faria

Que sempre me apoiaram, são minha inspiração, orgulho e exemplo de vida.

Dedico.

"Alguns traziam uns ouriços verdes, de árvores, que, na cor, queriam parecer de castanheiros, embora mais pequenos. E eram cheios duns grãos vermelhos pequenos, que, esmagando-os entre os dedos, faziam tintura muito vermelha, de que eles andavam tintos. E quanto mais se molhavam, tanto mais vermelhos ficavam" (A Carta de Pero Vaz de Caminha, 1500).

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BIOGRAFIA

Daniele Vidal Faria é filha de Marilda de Souza Vidal de Faria e José Virgílio de Faria. Natural de Guaçuí, Sul do estado do Espírito Santo, onde em 1997 se matriculou na escola rural da Fazenda Aparecida. Em 2000 continuou seus estudos na Escola Estadual Antônio Carneiro Ribeiro. No ano de 2004 realizou ensino médio na Escola Monsenhor Miguel de Sanctis também em Guaçuí, onde decidiu a carreira que seguiria. Em 2008 ingressou no curso de Bacharelado em Ciênciass Biológicas da Universidade Federal do Espírito Santo, no campus de Alegre, onde nasceu seu interesse pela biologia vegetal e realizou iniciação científica e publicação de trabalhos científicos na área de cultura de tecidos vegetais, buscando enriquecer seus conhecimentos e experiencia profissional. Em setembro de 2012 iniciou mestrado em Fisiologia Vegetal na Universidade Federal de Viçosa. Em março de 2015 a fim de ampliar seus conhecimentos em biologia vegetal ingressou no doutorado em Botânica também na Universidade Federal de Viçosa, defendendo sua tese em fevereiro de 2019.

SUMÁRIO

ABSTRACT

FARIA, Daniele Vidal, D.Sc., Universidade Federal de Viçosa, February, 2019. **Bixin biosynthesis in annatto (*Bixa orellana* L.) leaves cultured *in vitro*: influence of irradiance, spectral light quality and the methyltransferase inhibitor 5-azacytidine**. Adviser: Wagner Campos Otoni. Co-Advisers: Diego Silva Batista and Marcelo Rogalski.

Annatto (*Bixa orellana* L.) is an important functional food and the unique exploited source for the production of natural dye bixin and norbixin, widely used in the food, pharmaceutical, cosmetic textile and othes industries. The bixin are produced in all plant tissues and accumulated in secretory structures, enabling use the *in vitro* tissue culture for micropropagation and study of the bixin pathway in the other tissues. However, its biosynthesis pathway and regulation are still not well understood. The genes carotene cleavage dioxygenase (*CCD4*), aldehyde dehydrogenase (*ALDH*) and *SABATH family methyltransferase* (*SABATH*) are involved with the bixin biosynthesis, since they are overexpressed in immature seeds and are related to carotenoids and apocarotenoids accumulation. Treatments with demethylating agents, such as 5-azacytidine (5-AZA) were previously related to an increased production of secondary compounds and used to study secondary metabolism pathways in plants. The light are characterized by irradiance, wavelength or quality and direction, all these factor can influence in development, growth and in several physiological processes such as in the production of plant secondary metabolites *in vitro*. Aiming to study the influence of DNA demethylating compound on the bixin pathway in *B. orellana* cv. Piave Vermelha leaves, we evaluated for the first time the effect of 5-AZA in the global methylation, the expression of some bixin pathway candidate genes, as well as the bixin content, chlorophylls and total carotenoids in the leaves of *B. orellana in vitro* grown plants. The temporary exposure of shoots apexes cultured *in vitro* to the demethylating compound 5-AZA did not affect the genome global methylation, chlorophylls and total carotenoid content in leaves of *B. orellana.* However, plants submitted to 5-AZA showed downregulation of SABBATH family genes and increased the bixin content in the leaves, suggesting that this methyltransferase inhibitor can direct regulate the biosynthesis pathway and the production of bixin in *B. orellana*. In addition, aiming to study the influence of different light conditions in the *in vitro* culture of *B. orellana*, we analysed for the first time the effect of irradiance and light quality on shoot apexes cultured *in vitro*.

The number of leaves per plant, stomatal density, leaf area and leaf expansion, chlorophylls, total carotenoids, malondialdehyde (MDA) and bixin content were analysed in the leaves of *B. orellana* 'Piave Vermelha' and 'UESB74' cultured *in vitro*. The fist produces 1.6-fold more bixin in leaves than the latter and visibly higher bixin content in the seeds. The stomata cells in the leaves of both cultivars were in paracytic arrangement, whit peltate trichomes predominant along the adaxial and abaxial surfaces of the leaf. For *in vitro* culture of 'Piave Vermelha' the blue/red LED light was the best, on the other hand for 'UESB74', the culture in fluorescent light is indicared, if the use fluorescent light is chosen for both varieties, are indicated the irradiance of 50 μmol m^{-2} s^{-1} is the must recommented. This work opens perspectives about the regulation of the pathway and the heterogeneity of the biosynthesis pathway of bixin.

RESUMO

FARIA, Daniele Vidal, D.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Biossíntese de bixina em folhas de plantas de urucum (*Bixa orellana* L.) cultivadas *in vitro*: influência de irradiâncias, qualidades espectrais da luz e do inibidor de metiltransferase 5-azacitidina**. Orientador: Wagner Campos Otoni. Coorientadores: Diego Silva Batista e Marcelo Rogalski.

O urucum (*Bixa orellana* L.) se destaca como importante alimento funcional e única fonte explorada para a produção do corante natural bixina, largamente utilizado nas indústrias alimentícia, farmacêutica, cosmética, têxtil e entre outras. A bixina é produzida em todos os tecidos da planta e acumulada em estruturas secretoras, possibilitando o uso da cultura de tecidos vegetais *in vitro* para micropropagação e estudo da via da bixina em outros tecidos. Todavia, sua via de biosíntese, assim como sua regulação, ainda não estão elucidadas. Sabe-se que os genes *desoxigenase da clivagem do caroteno* (*CCD4*), *aldeído desidrogenase* (*ALDH*) e *metiltransferase* da família *SABATH* (*SABATH*) estão envolvidos com a biosíntese de bixina, pois são superexpressos em sementes imaturas e relacionados com o acúmulo de carotenoides e apocarotenoides. O tratamento com composto demetilante, como 5-azacitidina (5-AZA) é relacionado ao aumento da produção de compostos do metabolismo secundário e utilizado no estudo da via de biossíntese do metabolismo secundário em plantas. A luz é caracterizada por irradiância, comprimento de onda e qualidade e direção, todos esses fatores podem influenciar no desenvolvimento, crescimento e em vários processos fisiológicos, como na produção de metabólitos secundários na cultura de células e tecidos vegetais. Objetivando estudar a influência do composto desmetilante do DNA na via da bixina em folhas de *B. orellana* cv. Piave Vermelha. avaliou-se pela primeira vez o efeito da 5-AZA na metilação global, a expressão de alguns genes candidatos à via da bixina, bem como o teor de bixina, clorofilas e carotenoides totais nas folhas de plantas de *B. orellana* cultivadas *in vitro*. O tratamento de ápices meristemáticos ao composto desmetilante 5-AZA não influenciou a metilação global do genoma, clorofilas e teor de carotenoides totais em folhas de *B. orellana* cultivados *in vitro*. No entanto, plantas submetidas a 5-AZA mostraram diminuição da expressão de genes da família SABATH e aumentaram o conteúdo de bixina nas folhas, sugerindo que a 5-AZA pode ter influenciado na regulação da via de biossíntese de bixina nas folhas. Além disso, com o objetivo de estudar a influência de diferentes condições de luz no cultivo *in vitro* de *B. orellana*,

analisou-se de forma inédita o efeito da irradiância e da qualidade da luz em ápices de caulinares cultivados *in vitro*. Também foi analisado o número de folhas por planta, densidade estomática, área foliar, expansão foliar, teor de clorofilas e carotenoides, malonaldeído e teor de bixina nas folhas de *B. orellana* 'Piave Vermelha' e 'UESB74' cultivadas *in vitro*. A 'Piave Vermelha' produz 1,6 vezes mais bixina nas folhas do que 'UESB74' e visivelmente maior teor de bixina nas sementes. Os estomatos nas folhas de ambas cultivares são paracíticos, com presença de tricomas peltados ao longo das superfícies adaxial e abaxial das folhas. Observou-se que para a cultura *in vitro* de 'Piave Vermelha' a luz LED azul/vermelha é indicada; por outro lado, para 'UESB74' é indicada o uso da luz fluorescente. Caso a luz fluorescente seja utilizada no cultivo, para ambas as variedades, é indicada a irradiância de 50 μmol m^{-2} s^{-1}. Este trabalho abre perspectivas sobre a regulação da via de biossíntese da bixina e sua heterogeneidade.

INTRODUCTION

Bixa orellana L. (annatto) is a woody tree species native to North, Central and South America (Moreira et al. 2015; Dequigiovanni et al. 2018). From their seeds is obtained the liposoluble apocarotenoid bixin, and one of the few natural hydrosoluble dyes: the norbixin (Giuliano et al. 2003; Rivera-Madrid et al. 2013), which vary in color ranging from yellow to brown, through red. Therefore, their seeds are used since pre-Columbian times as textile and food dye and body painting (Giuliano et al. 2003). Currently, they are used worldwide in various foods, pharmaceutical and cosmetics branches, among others (Marcolino et al. 2011; Shahid-ul-Islam et al. 2016; Rangel et al. 2017), being an important functional food (Rahmatullah et al. 2009). The bixin are produced and stored in large quantities in seeds (Preston and Rickard 1980; Giuliano et al. 2003) and in other tissues such as roots, stalk, flower and leaves (Mahendranath et al. 2011; Rodríguez-Ávila et al. 2011; Rivera-Madrid et al. 2013).

B. orellana seeds are the only source of commercially available bixin, despite other species also produce bixin as *Costus pictus*, grape and saffron, but in lower concentrations (Ramamoorthy et al. 2010; Annadurai et al. 2012). The demand for *B. orellana* seeds has grown, mainly after the prohibition of the use of synthetic dyes in the European market, since they be toxic or carry a carcinogen potential (Filho 2004; Guesmi et al. 2013).

Brazil is one of the main producing countries that supply the world market for processed seeds and derivatives of *B. orellana*. In 2008 17,500 tons of *B. orellana* seeds were produced, and from this amount, approximately 12 tons were produced by Brazil (ANVISA, 2009). There are many cultured of *B. orellana* cultivars in the world (Rivera-Madrid et al. 2006; Akshatha et al. 2011), amongst them, Piave Vermelha stands out for its good productivity associated with high levels of bixin, ranging from 3.5 to 5.0%, which promotes greater acceptance of these seeds in the market (Lemos et al. 2011). There are several cultivars of *B. orellana* that produce from low to high bixin content in the seeds (Rivera-Madrid et al. 2006; Akshatha et al. 2011), and these different phenotypes allow genetic and physiological studies aiming the development of elite cultivars and understanding the bixin biosynthesis pathway. The cultivar UESB74, for example, produces low bixin content leading to changes in seed phenotype, which shows low red dye.

Despite the importance of the natural dye bixin, its biosynthetic pathway as well as its regulation has not been elucidated yet. Bouvier et al. (2003) proposed a model for the bixin synthesis pathway using a heterologous expression system in *Escherichia coli*. Three genes encoding enzymes required for bixin synthesis derived from lycopene were assayed: *carotene cleavage dioxygenase* (*BoLCD*), *bixin aldehyde dehydrogenase* (*BoADH*) and *norbixinmethyltransferase* (*BonBMT*). However, this proposed pathway did not explain bixin biosynthesis at all. Subsequent studies indicated other candidate genes related to the accumulation of carotenoids and apocarotenoids in immature seeds and leaves, such as the *caffeic acid O-methyltransferase* (*COMT*), *1-Deoxy-D-xylulose-5-phosphate synthase* (*DXS*), *carotene cleavage dioxygenase4* (*CCD4*), *aldehyde dehydrogenase* (*ALDH*) and *SABATH methyltransferase family* (*SABATH*) (Rodríguez-Ávila et al. 2011; Soares et al. 2011; Rivera-Madrid et al. 2013; Cárdenas-Conejo et al. 2015).

The *SABATH* methyltransferases family catalyze the transfer of a methyl group from S-adenosyl-L-methionine to the carboxyl or nitrogen group of the substrate, forming small methylesters molecules such as bixin or N-methylated compounds such as caffeine (D'Auria et al. 2003). The *SABATH* family genes may methylate norbixin, producing bixin at the end of the pathway (Cárdenas-Conejo et al. 2015). *SABBATH* methylates small molecules relating to the plant defense in the primary and secondary metabolism (Qin et al. 2005; Köllner et al. 2010; Abbas et al. 2018; Bulman et al. 2018).

DNA methylation by methyltransferases is a mechanisms that define the epigenetic landscape and confer mitotically and genetic heritable alterations in gene activity and may play a major role in the expression of several eukaryotic genes (Law and Jacobsen et al. 2010). The DNA methylation manipulation by hypomethylating chemicals may be a powerful biotechnological tool to increase crop quality (Fieldes et al. 2005; Akimoto et al. 2007).

The 5-azacytidine (5-AZA) is a hypomethylating compound that generally cause demethylation via blocking the activity of DNA methyltransferases (Santi et al. 1984). The 5-AZA is used in plants to study programmed cell death (Betekhtin et al. 2018), mechanisms of resistance to stresses (Zhong et al. 2010), food enrichment (Zhu et al. 2018), reactivation of silenced transgenes plants (Tyč et al. 2017), and recovering of embryogenic competence (Pila et al. 2017). This compound is also

used in studies of secondary metabolism pathways and even to increase the production of these compounds (Kiselev et al. 2011; Yang et al. 2018).

Since the bixin is produced throughout the plant tissues, the *in vitro* tissue culture can be used for micropropagation and the study of the bixin pathway all over the tissues. *In vitro* culture of plants has advantages, such as restricted external factors, control of the culture environment, and the possibility to use elicitors for the metabolic pathway of interest, no needing to use an adult plant, which would spend more time (Hussain et al. 2012; Murthy et al. 2014).

Light is an abiotic factor characterized by its irradiance, wavelength and direction, being a fundamental factor for the plant development, as an energetic source for photosynthesis and controlling a plethora of biological activities (Muneer et al. 2014; Batista et al. 2018; Plantenga et al. 2019). The biological responses to the irradiance variation throughout the day and seasons depend on the genotype and the life cycle of the plant through mechanisms that may involve the whole plant, starting from molecular to cellular levels and involving responses such plant length, leaf area, chlorophyll content, among others (Givnish 1988; Ruban 2015). Specific irradiances in plant tissue culture can increase the development (Muneer et al. 2014), micropropagation (Gupta et al. 1997), healing after grafting (Muneer et al. 2015) and the production of secondary compounds (Batista et al. 2016; Irshad et al. 2018).

Cultured cells and tissues are influenced by many factors being the light quality is one of them, since it affects *in vitro* morphogenetic responses and secondary metabolite production (Batista et al. 2018). The *in vitro* culture of *B. orellana* is commonly performed under fluorescent lights (Paiva Neto et al. 2003 a and b; Carvalho et al. 2005; Cruz *et al.* 2014; Cruz et al. 2015; Mohammed *et al.* 2015), which has less duration and offer inferior lighting conditions and also generate more waste products compared to light-emitting diodes (LEDs) (Batista et al. 2018). Light quality is known for modulating plant metabolic proceses such as carotenoid production, growth and development (Li et al. 2010; Lin et al. 2013; Kopsell et al. 2014; Amoozgar et al. 2017).

Based on the benefits of using better light qualities in plant tissue culture, the LED technology implementation in the *in vitro* culture of *B. orellana* is interesting due its great commercial value. It is known that *B. orellana* cultured under ultraviolet radiations (UV-B e UV-CAs) increase the β-carotene content and decrease the total carotenoids, not affecting the bixin content in leaves (Sankari et al. 2017). Therefore,

to the LED technology implementation in the *in vitro* culture of *B. orellana* it is necessary to evaluate the appropriate conditions to increase growth and bixin production.

In this work, the irradiance, the spectral quality as well as the use of a demethilator compound in the *in vitro* tissue culture of annatto (*Bixa orellana* L.) is assayed, in order to understand the effect of these factors on growth and bixin biosynthesis.

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CHAPTER I

5-AZACYTIDINE DOWNREGULATED METHYLTRANSFERASE SABATH GENES AND AUGMENTED BIXIN CONTENT IN ANNATTO (*Bixa orellana* L.) LEAVES

ABSTRACT

Bixa orellana L. seeds are important sources of the liposoluble apocarotenoid bixin and the natural hydrosoluble dye norbixin. These natural dyes are used worldwide in various food, pharmaceutical and cosmetic branches. Treatments with demethylating agents, such as 5-azacytidine (5-AzaC) were previously related to an increased production of secondary compounds and used to study secondary metabolism pathways in plants. Here, we evaluated for the first time the effect of 5-AzaC in the global DNA methylation, the expression of some bixin pathway candidate genes, as well as the bixin content, chlorophylls and total carotenoids in the leaves of *B. orellana* plants cultured *in vitro*. The temporary exposure of shoots apexes cultivated in vitro to the demethylating compound 5-AzaC did not affect the genome global methylation, chlorophylls and total carotenoid content in leaves of *B. orellana*. However, plants submitted to 5-AzaC showed downregulation of the SABATH family genes and increased the bixin content in the leaves, suggesting that this methyltransferase inhibitor can selectively regulate the biosynthesis pathway and the production of bixin in *B. orellana*.

Key words: Anatto, bixin pathway, methyltransferase inhibitor, 5-azacytidine, SABATH genes.

INTRODUCTION

Bixa orellana L. (annatto) is tree native to tropical North, Central and South America, (Moreira et al.; Dequigiovanni et al. 2018). From *B. orellana* seeds is obtained the liposoluble apocarotenoid bixin, and one of the few natural hydrosoluble dyes: the norbixin (Giuliano et al. 2003; Rivera-Madrid et al. 2013), which vary in color ranging from yellow to brown, through red. Therefore, seeds are used since pre-Columbian times as textile and food dye and body painting (Giuliano et al. 2003). Currently, they are used worldwide in various foods, pharmaceutical and cosmetics branches, among others (Marcolino et al. 2011; Shahid-ul-Islam et al. 2016; Rangel et al. 2017).

The only available source of commercially bixin is the *B. orellana* seeds. Other plant species also produce bixin such as *Costuspictus*, grape and saffron, but in lower concentrations (Ramamoorthy et al. 2010b; Annadurai et al. 2012). The bixin is produced and stored in large quantities in seeds (Preston and Rickard 1980; Giuliano et al. 2003), and in other tissues such roots, stalk, flower and leaves (Mahendranath et al. 2011; Rodríguez-Ávila et al. 2011 b; Rivera-Madrid et al. 2013). Seeds are most often the main target for studies of the biosynthetic pathway of bixin, but little is known about this pathway or the genes involved in other organs of the plant such as leaves.

Despite the importance of the natural dye bixin, its biosynthetic pathway as well as its molecular regulation, have not been elucidated yet. Bouvier et al. (2003) proposed a model for the bixin synthesis pathway using a heterologous expression system in *Escherichia coli*. Three genes encoding enzymes required for bixin synthesis derived from lycopene were assayed: *carotene cleavage dioxygenase* (*BoLCD*), *bixin aldehyde dehydrogenase* (*BoADH*) and *norbixinmethyltransferase* (*BonBMT*). However, this proposed pathway did not explain bixin biosynthesis. Subsequent studies indicated other candidate genes related to the accumulation of carotenoids and apocarotenoids in immature seeds and leaves such as the *caffeic acid O-methyltransferase* (*COMT*), *1-Deoxy-D-xylulose-5-phosphate synthase* (*DXS*), *carotene cleavage dioxygenase4* (*CCD4*), *aldehyde dehydrogenase* (*ALDH*) and S-adenosyl-l-methionine dependent methylation from *SABATH methyltransferase family* (*SABATH*) (Rodríguez-Ávila et al. 2011b; Soares et al. 2011; Rivera-Madrid et al. 2013; Cárdenas-Conejo et al. 2015).

The methyl transferases of the *SABATH* family catalyze the transfer of a methyl group from S-adenosyl-L-methionine to the carboxyl or the nitrogen group of the substrate, forming small methyl ester molecules such as bixin or N-methylated compounds such as caffeine (D'Auria et al. 2003). The genes of the *SABATH* family may methylate norbixin and produce bixin at the end of the pathway (Cárdenas-Conejo et al. 2015). *SABBATH* methylates small molecules relating to the defense of plant in the primary and secondary metabolism (Qin et al. 2005; Köllner et al. 2010; Abbas et al. 2018; Bulman et al. 2018).

DNA methylation by methyltransferases is a mechanism that define the epigenetic landscape and confer mitotically and genetic heritable alterations in gene activity and may play a major role in the expression of several eukaryotic genes (Law and Jacobsen et al. 2010). The manipulation of DNA methylation by hypomethylating chemicals may be a powerful biotechnological tool to increase crop quality (Fieldes et al. 2005; Akimoto et al. 2007; Tyč et al. 2017).

The 5-azacytidine (5-AzaC) is a hypomethylating compound, generally regarded to cause demethylation via blocking the activity of maintenance DNA methyltransferases (Santi et al. 1984). The 5-AzaC is used in plants to study programmed cell death (Betekhtin et al. 2018), mechanisms of resistance to stresses (Zhong et al. 2010), defense responses against pathogens (Kiselevet al.2013), food enrichment (Zhu et al. 2018), reactivation of silenced transgenes in transgenic plants (Tyč et al. 2017), and recovering or increasing embryogenic competence (Pila et al., 2017; Osorio-Montalvo et al., 2018).

This demethylating compound is also used in the study of secondary metabolism pathways and is also related to an increased production of secondary compounds (Kiselev et al. 2011, Yang et al. 2018). Here we tested for the first time the effect of 5-AzaC on global DNA methylation and expression of the bixin pathway candidate genes, as well as the bixin, chlorophylls and carotenoid contents in the leaves of *B. orellana* plants cultured *in vitro*.

MATERIALS AND METHODS

Plant material and in vitro *culture*

Seeds of the cultivar PiaveVermelha (donated by Christian Hansen) were scarified and sterilized, according to Carvalho et al. (2005), and germinated in test tube containing 10 mL of MS medium (Murashige and Skoog 1962) with half of the salt composition (MS ½), supplemented with B5 medium vitamins (Gamborg 1968). All the other experiments were conducted using flask (6.5 cm of diameter x 10 cm of length) containing 35 mL of JADS medium (Correia 1995). Both media formulation were supplemented with 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol and solidified with 5.0 g L^{-1} agar (PhytoTechnologies®). The pH was adjusted to 5.7 ± 0.1 and the medium autoclaved at 120 °C, 108 kPa for 20 min and kept in growth room at 25 ± 2 °C, 16 h of photoperiod and irradiance of 50 µmol m^{-2} s^{-1}. After 30 days shoot apexes from germinated plants were growth in JADS medium (Correia 1995) with 4.56 µM zeatin during 45 days for plant multiplication then, shoot apexes were cultivated in medium JADS supplemented with 5 µM indole-3-butyric acid (IBA) and 5-AzaC (0, 1 and 100 µM) for 48 h (these experimental conditions was delimited on the basis of further experiments varying the exposure time and concentration of 5-Azac). According to Tyč et al. (2017) the 5-AzaC half-life in plant tissue culture conditions is about two days, therefore, after 48h in presence with 5-AzaC, the shoot apexes were transferred to JADS medium with 5 µM IBA.

After 60 days of culture, bixin (µg g^{-1} Dry Weight (DW)) and chlorophyll *a* and *b* (µg g^{-1} DW) contents, global DNA methylation (%5mdC), total carotenoids (µg g^{-1} DW) and the expression of genes related to bixin synthesis pathway were evaluated. Experiments were conducted on a completely randomized design with ten replicates, composed by a flask with five explants each.

Bixin content

The bixin content evaluation was performed according to Napoleão et al. (2017). Approximately 10 mg of freeze-dried leaves were grounded in liquid nitrogen followed by two extractions with 300 µL of methanol: isopropanol: acetic acid (20:79:1; v/v/v). The samples were vortexed (4 times for 20 s), sonicated (10 min), kept on ice (30 min) and centrifuged (20,000 *g*, 10 min at 4 °C) to collect the supernatant. Then, the supernatant was filtered (Econofltr PVDF 13 mm and 0.2 µm;

Agilent Technologies, Santa Clara, CA, USA) and used for liquid chromatography-mass spectrometry (LC-MS) analysis.

Samples of the final supernatant (5 µL) were automatically injected into the LC-QqQ MS (Triple Quadrupole 6430, Agilent Technologies, Waldbronn, Germany) equipped with an Eclipse Plus C18 (2.1 × 50 mm, 1.8 µm) column and guard column Zorbax SB-C18 (1.8 µm, Agilent) at 26 °C. The mobile phase consisted of 0.02% acetic acid in water (solvent A) and 0.02% acetic acid in acetonitrile (solvent B) at a constant flow rate of 300 µL min^{-1}. A linear gradient was applied as follow: 0–3 min and 2%–60% of B, 3–8 min and 60%–99% of B, 8–11 min and 99% of B, 11–12 min and 99% to 2% of B and 12–15 min and 2% of B. A mass spectrometer ionization source ESI (Electrospray Ionisation) was used with the following conditions: gas temperature of 300 °C, nitrogen flow rate of 10 L min^{-1}, nebulizer pressure of 35 psi and capillary voltage of 4000 V. Analysis of bixin were performed on Multiple Reaction Monitoring (MRM) of ion pairs to use the mass transitions (395/157). The bixin were scanned as positive and quantified via calibration curves using authentic standards (1 to 200 µg and 0.1 to 500 ng, respectively) (Sigma-Aldrich, St. Louis, Missouri, USA). The data generated were analyzed in Mass Hunter Workstation software and results are expressed on a dried weight (DW) basis.

Carotenoids and chlorophyll content

Total carotenoid and chlorophyll were extracted from 20 mg of leaves freeze-dried with 500 µL of acetone 80% in a 1.5 mL microcentrifuge tube. Tubes were vortexed three times for 30 seconds and centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was transferred to a new microcentrifuge tube and to the remaining pellet was added 500 µL of acetone 80%, vortexed and centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was transferred to the microcentrifuge tube contain the previously obtained supernatant. The 200 µL of supernatant was added into a microplate from Multiskan FC, Thermo Scientific® and read in absorption spectra of 663 nm (chlorophyll *a*), 646 nm (chlorophyll *b*) and 470 nm (total carotenoids). The absorption spectra values were used in equations (Chlorophyll *a* (C_a) = 12.21 A_{663} – 2.81 A_{646}; Chlorophyll *b* (C_b) = 20.13 A_{646} – 5.03 A_{663} and total carotenoids = (1000 A_{470} – 3.27 C_a – 104 C_b)/198) to get to total carotenoids and chlorophyll content according to Lichtenthaler (1987). The experimental design was completely randomized scheme with four replicates, composed by leaves from ten plants per

replicate. The data were submitted to ANOVA, and the means compared by the Tukey's test ($P \geq 0.05$).

Global DNA methylation

Genomic DNA was extracted from leaves of 10 plants per treatment, using Doyle and Doyle (1987) methodology with addition of polyvinylpyrrolidone (PVP) 2% to CTAB buffer. The analysis of DNA integrity and quality was made in agarose gel denatured 1.5% (w/v), stained with gel red (Biotium) and quantified in NanoDrop™ 2000/2000c spectrophotometers of Thermo Scientific™. The RNA was digestion in DNA samples using RNA A (Sigma-Aldrich®) according to fabricant and after that, the DNA samples was digestions with nuclease P1 and alkaline phosphatase (Sigma-Aldrich®) were performed according Fraga et al. (2012). The HPLC/MS/MS analysis were performed to according described by Fraga et al. (2012) using a Shim-pack XR-ODS 2.2 lm C18 column (30 mm 9 2 mm) (Shimadzu®) and an Agilent 1200® HPLC with a quaternary pump coupled to a mass spectrometer 3200 Q TRAP® LC/MS/MS (Applied Biosystems®) were used.

The experimental design was in a randomized scheme with five replications, composed by DNA extracted from leaves of ten plants per replicate. The identification of each nucleoside was made according to the known mass of each (5mdC: 242.1/126.3; dC: 228.2/112.2; dG: 268.1/152.3; dA: 252.3/135.9; dT: 243.3/127.2, in Daltons). 5mdC quantification (%) was performed according formula: 5 mdC concentration/((5 mdC concentration + dC concentration).100). The peak area obtained was analyzed by Analyst 1.5.1 (Applied Biosystems®). Data were analyzed by Statistica® (Statsoft Inc., Tulsa, USA) for Windows® and submitted to ANOVA. The means were compared by the Tukey's test ($p \geq 0.05$).

Real time RT-qPCR analysis

Total leaves of the ten plants per treatment (two flasks) were used to RNA extraction. Fresh leaves were powdered in liquid nitrogen with a mortar and pestle, then 300 µL of phenol: chloroform: isoamyl alcohol (25: 24: 1) and 450 µL of lysis buffer NTES (NaCl 0.1 M, Tris-HCl 0.01 M, EDTA 1 mM, SDS 1%, Diethyl pyrocarbonate (DEPC) water (0.1%), were added into 100 mg of powdered leaves and centrifuged 10,000 *g* for 10 min. The aqueous phase was transferred to a new

tube to which was added NaOAc 1:10 (v/v) (3 mM, pH 5.4), ethanol 2:1 (v/v) homogenized and incubate at -20 °C for at 1 h.

The samples were centrifuged at 10,000 *g* for 15 min and the aqueous phase was discarded and pellet was suspended in 100 µL of DEPC water (0.1%) and 100 µL of lithium chloride (4M) overnight. After this, samples were centrifuged at 10,000 *g* for 15 min at 4°C and the aqueous phase was discarded and pellet was suspended in 100 µL of DEPC water (0.1%) 10 µL NaOAc (3 mM, pH 5.4), 200 µL of ethanol homogenized and incubate at -20 °C for at 1 h. Then samples were centrifuged at 10,000 g for 20 minutes at 4°C, the aqueous phase was discarded and pellet was washed with 800 µL of ethanol 70% and centrifuged at 10,000 g for 3 minutes at 4°C. The aqueous phase was discarded and the pellets were dried at room temperature and resuspended in 30 µL of DEPC water (0.1%).

The RNA was digested with Ambion™ DNase I for degradation of DNA remaining from RNA extraction according to the manufacturer's specifications. The analysis of RNA integrity and quality was made in agarose gel denatured 1.5% (w/v), stained with gel red (Biotium®) and quantification in NanoDrop™ 2000/2000c Spectrophotometers of Thermo Scientific™. For the cDNA synthesis, it was used 1 µg of total RNA, dNTPs and reverse transriptase M-MLV (Invitrogen®), according to the manufacturer's specifications.

Real-time RT-PCR was performed on a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD), using Applied Biosystems SYBR Green Master Mix (Bio-Rad®). The experimental design was in a randomized scheme with three replications, composed by RNA extracted of leaves from ten plants. Genes expression of *BoCCD4-2(*KT359023), *BoCCD4-3*(KT359024), *BoALDH3H1-1* (KT359033), *BoSABATH1*(KT359049), *BoSABATH3*(KT359051) and *BoSABATH4*(KT359052) were evaluated with primers reported by Cárdenas-Conejo et al. (2015) (Table S1). Gene expression profile was normalized to the *Bo18S* level expression (Cárdenas-Conejo et al. 2015) and the calculations for the values were made using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen 2001), with three biological replicates and two technical replicates and the means were compared using confidence interval by the Dunnett's test ($P \leq 0.01$).

Pearson correlation analysis was performed between genes expression *BoCCD4-2*, *BoCCD4-3*, *BoALDH3H1-1*, *BoSABATH1*, *BoSABATH3* and

BoSABATH4 with bixin content. The values were normalized to log2 and illustrated in a heat map plotted in the Metaboloanalyst online program (Chong et al. 2018).

RESULTS

B. orellana produces bixin in the leaves and stores in secretory structures that are distributed along the leaves and others organs of the plant (Freitas 2009, Metcalfe and Chalk, 1957). The bixin storage occurs in both adaxial and abaxial leaf surfaces, being more evident on the abaxial (Figure 1D).

After 60 days of the exposure of *B. orellana* shoot apexes to 5-AzaC, it was observed the formation of leaves from the shoot meristem, and roots from the basal region of explant (Figure 1 A to C). The exposure of shoot apexes to 1 and 100 µM of 5-AzaC for 48 h resulted in different root development (Figure 1 A to C). For instance, shoot apexes in presence of 1 µM of 5-AzaC (Figure 1 B) which showed less root development than the control (Figure 1 A).

Leaves from shoot apexes exposed to 1 and 100 µM 5-AzaC for 48 h did not influence the production of chlorophyll *a* and *b* (Figure 2 A). The total carotenoids in the leaves of explants exposed to 5-AzaC show concentration-dependent behavior. As the concentration of 5-AzaC increases, the total carotenoids' concentration increase although this increment was not statistically significant (Figure 2 B). Also, the global DNA methylation on the leaves formed after 60 days of the exposure to 1 and 100 µM of 5-AzaC did not varied compared to the control (Figure 2 C).

In the neoformed leaves from *B. orellana* stem apexes, 5-AzaC did not affect the expression of the bixin biosynthesis candidate genes *BoCCD4-3*, *BoCCD4-2*, *BoALDH3H1-1* and *BoSABATH3*. The exposure of plants to 100 µM 5-AzaC significantly reduced the expression of *BoSABATH4* by 1.8-fold (Figure 3 E) and *BoSABATH1* by 1.7-fold (Figure 3F). The bixin content was augmented inleaves of plants under 1 µM 5-AzaC, compared to the control, while in 100 µM treatment the leaves had a intermediary content of bixin (Figure 3 G).

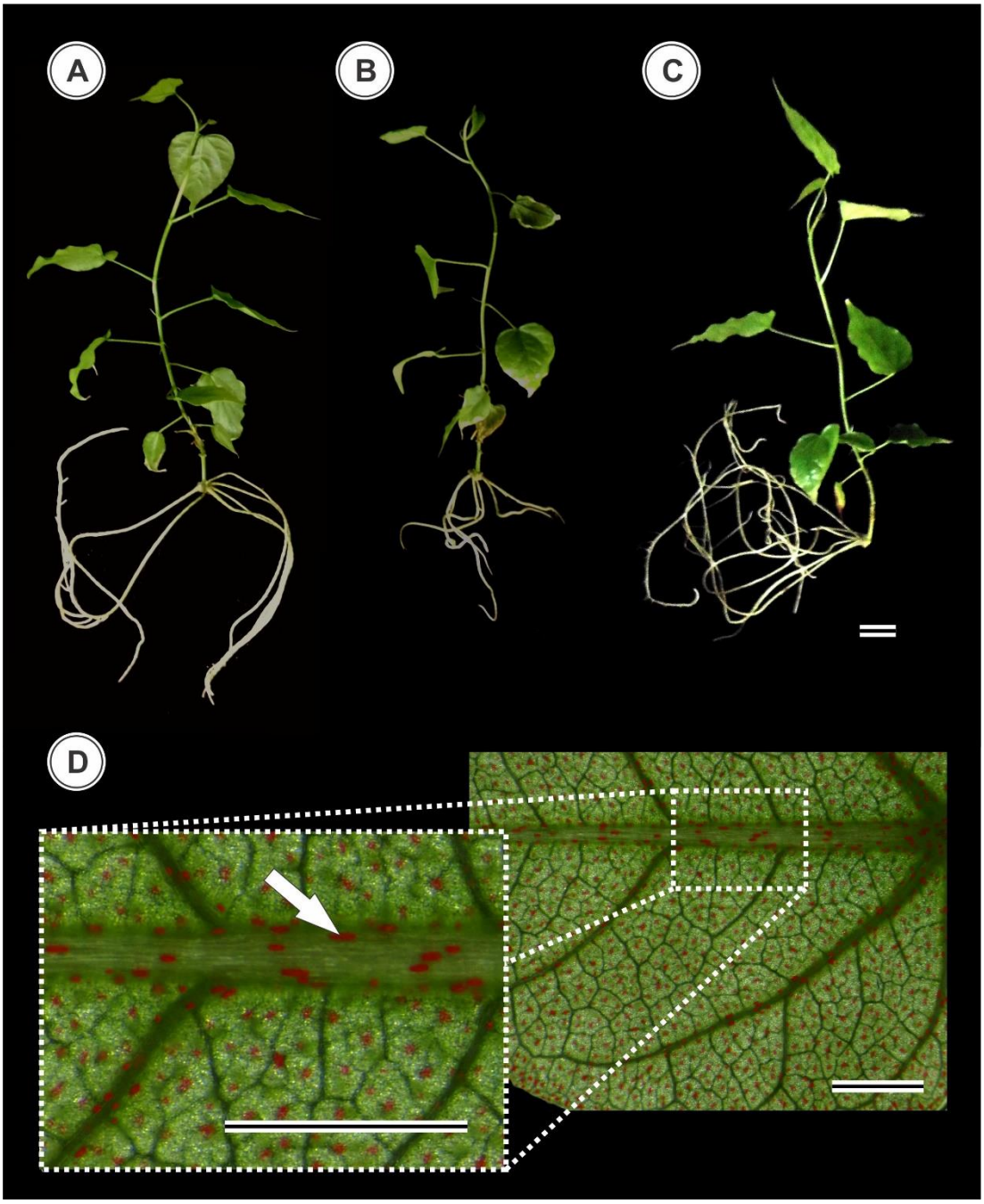

Figure 1. Plants of *Bixa orellana* L. after 60 days submitted to different concentrations of 5-azacytidine. **A** – 0, **B** – 1, and **C** – 100 µM 5-azacytidine. **D** - Leaf showing carotenoid storage cells. Bars: A – C = 10 mm; D = 1 mm.

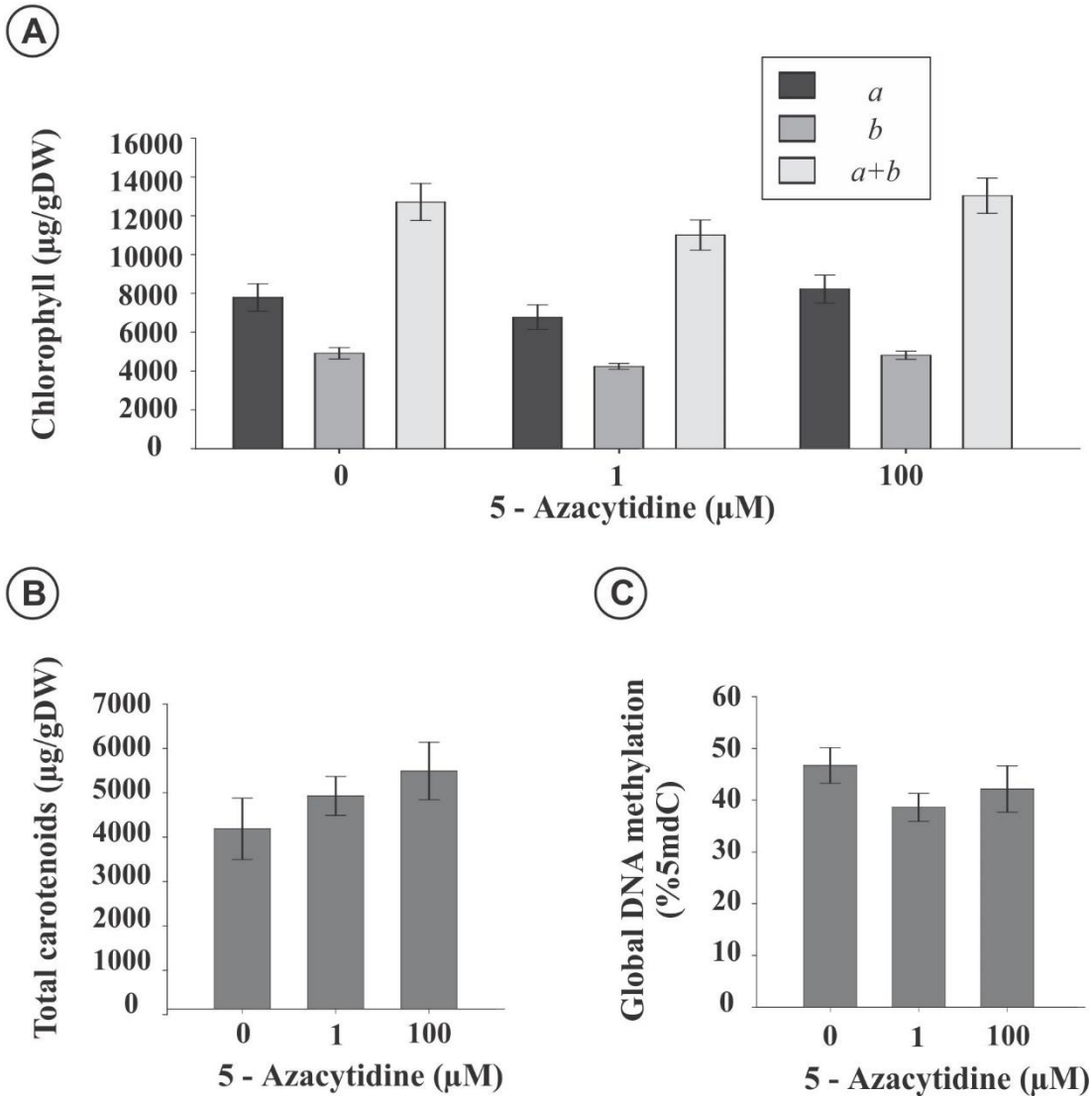

Figure 2. Influence of 5 – azacytidine in leaves of *Bixa orellana* L. cultured *in vitro*. **A** – Chlorophylls (µg g DW^{-1}); **B** – Total carotenoids; **C** - Global DNA methylation. Abbreviation: *DW* – Dry Weight. **P*≤0.05 by Tukey's test.

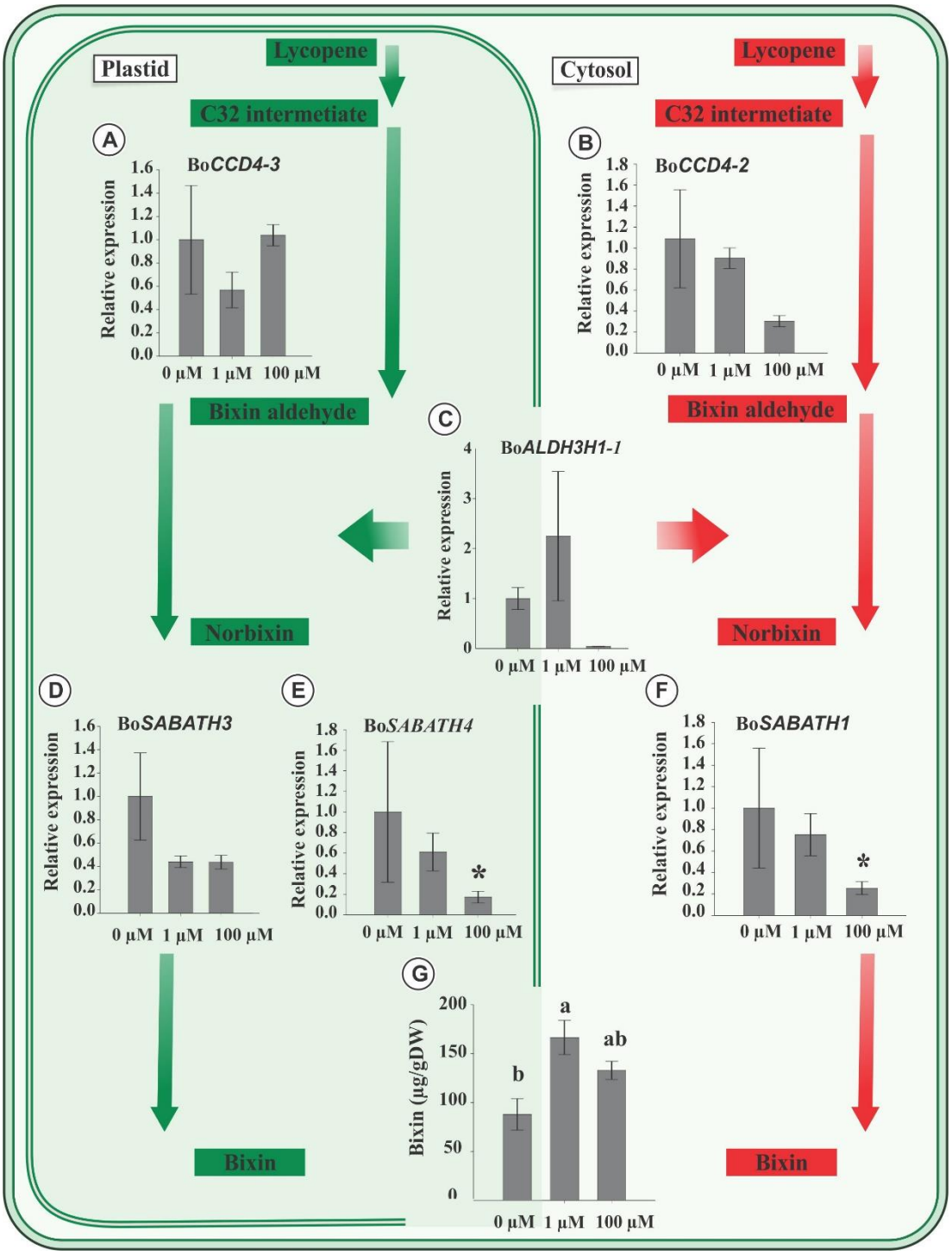

Figure 3. Relative expression of bixin pathway candidate genes in leaves of *Bixa orellana* L. after 60 days submitted to different concentrations of 5-azacytidine. **A** – *BoCCD4-3*; **B** – *BoCCD4-2*; **C** – *BoALDH3H1-1*; **D** – *BoSABATH3;* **E** – *BoSABATH4;* **F** – *BoSABATH1*. Gene expression relative to *Bo18S*. Data presented as mean ± standard deviation (n=3), **P*≤0.01 by Dunnett's test. **G** – Synthesis of bixin (µg g DW^{-1}). Abbreviation: *DW* – Dry Weight, means jallowed by the same letters do not differ by Tukey's test (*P*≤0.05).

The correlation analyzes of bixin pathway candidate genes showed significant positive correlation between the genes *BoSABATH4* x *BoSABATH1*, *BoALDH3H1-1* x *BoSABATH1*, *BoALDH3H1-1* x *BoSABATH4*, *BoCCD4-2* x *BoSABATH1*, *BoCCD4-2* x *BoSABATH4* and *BoCCD4-2* x *BoALDH3H1-1* (Figure 4). These results evidenced the coexpression of these four bixin pathway candidate genes, with genes encoding enzymes for both plastid and cytosol (Figure 3) whit correlated expression patterns (Figure 4).

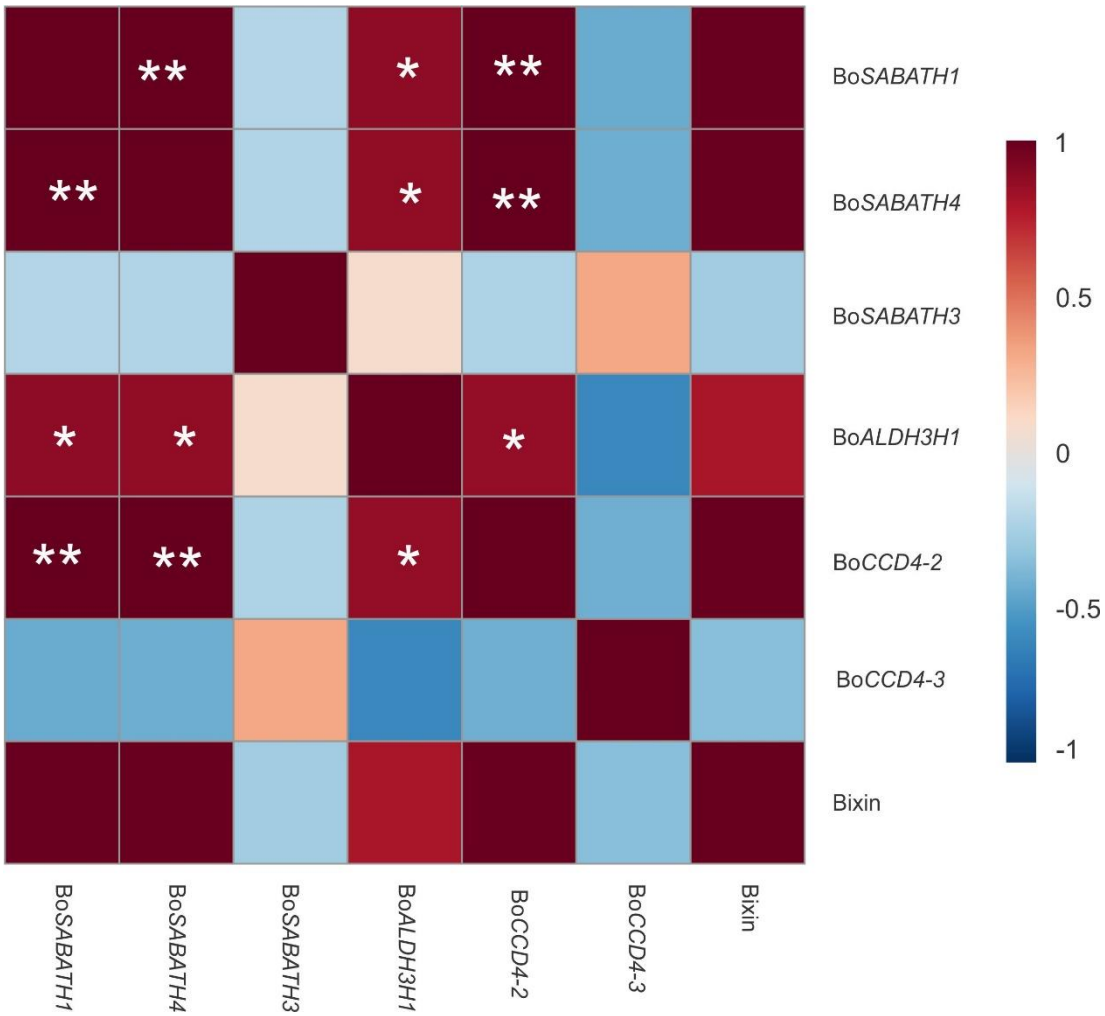

Figure 4. Pearson correlation heat map between genes *BoCCD4-3*; *BoCCD4-2*; *BoALDH3H1-1*; *BoSABATH3; BoSABATH4; BoSABATH1* and bixin content. **P*<0.05 and ** *P*<0.01 by Tukey's test.

Notwithstanding the 5-AzaC treatment induced increased bixin content in leaves (Figure 3 G), no significant correlations between the bixin content and the expression of the bixin pathway candidate genes were observed (Figure 4). However,

there was a positive correlation trend (although not significant) between the bixin content and the genes evaluated, except for the genes *BoCCD4-3* and *BoSABATH3*, demonstrating a probably involvement of these genes with the bixin pathway (Figure 3).

DISCUSSION

DNA methylation is conserved in plants and mammals and precise patterns of genomic DNA methylation are crucial for development (Song and Chen 2015; Satge et al. 2016). The demethylation of genomic DNA can result in the reactivation on developmentally silenced genes (Tyč et al. 2017). The methylation of DNA is maintained by methyltransferases, that recognizes hemi-methylated CG dinucleotides following DNA replication and methylates the unmodified cytosine in the daughter strand. The 5-AzaC generally causes demethylation via blocking the activity of maintenance DNA methyltransferases (Santi et al. 1984; Chiappinelli et al.2015; Cai et al. 2017). DNA demethylation caused by 5-AzaC requires repeatedly DNA replication, because only newly synthesized DNA strands can be non-methylated (Tyč et al. 2017).

In our experiments, 5-AzaC did not affect the total DNA methylation on the leaves formed 60 days after the exposure to this compound. Possibly the exposure time to 5-AzaC was not sufficient for altering total DNA methylation on meristematic tissue of the stem apexes used as explant. Stem apexes explant can limited accessibility of plant meristems reduce the possibility to affect DNA methylation in a whole plant level (Tyč et al. 2017). However, neoformed leaves from *B. orellana* stem apexes after 60 days exposed 48 hours to 5-AzaC increased the bixin content and showed differential gene expression of the *SABATHs* family genes.

The plant phenotype in response to 5-AzaC depends on the concentration and the species and may cause no significant effect or even induce programmed cell death in plant tissue culture (Betekhtin et al. 2018). Here we observed that low concentrations influenced in incrased bixin continent in leaves after 60 days of exposed 48 hours to 5-AzaC, however, not influence the total DNA methylation, there is a possibility that the DNA has been methylated again over 60 days after exposure to 5-Azac. The treatment with 5-AzaC in other plant species can induces

the expression of genes encoding transcription factors, up-regulation of a wide range of transposable elements and increase the production of secondary metabolites (Kiselev et al. 2011; Xu et al. 2017; Yang et al. 2018). Here, we observed only changes in the secondary metabolism, i.e., bixin production, not altering primary metabolism features such as chlorophylls *a* and *b* contents.

In cultured calli of *Citrus paradisi*, the treatment with 5-AzaC induced carotenoid degradation with strong activation of *carotenoid cleavage dioxygenases 1* (*CCD1*), which has strong catalytic activities toward zeaxanthin, b-carotene and lycopene, producing apocarotenoids (Xu et al. 2017). In *B. orellana,* the leaves and immature seeds show increased expression of the *BoCCD1* gene, and was localized by *in situ* reverse transcriptase-mediated PCR on bixin stored cell in leaves, stalk and seeds indicating that this gene may be involved with the bixin biosynthetic pathway (Rodríguez-Ávila et al. 2011b; Rivera-Madrid et al. 2013).

The seeds are commonly studied in the bixin pathway, and so far little is known about the biosynthesis routes in other organs of the plant. Leaves produce lower bixin content than seeds (Rodríguez-Ávila et al. 2011a and b) and several works relate the absence of lycopene, known as a bixin pathway precursor in *B. orellana* leaves (Rodríguez-Ávila et al. 2011b; Rivera-Madrid et al. 2013). These results suggest that the bixin can be synthesized from other yet unknown precursors or in alternative biosynthetic pathway from the mevalonate (MVA) pathway in the cytosol (Rodríguez-Ávila et al. 2011b; Rivera-Madrid et al. 2013; Cárdenas-Conejo et al. 2015; Louro and Santiago 2015).

There are many gaps around the bixin pathway, based on the assumption that this biosynthetic route occurs in different cellular compartments: plastids and cytosol, we elected the genes *BoCCD4-2*, *BoCCD4-3*, *BoALDH3H1-1*, *BoSABATH1*, *BoSABATH3* and *BoSABATH4,* which according to Cárdenas-Conejo et al. (2015) can be involved on these pathways (Figure 3). In our results, it seems to be a pattern of coexpression between *BoCCD4-2, BoALDH3H1-1, BoSABATH1* and *BoSABATH4* genes in the leaves from *B. orellana*. Cárdenas-Conejo et al. (2015) also observed that in both immature and mature seeds the expression of genes *BoSABATH3, BoSABATH4, BoCCD4-2* and *BoALDH3H1-1* have similar patterns.

The mature seed is the developmental stage of the seed with the highest bixin content in *B. orellana* (Rodríguez-Ávila et al. 2011a and b). In the mature seed as mentioned above the *BoSABATH* genes reduce in their expression. A similar result

was observed in the neoformed leaves from meristematic apexes exposed to 5-AzaC, where the reduction of *BoSABTH* expression resulted in higher bixin accumulation.

CONCLUSIONS

The temporary exposure of shoot apexes cultivated *in vitro* to the demethylating compound 5-AZA did not affect the genome global methylation, chlorophylls and total carotenoid content in leaves of *B. orellana.* However, plants submitted to 5-AZA induced the downregulation of the SABATH family genes and increased the bixin content in the leaves, suggesting that this methyltransferase inhibitor can direct regulate the biosynthesis pathway and the production of bixin in *B. orellana*. Due to the complexity the bixin biosynthetic route, the relation among DNA methylation, gene regulation and bixin production needs to be further explored. In addition, this work opens new perspectives on the influence of 5-AzaC, and hence the levels of methylation and demethylation, on bixin biosynthesis and accumulation during the plant development, knowledge that can be a useful tool for the production of *B. orellana* plants with higher bixin content.

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SUPPLEMENTARY MATERIAL

Table S1. *Bixa orellana* L. primers used to RTq-PCR of bixin biosynthesis pathway genes.

RT-qPCR primers to *Bixa orellana* L.	
Gene ID	**Sequence**
F-CCD4-2	5' – GATTCCCACCTCTCTGGA – 3'
R-CCD4-2	5' – AACATATTGGGCATGCGA – 3'
F-CCD4-3	5' – ATGAGGACACCAAGGACG – 3'
R-CCD4-3	5' – CTAGCATCATTTTGGCAACG – 3'
F-ALDH3H1	5' – TGGTAGATACTTTCAGAGAGG – 3'
R-ALDH3H1	5' – GTTGGAGCAATCTTCAGC – 3'
F-SABATH1	5' – ACGGAGGAAGCTATCACCA – 3'
R-SABATH1	5' – GACGTGATATTCAGGGGATC – 3'
F-SABATH3	5' – TTATGGCCGTCTCTGAGGT – 3'
R-SABATH3	5' – ACCTGGCACTCCTGATACG – 3'
F-SABATH4	5' – TCCCGTCTACAAATGGCA – 3'
R-SABATH4	5' – AATTTCGGAGATGACCAGG – 3'
F-18S	5' – CGGCTACCACATCCAAGGAA– 3'
R-18S	5' – GCTGGAATTACCGCGGCT– 3'

CHAPTER II

INFLUENCE OF IRRADIANCE AND LIGHT QUALITy SPECTRA IN TWO CULTIVAR OF ANNATTO (*Bixa orellana* L.) WITH CONTRASTING BIXIN PRODUCTION CULTURED *in vitro*

ABSTRACT

Bixin and norbixin are important natural dyes, used worldwide in various foods, pharmaceutical and cosmetics branches, among others, obtained from seeds of *Bixa orellana* L. (annatto) a tree native to tropical North, Central and South America. The bixin are produced in all tissues of the plant and accumulated in bixin-secretory structures. The *in vitro* plant tissue culture can be used for micropropagation and study of the bixin pathway. The light is one of the many factors in plant in vitro culture that can influence the development, growth and in several physiological processes, as in the production of secondary metabolites. The light is characterized by irradiance, wavelength, quality and direction. In this work, we analyze for the first time the effect of irradiance and light quality in the developmental of shoot apexes of *B. orellana in vitro*. The number of leaves per plant, stomatal density, leaf area, leaf expansion, chlorophylls and carotenoids contents, malondialdehyde (MDA) and bixin content were analyzed in the leaves of Piave vermelha and UESB74 cultivars of *B. orellana* cultured *in vitro*. The cultivar Piave vermelha produced 1.6-fold more bixin than the UESB74 in leaves and visibly higher bixin content in the seeds. The stomata cells in the leaves of both cultivars were in paracytic arrangement whit peltate trichomes predominant along the adaxial and abaxial surfaces of the leaf epidermis. For *in vitro* culture of cultivar Piave vermelha the blue/red LED light was the best, on the other hand for UESB74 the culture in fluorescent light is indicated. If the use fluorescent light is chosen, for both varieties, the irradiance of 50 μmol m^{-2} s^{-1} is indicated. This work opens new perspectives on the regulation and the heterogeneity of the bixin biosynthesis pathway.

Key words: Annatto, bixin pathway, irradiance, light quality.

INTRODUCTION

Bixa orellana L. (annatto) is a woody tree species native to tropical North, Central and South America (Moreira et al. 2015; Dequigiovanni et al. 2018) and an important source of natural dyes liposoluble apocarotenoid bixin, and one of the few natural hydrosoluble dyes: the norbixin which vary in color ranging from yellow to brown, through red (Giuliano et al. 2003; Rivera-Madrid et al. 2013). Therefore, are used since pre-Columbian times as textile and food dye and body painting (Giuliano et al. 2003). Currently, they are used worldwide in various foods, pharmaceutical and cosmetics branches, among others (Marcolino et al. 2011; Shahid-ul-Islam et al. 2016).

The only available source of commercially bixin is the *B. orellana* seeds, where the bixin is produced and stored in large quantities (Preston and Rickard 1980; Giuliano et al. 2003). Nevertheless in other tissues such roots, stalk, flower and leaves there are bixin-secretory structures (Freitas 2009; Metcalfe and Chalk, 1957; Mahendranath et al. 2011; Rodríguez-Ávila et al. 2011; Rivera-Madrid et al. 2013). Other species also produce bixin such as *Costus pictus*, grape and saffron, but in lower concentrations (Ramamoorthy et al. 2010; Annadurai et al. 2012). The seeds are commoly used to study of the bixin biosynthesis pathway; however, bixin is produced throughout the plant tissues that enable the in vitro plant tissue culture techniques to be used for culture and study of the bixin biosynthesis pathway in the other tissues. *In vitro* culture of plants has advantages, such as restricted external factors, control of the culture environment, and the possibility to use elicitors for the metabolic pathway of interest and without the need to use an adult plant, which would take longer (Hussain et al. 2012; Murthy et al. 2014).

Light is an abiotic factor characterized by its irradiance, wavelength, duration and direction being fundamental for the development of the plant as an energetic source for photosynthesis, and control of various biological activities (Muneer et al. 2014; Batista et al. 2018; Plantenga et al. 2019). A plethora of biological responses to the variation of light irradiance throughout the day and seasons depend on the genotype and the life cycle of the plant besides mechanisms that may involve the whole plant, starting from molecular levels, cellular, plant length, leaf area, chlorophyll content and others (Givnish, 1988; Ruban, 2015). Specific irradiances in plant tissue culture can improve the development (Muneer et al. 2014),

micropropagation (Gupta et al. 1997), healing after grafting (Muneer et al. 2015) and the production of secondary compounds (Batista et al. 2016; Irshad et al. 2018).

The light quality is another factor that affects *in vitro* growth, development, morphogenetic responses, secondary metabolite production and carotenoid concentrations in plants (Gupta et al. 1997; Li et al. 2010; Lin et al. 2013; Kopsell et al. 2014; Kwon et al. 2015; Amoozgar et al. 2017; Batista et al. 2018). Genes of the carotenoid biosynthesis pathway have their expression modulated by specific spectral bands, such as white, blue and red, which may lead to an increase or decrease in the production of carotenoids (Tuan et al. 2013; Amoozgar et al. 2017). The carotenoid biosynthesis pathway is also regulated by the redox potential plastoquinone pool caused by exposure of the photosynthetic system to increased irradiance (Woitsch and Römer, 2003).

The *in vitro* culture of *B. orellana* is commonly performed under fluorescent light (Paiva Neto et al. 2003 a and b; Carvalho et al. 2005; Cruz et al. 2014; Cruz et al. 2015; Mohammed et al. 2015), which is less durable offer inferior lighting conditions and generate more waste products compared to light-emitting diodes (LEDs) (Batista et al. 2018). Based on the benefits of using better light qualities in plant tissue culture, the implementation of LED technology in the *in vitro* culture of *B. orellana* is interesting due its great commercial value. It is known that *B. orellana* cultured under ultraviolet radiations (UV-B e UV-CAs) increased the β-carotene content, decreased the total carotenoids and not affected the bixin content in the leaves (Sankari et al. 2017).

Therefore, to LED technology implementation in the *in vitro* culture of *B. orellana* it is necessary to evaluate the appropriate conditions that will promote an increased growth and bixin production. There is a huge diversity of *B. orellana* cultivars around the world (Rivera-Madrid et al. 2006; Akshatha et al. 2011; Dequigiovanni et al. 2018), among them, 'Piave Vermelha' stands out due its good productivity, associated with high levels of bixin, ranging from 3.5 to 5.0%, which promotes great acceptance in the market of these seeds (Figure 1 A) (Lemos et al. 2011). There are several varieties of *B. orellana* that produce from low to high bixin content in the seeds (Rivera-Madrid et al. 2006; Akshatha et al. 2011;), such different phenotypes allow genetic and physiological studies that aid in the development of elite varieties and in the study of bixin biosynthesis pathway.

The cultivar UESB74 is an example of low bixin content, which leads to changes in seed phenotype that do not show the red dye (Figure 1 B). Here, we tested for the first time the effect of irradiance and light quality in growth and chlorophylls, carotenoids and bixin contents in leaves of plants induced *in vitro* from shoot apexes of two *B. orellana* cultivars with contrasting phenotypes regarding bixin production.

MATERIALS AND METHODS

Plant material and in vitro *culture*

Seeds of the cultivars Piave Vermelha (donated by Christian Hansen) and UESB74 (donated by State University of the Southwest Bahia– UESB Germplasm Bank, Campus Vitória da Conquista) (Figure 1 A and B) were scarified and sterilized, according to Carvalho et al. (2005 b), and germinated in test tube containing 10 mL of MS medium (Murashige and Skoog, 1962) with half of the salt composition (MS ½), supplemented with B5 medium vitamins (Gamborg et al. 1968). All the other experiments were conducted using flask (6.5 cm of diameter x 10 cm of length) containing 35 mL of JADS medium (Correia 1995). Both media formulation were supplemented with 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol and solidified with 5.0 g L^{-1} agar (PhytoTechnologies®). The pH was adjusted to 5.7 ± 0.1 and the media autoclaved at 120 °C, 108 kPa for 20 min and kept in growth room at 25 ± 2 °C, 16 h of photoperiod and irradiance of 50 µmol m^{-2} s^{-1}.

After 30 days of culture, shoot apexes from germinated plants were inoculated in JADS medium with 4.56 µM zeatin for 60 days, then they were transferred to JADS medium with 5 µM IBA and kept in the growth room at 25 ± 2 °C for 45 days, in two different conditions of irradiance and light quality. To evaluate the influence of the irradiance, shoot apexes were cultured under 50, 150 and 200 µmol m^{-2} s^{-1} for 45 days, and other set of shoot apexes were grown under 50 µmol m^{-2} s^{-1} for 15 days, then transferred to 50, 150 and 200 µmol m^{-2} s^{-1} irradiance, where remained for 30 days. Both conditions were provided by tubular fluorescent lamps (HO Sylvania T12, 110W, São Paulo, Brazil) (Figure 1 C).

In order to evaluate the influence of light quality, the shoot apexes were cultivated under 50 µmol m^{-2} s^{-1} using: two fluorescent lamps (HO Sylvania T12, 110W, São Paulo, Brazil); two white light emitting diode (LED) lamps (SMD 100, 18 W, Vilux®, Vitória, ES, Brazil); and two lamps LED blue/red (Tec-Lamp-

660/450-120 cm, 36 W, Tecnal®, Piracicaba, Brazil). The absorption spectra at wavelengths from 350 to 800 nm were obtained by spectrophotometer using Ocean Optics Spectra-Suite software (OceanOptics®, Dunedin, FL, SP100 Version 1.0.0.1 Photo System Instruments, Czech Republic) (Figure 1 D).

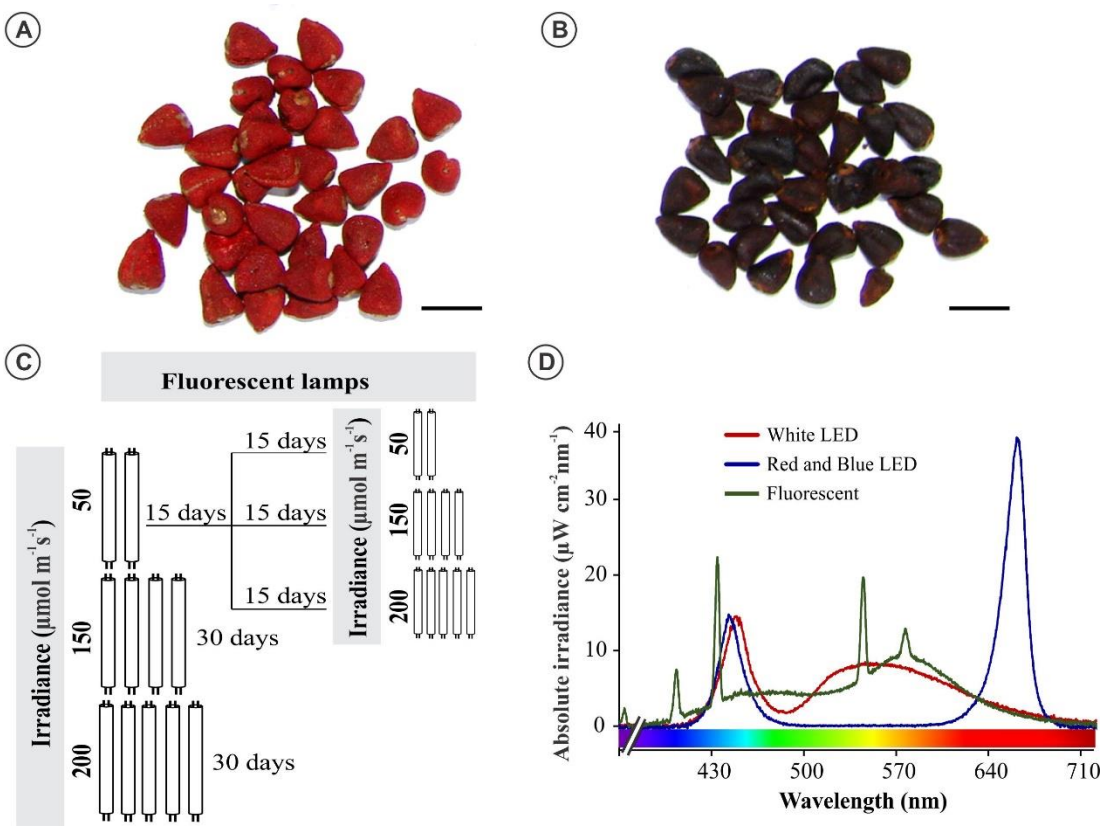

Figure 1. Plant material and light quality conditions used. A – Piave Vermelha seeds; B – UESB74 seeds; C – Irradiance (µmol m^{-1} s^{-1}) emitted by fluorescent lamps used in the light irradiance experiment; D - Absolute irradiance (µW cm^{-2} nm^{-1}) emitted by fluorescent, white LED and blue/red LED lamps used in the light quality experiment. Bar = 0.5 cm.

After 45 days of culture, stem length (mm), number of roots/plant, root length (mm), rooting frequency (%), fresh weight (g), dry weight (g), number of leaves/plant, leaf expansion (cm^2), leaf area/plant (cm^2) (calculated using imageJ software (Schneider et al. 2012)), stomatal density in the abaxial and adaxial leaf surfaces (mm^2), chlorophyll *a* and *b* (µg g^{-1} dry weight (DW)), total carotenoids (µg g^{-1} DW), lipid peroxidation through quantification of malondialdehyde (MDA) (ηmol g^{-1} DW) and bixin content (µg g^{-1} DW) were evaluated. All experiments were conducted on a completely randomized design (CRD) in a factorial scheme with five

replications, composed by a flask containing five explants, except for analyses of chlorophyll, carotenoids and bixin where it was used four replicates per treatment. The data of all analyzes were submitted to ANOVA, and the means were compared by the Tukey's test ($P \geq 0.05$).

Stomatal density

Full expanded leaves from plants grown *in vitro* under different irradiances and light qualities were diaphanized according Johansen (1940) with modifications and dehydrated in ethanolic series (50%, 70% and 100%, for 30 min each), After, leaves were clarified and stained in alcoholic fuchsin solution. Slides were mounted using Permount. Five leaves per treatment and three different regions in each leaf surface were analyzed: proximal, central and distal. The images were obtained with a 40x objective using a fluorescence microscope and the images were obtained using a photomicroscope. Stomatal density was calculated by the average of three leaf regions.

Lipid peroxidation

Lipid peroxidation in the leaf tissue was measured in malondialdehyde (MDA, a product of lipid peroxidation) content determined by the thiobarbituric acid (TBA), performed according to Heath and Packer (1968) with modifications. Fifty mg leaf sample sprayed in liquid nitrogen were homogenized in 1 mL trichloroacetic acid (TCA) (1% w/v), then centrifuged at 12,000 x *g* for 15 min at 4 °C. 500 µL of supernatant was collected and added to 500 µL of TCA (20% w/v) + thiobarbituric acid (TBA) (0.5% w/v). The mixture was heated at 95 °C for 30 min and then kept on ice-bath for one minute. After centrifuge at 12,000 x *g* for 10 min, 200 µL of the supernatant was add in a microplate from Multiskan FC, Thermo Scientific® and read in absorption spectra at 532 nm and 600 nm. The concentration of MDA was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968). The design of the analyses was in a CRD with five replications, composed by leaves from ten plants per replicate.

Total carotenoids and chlorophyll content

Total carotenoids and chlorophylls were extracted from 20 mg of leaves freeze-dried with 500 µL of acetone 80% in a 1.5 mL microcentrifuge tube. Tubes

were vortexed thrice for 30 seconds and centrifuged at 10,000 x *g* for 10 min. The supernatant was transferred to a new microcentrifuge tube and to the remaining pellet was added 500 µL of acetone 80% vortexed and centrifuged at 10,000 x *g* for 10 min, the supernatant was transferred to microcentrifuge tube with the previously obtained supernatant. 200 µL of supernatant was add in a microplate from Multiskan FC, Thermo Scientific® and read in absorption spectra of 663 nm (chlorophyll *a*), 646 nm (chlorophyll *b*) and 470 nm (total carotenoids). The absorption spectra values were used in equations (Chlorophyll *a* (C_a) = 12.21 A_{663} – 2.81 A_{646}; Chlorophyll *b* (C_b) = 20.13 A_{646} – 5.03 A_{663} and total carotenoids = (1000 A_{470} – 3.27 C_a – 104 C_b)/198) get to total carotenoids and chlorophyll content according to Lichtenthaler (1982). The design was in a CRD with four replications, composed by leaves from ten plants per replicate.

Bixin content

Bixin content was performed according to Napoleão et al. (2017). Approximately 10 mg of freeze-dried leaves were grounded in liquid nitrogen followed of extraction by twice 300 µL of methanol: isopropanol: acetic acid (20:79:1; v/v/v). The samples were vortexed (4 times for 20 s), sonicated (10 min), kept on ice (30 min), centrifuged (20,000 x *g*, 10 min at 4 °C), and the supernatant was collected. Then, the supernatant was filtered (Econofltr PVDF 13 mm and 0.2 µm; Agilent Technologies, Santa Clara, CA, USA) and used for liquid chromatography-mass spectrometry (LC-MS) analysis.

Samples of the final supernatant (5 µL) were automatically injected into the LC-QqQ MS (Triple Quadrupole 6430, Agilent Technologies, Waldbronn, Germany) equipped with an Eclipse Plus C18 (2.1 × 50 mm, 1.8 µm) column and guard column Zorbax SB-C18 (1.8 µm, Agilent) at 26 °C. The mobile phase consisted of 0.02% acetic acid in water (solvent A) and 0.02% acetic acid in acetonitrile (solvent B) at a constant flow rate of 300 µL min^{-1}. A linear gradient was applied as follow: 0–3 min and 2%–60% of B, 3–8 min and 60%–99% of B, 8–11 min and 99% of B, 11–12 min and 99% to 2% of B and 12–15 min and 2% of B. A mass spectrometer ionization source ESI (Electrospray Ionisation) was used with the following conditions: gas temperature of 300 °C, nitrogen flow rate of 10 L min^{-1}, nebulizer pressure of 35 psi and capillary voltage of 4000 V. Analysis of bixin were performed on Multiple Reaction Monitoring (MRM) of ion pairs to use the mass transitions (395/157). The

bixin were scanned as positive and quantified via calibration curves using authentic standards (1 to 200 µg and 0.1 to 500 ng, respectively; Sigma-Aldrich, St. Louis, Missouri, USA). The data generated were analyzed in Mass Hunter Workstation software and results are expressed on a dried weight (DW) basis.

RESULTS

The cultivars analyzed in this work showed different phenotypes regarding bixin content in leaves (Table 2 and 4) and in seeds (Figure 1 A and B). The cultivar Piave Vermelha produces 1.6-fold more bixin than the UESB74. The leaves of both cultivars of *B. orellana* showed stomata predominant in the abaxial surface, but was also found in the adaxial surface in lower number. The stomata cells were in paracytic arrangement whit peltate trichomes predominant along the adaxial and abaxial surfaces of the leaf epidermis (Figure 2 A and B).

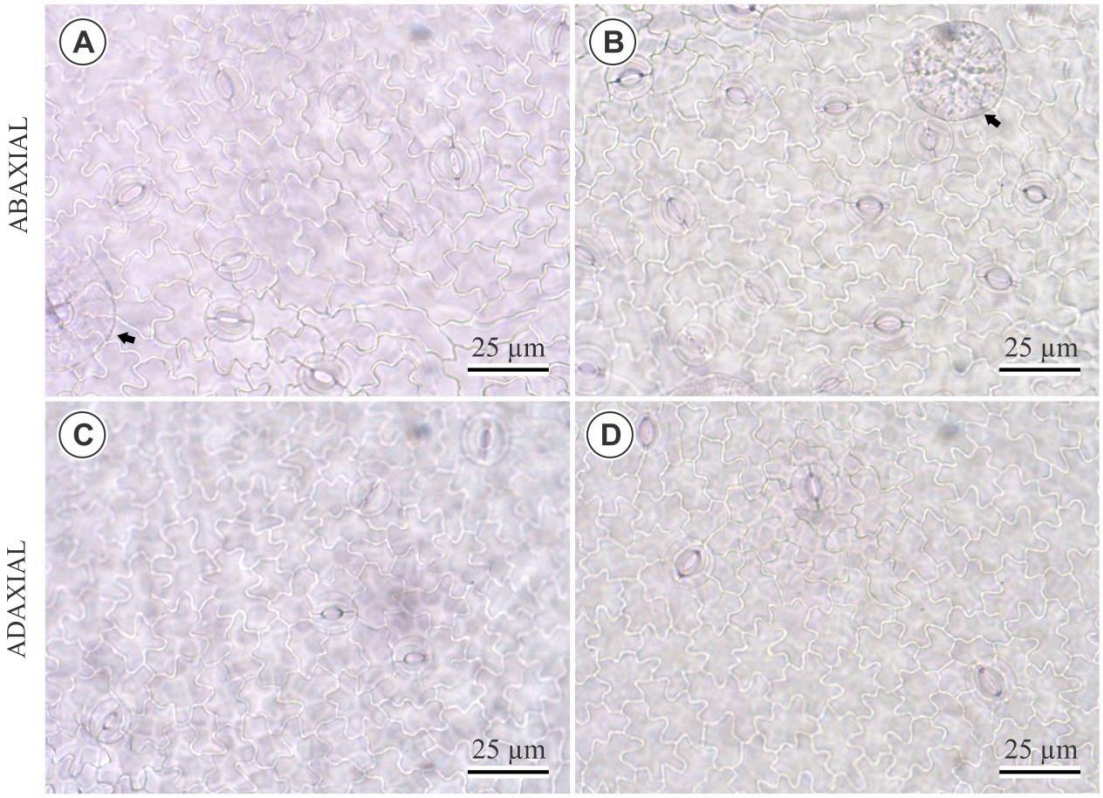

Figure 2. Leaves of *Bixa orellana* L. cultivars Piave Vermelha and UESB74 submitted to different irradiance and quality of light, after 45 days of *in vitro* culture. A – 'Piave Vermelha' under White LED in abaxial surface; B – 'UESB74' under White LED in abaxial

surface; C – ‘Piave Vermelha’ under White LED in abaxial surface; D - 'UESB74’ under White LED in adaxial surface. Black arrow indicate peltate trichomes. Bar = 25 µm.

Irradiance

Plants of B. orellana grown in vitro under different irradiances significant altered the stem length, number of roots/plant, root length, stomatal density in adaxial surface, MDA, chlorophyll *a* and bixin content (Table S1).

The irradiances of 50, 150 and 50+150 µmol m^{-2} s^{-1} reduced growth in shoot apexes of UESB74 (Figure 3 B), when compared to Piave Vermelha (Figure 3 A). Higher irradiances in induced reduction in stem length and number of roots in ‘Piave Vermelha’, whereas in ‘UESB74’ no differences were observed for these variables. Also, no influence of irradiance was observed for both cultivars in root length, root frequency, fresh weight, dry weight (Table 1), number of leaves per plant, leaf expansion, leaf area and stomatal density (Table 2). However, there was significant difference for chlorophylls, total carotenoids and MDA (Table 2).

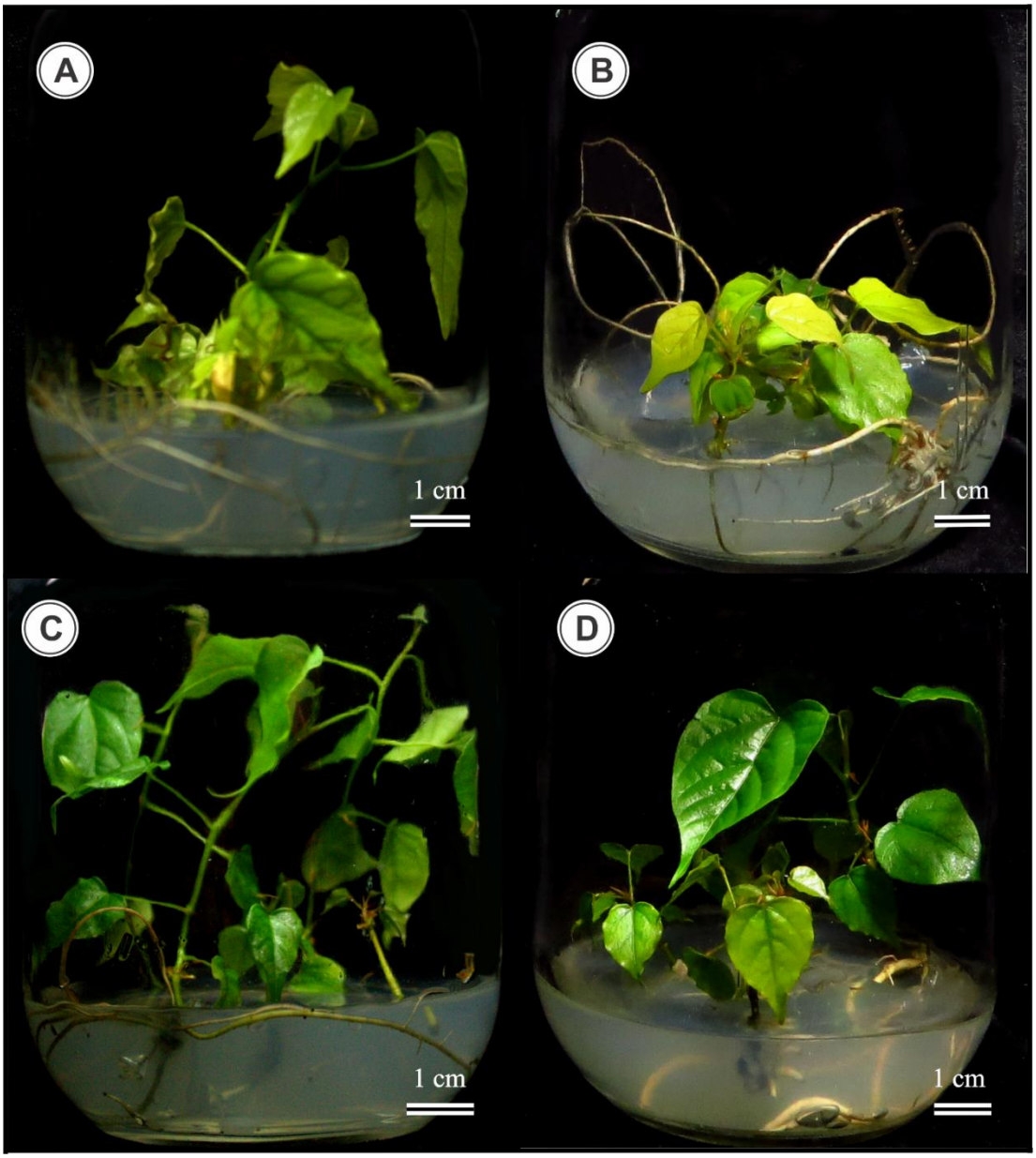

Figure 3. Plants of *Bixa orellana* L. cultivars Piave Vermelha and UESB74 grown *in vitro* from shoot apexes after 45 days submitted to different irradiance and light quality. A – Piave Vermelha grown under 150 µmol m^{-2} s^{-1}; B – UESB74 grown under 150 µmol m^{-2} s^{-1}; C – 'Piave Vermelha' grown under fluorescent lights (50 µmol m^{-2} s^{-1}); D – 'UESB74' in fluorescent light. Bar = 1 cm.

Table 1. Influence of light irradiance on stem length, number of roots/plant, root length, rooting frequency, fresh weight and dry weight of plants *of Bixa orellana* L. cultivars Piave Vermelha and UESB74 derived from shoot apexes cultured 45 days *in vitro*.

Irradiance (µmol m^{-2} s^{-1})	Stem length (mm)		Number of roots/plant		Root length (mm)	
	Piave Vermelha	UESB74	Piave Vermelha	UESB74	Piave Vermelha	UESB74
50	35.00 Aa	22.97 Ba	4.72 Aa	3.28 Aa	19.34 Aa	17.42 Aa
150	28.35 Aab	20.64 Ba	1.12 Ab	1.08 Aa	19.09 Aa	7.46 Aa
50+150	33.39 Aa	23.69 Ba	3.52 Aab	2.20 Aa	28.20 Aa	20.68 Aa
200	19.14 Ab	21.34 Aa	0.44 Ab	1.88 Aa	11.81 Aa	9.18 Aa
50+200	26.40 Aab	22.20 Aa	3.16 Aab	3.28 Aa	17.78 Aa	25.90 Aa
	Rooting frequency (%)		**Fresh weight (g)**		**Dry weight (g)**	
	Piave Vermelha	**UESB74**	**Piave Vermelha**	**UESB74**	**Piave Vermelha**	**UESB74**
50	64.00 Aa	64.00 Aa	0.52 Aa	0.77 Aa	0.09 Aa	0.14 Aa
150	32.00 Aa	40.00 Aa	0.47 Aa	0.96 Aa	0.08 Aa	0.09 Aa
50+150	64.00 Aa	56.00 Aa	1.01 Aa	0.744 Aa	0.12 Aa	0.09 Aa
200	32.00 Aa	64.00 Aa	0.17 Aa	0.36 Aa	0.05 Aa	0.06 Aa
50+200	68.00 Aa	70.00 Aa	0.90 Aa	1.15 Aa	0.11 Aa	0.07 Aa

Means followed by the same capital letter in the rows and small letter in the columns are not statistically significant by Tukey's test ($p \geq 0.05$).

Comparing the cultivars, the treatment of 50 µmol m^{-2} s^{-1} decreased chlorophyll *a*, *b* and total chlorophyll content in UESB74, while the irradiance of 50+200 µmol m^{-2} s^{-1} decreased chlorophyll content and chlorophyll *a/b* in 'Piave Vermelha'. The chlorophylls decreased as the irradiance increased in 'Piave Vermelha', and the same were observed in 'UESB74 with the exception of chlorophyll *b*, which did not vary among the irradiances (Table 2). The 'Piave Vermelha' plants reduced the MDA content in 150 µmol m^{-2} s^{-1} treatment and showed an increase in MDA in 50+200 µmol m^{-2} s^{-1} compared to 'UESB74'. The MDA increased with increasing irradiance in 'Piave Vermelha', while in 'UESB74' showed the opposite, increasing with a decrease in the irradiance (Table 2). For *in vitro* culture of both cultivars, the irradiance of 50 µmol m^{-2} s^{-1} is indicated, due to the reducting of plant stress, increase of stem length, chlorophylls and total carotenoids.

Table 2. Influence of light irradiance in leaves of plants of *B. orellana* L. cultivars Piave Vermelha and UESB74 after 45 days of culture *in vitro* in number of leaves/plant, leaf expansion, leaf area/plant , stomatal density in the abaxial and adaxial surfaces, chlorophylls *a* and *b* , total carotenoids , malonodialdehyde (MDA) and bixin content.

Irradiance (µmol m^{-2} s^{-1})	Stomatal density				Number of leaves/plant		Leaf expansion (cm^2)	
	Abaxial surface (mm^2)		Adaxial surface (mm^2)					
	Piave Vermelha	UESB74	Piave Vermelha	UESB74	Piave Vermelha	UESB74	Piave Vermelha	UESB74
50	415.33 Aa	340.46 Aa	90.90 Aa	65.95 Aa	4.76 Aa	4.20 Aa	0.75 Aa	0.80 Aa
150	397.50 Aa	406.42 Aa	151.51 Aa	126.56 Aa	4.80 Aa	3.24 Aa	0.63 Aa	0.74 Aa
50+150	360.07 Aa	500.89 Aa	101.60 Aa	96.26 Aa	5.04 Aa	4.28 Aa	0.81 Aa	0.69 Aa
200	381.46 Aa	429.59 Aa	119.42 Aa	58.82 Aa	4.12 Aa	3.76 Aa	0.43 Aa	0.40 Aa
50+200	390.374 Aa	475.93 Aa	139.04 Aa	103.39 Aa	4.08 Aa	4.08 Aa	0.84 Aa	0.56 Aa
	Leaf area/plant (cm^2)		Chl *a* (µg g^{-1} DW)		Chl *b* (µg g^{-1} DW)		Chl *a/b* (µg g^{-1} DW)	
	Piave Vermelha	UESB74	Piave Vermelha	UESB74	Piave Vermelha	UESB74	Piave Vermelha	UESB74
50	3.76 Aa	4.01 Aa	8146.69 Aa	6259.21 Ba	4947.17 Aa	4128.10 Ba	1.64 Aa	1.51 Aa
150	3.15 Aa	3.72 Aa	4480.04 Abc	4141.61 Abc	4285.58 Aab	3527.01 Ba	1.04 Ab	1.16 Ab
50+150	4.03 Aa	3.46 Aa	4809.72 Ab	5057.29 Aab	4045.69 Aab	4015.84 Aa	1.18 Ab	1.25 Aab
200	2.17 Aa	2.04 Aa	2762.80 Ac	2727.11 Ac	2980.23 Ac	3297.46 Aa	0.92 Ab	0.83 Ac
50+200	4.20 Aa	2.83 Aa	3293.61 Bbc	4952.77 Aab	3452.27 Abc	3867.90 Aa	0.95 Bb	1.26 Aab
	Total chll (µg g^{-1} DW)		Total carotenoids (µg g^{-1} DW)		MDA (ηmol g^{-1} DW)		Bixin (µg g^{-1} DW)	
	Piave Vermelha	UESB74	Piave Vermelha	UESB74	Piave Vermelha	UESB74	Piave Vermelha	UESB74
50	13093.86 Aa	10387.31 Ba	1906.44 Aa	1286.82 Ba	3.28 Ab	4.33 Aab	13.02 Aa	7.32 Ba
150	8765.61 Ab	7668.62 Aab	1486.97 Aab	1075.50 Aab	3.93 Bb	5.78 Aa	11.83 Aa	7.66 Ba
50+150	8855.42 Ab	9073.14 Aab	1496.62 Aab	1262.79 Aab	3.48 Ab	4.63 Aab	12.08 Aa	7.71 Ba
200	5743.03 Ac	6024.58 Ab	923.21 Ab	515.60 Ab	4.62 Aab	5.66 Aab	11.51 Aa	7.01 Ba
50+200	6745.89 Bbc	8820.67 Aab	1099.37 Ab	1155.48 Aab	5.77 Aa	3.92 Bb	10.46 Aa	6.92 Ba

Means followed by the same capital letter in the rows and small letter in the columns are not statistically significant by Tukey's test ($p \geq 0.05$). Abbreviations: *Chl* = Chlorophyl; *DW* = Dry Weight; *MDA* = malondialdehyde.

Light quality

The different light qualities affected stem length and dry weight of the plants and stomatal density in adaxial surface, chlorophyll *a*, b, total chlorophyll, total carotenoids and bixin content in leaves from shoot apexes of *B. orellana* cultured *in vitro* (Tabela S2).

Fluorescent light decreased stem length in plants of UESB74 when compared to Piave Vermelha, whereas in white and blue/red LEDs, both cultivars showed similar growth. The light qualities after 45 days of culture also did not influence in both cultivars in number of roots/plant, root length, rooting frequency, fresh weight (Table 3), stomatal density in abaxial surface and number of leaves/plant (Table 4).

Table 3. Influence of light quality on stem length, number of roots/plant, root length, rooting frequency, fresh weight and dry weight in plants cultured *in vitro* of *Bixa orellana* L. cultivars Piave Vermelha and UESB74, for 45 days.

Light quality	Stem length (mm)		Number of roots/plant		Root length (mm)	
	Piave Vermelha	**UESB74**	**Piave Vermelha**	**UESB74**	**Piave Vermelha**	**UESB74**
FLU	31.58 Aa	22.98 Ba	3.24 Aa	3.28 Aa	22.06 Aa	17.42 Aa
W LED	28.12 Aa	22.97 Aa	2.88 Aa	2.56 Aa	23.03 Aa	12.90 Aa
B/R LED	25.87 Aa	21.43 Aa	4.16 Aa	2.68 Aa	21.65 Aa	16.04 Aa
	Rooting frequency (%)		**Fresh weight (g)**		**Dry weight (g)**	
	Piave Vermelha	**UESB74**	**Piave Vermelha**	**UESB74**	**Piave Vermelha**	**UESB74**
FLU	68.00 Aa	68.00 Aa	0.53 Aa	0.78 Aa	0.09 Ba	0.14 Aa
W LED	60.00 Aa	60.00 Aa	0.55 Aa	0.56 Aa	0.07 Aa	0.09 Ab
B/R LED	60.00 Aa	64.00 Aa	0.36 Aa	0.55 Aa	0.06 Aa	0.09 Aab

Means followed by the same capital letter in the rows and small letter in the columns are not statistically significant by Tukey's test ($p \geq 0.05$). Abbreviations: *FLU* = fluorescent; *W LED* = White LED; *B/R LED* = Blue/Red LED.

Shoot apexes of 'Piave Vermelha' cultured on fluorescent light decreased dry weight compared to 'UESB74', whereas other light qualities evaluated did not influence the dry weight of the former. However, shoot apexes of 'UESB74' grown on white LED and blue/red LED decreased the dry weight (Table 3). The 'UESB74' grown on white LED reduced the stomatal density in adaxial surface of leaves compared to 'Piave

Vermelha' (Figure 2 C and D). The white LED and blue/red LED increased the stomatal density in adaxial surface of 'Piave Vermelha', while in 'UESB74' the light quality did not influenced stomatal density. Regarding the stomatal density, the blue/red LED lamp decreased the expansion and leaf area of 'Piave Vermelha' compared to 'UESB74', but did not altered in the other light qualities (Table 4).

The light quality influenced in chlorophyll content, total carotenoids and bixin content in leaves (Table 4). Plants of 'UESB74' reduced chlorophylls *a*, *b* and total chlorophyll content grown under fluorescent light compared to 'Piave Vermelha'. The chlorophylls content in 'UESB74' was not influenced for light quality. On the other hand, in 'Piave Vermelha', light quality decreased chlorophylls *a*, *b* and total chlorophyll content, with the exception of chlorophyll *a/b,* which was not affected. Total carotenoids in both cultivars were similar in different light qualities. Only plants of 'Piave Vermelha' increased the total carotenoids under fluorescent lamps compared to white and blue/red LEDs, while 'UESB74' showed the opposite result, increasing total carotenoids in blue/red LED lamp compared to fluorescent and white LEDs (Table 4).

MDA content was considerably lower in 'Piave Vermelha' than 'UESB74' cultured under blue/red LEDs. Despite in 'Piave Vermelha' the light quality did not influence in MDA content, in 'UESB74' the blue/red LEDs induced an increase in MDA compared to fluorescent and white LEDs.

The different light qualities did not alter the bixin content in the cultivar UESB74, since for Piave Vermelha plants grown under blue/red LEDs increased the content of bixin relative to fluorescent and white LEDs. In all light qualities, the bixin content in Piave Vermelha was higher than UESB74 (Table 4). The blue/red LED light is indicated for the *in vitro* culture of cultivar Piave Vermelha, due inducing less stress, increasing chlorophyll, total carotenoids and bixin in leaves. On the other hand, for 'UESB74' the culture under fluorescent lights is indicated, for increase the dry mass and total carotenoids in leaves.

Table 4. Infuence of light quality in leaves of plants of *B. orellana* cultivars Piave Vermelha and UESB74, after 45 days cultured *in vitro* in number of leaves/plant, leaf expansion (cm^2), leaf area/plant (cm^2), stomatal density in the abaxial and adaxial surfaces, chlorophylls *a* and *b*, total carotenoids , MDA and bixin content.

Light quality	Stomatal density							
	Abaxial surface (mm^2)		Adaxial surface (mm^2)		Number of leaves/plant		Leaf expansion (cm^2)	
	Piave Vermelha	UESB74	Piave Vermelha	UESB74	Piave Vermelha	UESB74	Piave Vermelha	UESB74
Fluorescent	415.33 Aa	340.46 Aa	90.91 Ab	65.95 Aa	4.56 Aa	4.20 Aa	0.75 Aa	0.80 Aa
White LED	426.02 Aa	406.42 Aa	140.82 Aa	81.99 Ba	5.24 Aa	4.60 Aa	0.60 Aa	0.63 Aa
Blue/Red LED	360.07 Aa	383.24 Aa	101.60 Aab	87.34 Aa	5.72 Aa	4.44 Aa	0.47 Ba	0.79 Aa
	Leaf area/plant (cm^2)		Chl *a* ($\mu g\ g^{-1}$ DW)		Chl *b* ($\mu g\ g^{-1}$ DW)		Chl *a/b* ($\mu g\ g^{-1}$ DW)	
	Piave Vermelha	UESB74	Piave Vermelha	UESB74	Piave Vermelha	UESB74	Piave Vermelha	UESB74
Fluorescent	3.76 Aa	4.01 Aa	8146.69 Aa	6259.21 Ba	4947.17 Aa	4128.10 Ba	1.64 Aa	1.51 Aa
White LED	3.01 Aa	3.18 Aa	5837.44 Bb	7739.00 Aa	4107.79 Ab	4528.79 Aa	1.42 Ba	1.69 Aa
Blue/Red LED	2.34 Ba	3.95 Aa	6687.11 Aab	6259.44 Aa	4324.62 Aab	4032.84 Aa	1.54 Aa	1.54 Aa
	Total chlorophyll ($\mu g\ g^{-1}$ DW)		Total carotenoids ($\mu g\ g^{-1}$ DW)		MDA ($\eta mol\ g^{-1}$ DW)		Bixin ($\mu g\ g^{-1}$ DW)	
	Piave Vermelha	UESB74	Piave Vermelha	UESB74	Piave Vermelha	UESB74	Piave Vermelha	UESB74
Fluorescent	13093.86 Aa	10387.31 Ba	1906.44 Aa	1286.81 Ab	3.28 Aa	4.33 Ab	13.02 Ab	7.32 Ba
White LED	9945.23 Ab	12267.80 Aa	1309.74 Ab	1729.55 Aab	4.86 Aa	5.70 Aab	13.70 Aab	10.09 Ba
Blue/Red LED	11011.73 Aab	10292.29 Aa	1612.61 Aab	1407.96 Aa	4.66 Ba	7.21 Aa	17.39 Aa	8.08 Ba

Means followed by the same capital letter in the rows and small letter in the columns are not statistically significant by Tukey's test ($p \geq 0.05$). Abbreviations: *Chl* = Chlorophyl; *DW* = Dry Weight; *MDA* = malondialdehyde.

DISCUSSION

Bixa orellana L. has a wide genetic diversity and cultivars with heterogeneous bixin content, due the relative recent domestication from *B.orellana* var. *urucurana* (Kuntze 1925, Rivera-Madrid et al. 2006; Valdez-Ojeda et al. 2008; Moreira et al. 2015; Dequigiovanni et al. 2018). The cultivars and the wild variety coexist with gene flow crossing occurring among them, which results in plants with different phenotypes for bixin content (Moreira et al. 2015). Here, leaves of 'Piave Vermelha' cultured *in vitro* produce 1.6-fold more bixin than the 'UESB74', and the same result are observated to other organs. The seeds of 'Piave Vermelha' have higher levels of bixin, ranging from 3.5 to 5.0% (Lemos et al. 2011), while 'UESB74' produces visibly low bixin content in seeds (Figure 1 B).

The *in vitro* cultivated *B. orellana* produces bixin in their leaves in the same way that plants grown in the field (Mahendranath et al. 2011; Rodríguez-Ávila et al. 2011; Rivera-Madrid et al. 2013). In the leaves, the stomata cells were in paracytic arrangement whit peltate trichomes predominant along the abaxial anda adaxial surfaces of the leaf epidermis (Figure 2 A and B) (Carvalho et al. 2005; Cruz et al. 2014), showing secretory structures of bixin distributed along the leaves. These structures are found in others plant organs of the plant (Freitas 2009; Metcalfe and Chalk 1957).

The irradiance affects the accumulation of biomas, this way is necessary to adjust the appropriate irradiance for *in vitro* culture to each plant material for optimum biomass accumulation (Ali et al. 2017; Irshad et al. 2018). High irradiance depending on the plant species, increase the leaf area and rooting (Fu et al. 2017). For others species, the reduced irradiance *in vitro* increased the accumulation of biomass due reduced risk of photoinhibition and photooxidation (Chan et al. 2010). The photoinhibition and photooxidation caused oxidative stress, decreasing the growth and the content of photosynthetic pigments and increasing the production of secondary metabolites (Silva et al. 2017; Zhu et al. 2017). In *B. orellana* cultivar 'Bico de Pato', the increase in irradiance reduced chlorophylls *a*, *b* and total chlorophylls, also the 150 μmol m^{-2} s^{-1} irradiance induced photoinhibition in leaves developed from shoot apexes cultured *in vitro* (Ortíz 2004).

The same results were observed here for both cultivars of *B. orellana*, where the high irradiance decreased chlorophylls content and total carotenoid. Only 'Piave Vermelha' reduced stem length and increased MDA content under high irradiances. The 'UESB74' showed better growth and similar MDA compared to 'Piave Vermelha' under the same growing conditions, which shows that probably 'UESB74' be more resistant and produce other secondary metabolites with antioxidant activity, as phenolic compounds (Zhu et al. 2017; Pérez-López et al. 2018; Irshad et al. 2018). On the other hand, the both LEDs light induced increased growth, MDA content, chlorophylls content and total carotenoids in 'UESB74', while for 'Piave Vermelha' the LEDs not affected MDA content and increased bixin in leaves. In the cultivars of *B. orellana* it was observed different responses to light stress under high irradiances (Ortíz 2004). On the other hand, the bixin has antioxidant activity *ex vitro* (Zhang et al. 2018; Tay-Agbozo et al. 2018), but there are no reports if the same occurs *in planta* for *B. orellana*, being further studies still needed.

The blue/red LEDs emit energy on the visible spectrum in the regions of interest for the photosynthesis (Batista et al. 2018), being most effective for the *in vitro* culture of Piave Vermelha. Following the results found here, other works show that the use of blue/red lights can increase chlorophyll content, grown, leaves fresh and dry weight (Kalaji et al. 2014; Batista et al. 2016; Ramírez-Mosqueda et al. 2017) and stomatal density (Liu et al. 2014).

Carotenoids in plants are accessory pigments susceptible to photo-oxidation (Britton 1995) since they absorb light at a higher intensity bands (450–550 nm) found in the reaction centres of PSI and PSII protection of photosystems to photooxidative stress (Lichtenthaler et al. 1982; Hashimoto et al.; 2016) and one of the factors that regulate carotenoid biosynthesis is the light (Ruiz-Sola et al.; 2012), which promotes the activation of gene expression of the methylerythritol 4-phosphate (MEP) pathway, carotenoids and phytoene synthase (PSY) (Woitsch and Romer, 2003; Toledo-Ortiz et al. 2010; Leivar and Monte, 2014; Bou-Torrent et al., 2015).

In this work, we showed for the first time that the use of white and blue/red LEDs *in vitro* to the increase of carotenoids in both cultivars and bixin in leaves of 'Piave Vermelha'. Blue and red LEDs emit more energy in the blue region (Figure 1 C).

The blue region absorb strongly carotenoid pigments as lutein and *b*-carotene (Wright and Shearer 1984). It is known that UV-B and UV-C radiations increase the β-carotene content and decrease the total carotenoids, however not influencing in bixin content in leaves (Sankari et al. 2017). JLikewise *B. orellana*, buckwheat and lettuce cultured under different light qualities such as white, blue and red LEDs, increase the production of carotenoids (Tuan et al. 2013; Amoozgar et al. 2017).

The increased carotenogenesis in *B. orellana* can be attributed to an up-regulation of *PSY*, *phytoene desaturase* (*PDS*), *carotenoid cleavage deoxygenize* (*CCD*), *epsilon- lycopene cyclase* (*LCY*) and *zeta-carotene* desaturase (*ZDS*) genes in response to UV light (Sankari et al. 2017). These genes are also related to the bixin pathway (Cárdenas-Conejo et al. 2015). Similar results were observed in tomato (Giuliano et al. 1993) buckwheat and Oncidium Gower Ramsey leaves in response to light (Tuan et al. 2013; Lee et al. 2003). The carotenoid concentration in leaves is a result of the biosynthesis and degradation, due to photo-oxidation and metabolic conversion (Simkin et al. 2013). Which may explain the carotenoid levels reduction in leaves of Piave Vermelha and UESB74 cultivars under the higher irradiance.

The *B. orellana* cultivars have genetic combinations resulting from the segregation of dominant and/or recessive genes (Rivera-Madrid et al. 2006, Valdez-Ojeda et al. 2008; Dequigiovanni et al. 2018), which may limit or favor the metabolic conversion of carotenoids to the bixin pathway. This may explain the increase of bixin in Piave Vermelha leaves under white and blue/red LEDs, while UESB74 did not vary

CONCLUSIONS

The cultivar Piave Vermelha produces 1.6-fold more bixin in leaves and visibly higher bixin content in the seeds as compared to UESB74. The stomata cells in the leaves of both cultivars followed paracytic arrangement, whith peltate trichomes predominant along the leaf adaxial and abaxial surfaces. The irradiance and light quality influenced growth, chlorophylls contents, carotenoids and MDA in the leaves. For the *in vitro* culture of the cultivar Piave Vermelha, the blue/red LEDs are indicated due inducing less stress, increase chlorophyll, total carotenoids and bixin content in leaves. On the other hand, for UESB74, the culture under fluorescent light (50 μmol m^{-2} s^{-1}) is indicated, since it increases the dry mass and total carotenoids in the leaves. This work opens perspectives for a better comprehension about the regulation of the bixin biosynthesis pathway and its genetic diversity.

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SUPPLEMENTARY MATERIAL

Table S1. Analysis of variance (ANOVA) of stem length , number of roots/plant, root length, rooting frequency, fresh and dry weight, number of leaves/plant, leaf expansion, leaf area/plant, stomatal density in the abaxial and adaxial surfaces, chlorophylls *a* and *b*, total carotenoids, MDA and bixin content in shoot apex of cultivars of *Bixa orellana* L. Piave Vermelha and UESB74 grown under light irradiance levels.

	Stem length (mm)	Number of roots/plant	Root length (mm)	Rooting frequency (%)	Number of leaves/plant	Fresh weight (g)
LI	0.630 **	0.159 **	4.958 *	6.484 ns	100.0 ns	100.0 ns
Cv	0.024 **	100.0 ns	100.0 ns	26.166 ns	5.626 ns	100.0 ns
LI x VA	5.679 ns	36.230 ns	38.593 ns	100.0 ns	100.0 ns	100.0 ns
CV (%)	21.857	71.73	64.154	36.162	27.51	975.574
		Stomatal density				
	Dry weight (g)	Abaxial surface (mm^2)	Adaxial surface (mm^2)	Leaf expansion (cm^2)	Leaf area/plant (cm^2)	MDA (nmol g^{-1} DW)
LI	100.0 ns	100.0 ns	5.848 ns	9.370 ns	9.369 ns	2.165 *
Cv	100.0 ns	20.328 ns	3.558 *	100.0 ns	100.0 ns	2.894 *
LI x VA	100.0 ns	29.709 ns	100.0 ns	100.0 ns	100.0 ns	0.214**
CV (%)	975.574	27.822	46.753	46.161	46.161	22.294
	Chl *a* (µg g^{-1} DW)	Chll *b* (µg g^{-1} DW)	Chl *a/b* (µg g^{-1} DW)	Total Chl (µg g^{-1} DW)	Total carotenoids (µg g^{-1} DW)	Bixin (µg g^{-1} DW)
LI	4.790 *	8.069 ns	6.165 ns	5.25 ns	5.830 ns	100.0 ns
Cv	100.0 ns	51.257 ns	67.780 ns	100.0 ns	50.909 ns	0.212 **
LI x VA	10.196 ns	100.0 ns	7.779 ns	24.455 ns	100.0 ns	100.0 ns
CV (%)	27.592	23.925	18.182	25.176	39.913	29.138

*$P \leq 0,05$ and **$P \leq 0,01$ ns = not significant by Tukey's test $P \leq 0,05$nby F test. Abbreviations: *LI* = Light irradiance; *Cv* = Cultivat of *Bixa orellana* L.; *CV* = coefficient of variation; *Chl* = Chlorophyll; *DW* = Dry weight; *MDA* = malonodialdehyde

Table S2. Analysis of variance (ANOVA) of stem length, number of roots/plant, root length, rooting frequency, fresh and dry weight, number of leaves/plant, leaf expansion, leaf area/plant, stomatal density in the abaxial and adaxial surfaces, chlorophylls, total carotenoids, MDA and bixin content in shoot apexes of the *Bixa orellana* L. cultivars Piave Vermelha and UESB74 under different light quality.

	Stem length (mm)	Number of roots/plant	Root length (mm)	Rooting frequency (%)	Number of leaves/plant	Fresh weight (g)
LQ	18.505 ns	100.0 ns	100.0 ns	100.0 ns	33.827 ns	17.377 ns
Cv	0.068 **	100.0 ns	5.118 ns	100.0 ns	6.799 ns	7.967 ns
LQ x VA	100.0 ns	100.0 ns	100.0 ns	100.0 ns	100.0 ns	100.0 ns
CV (%)	16.723	63.429	48.059	33.373	22.719	40.862
	DW (g)	Stomatal density Abaxial surface (mm^2)	Adaxial surface (mm^2)	Leaf expansion (cm^2)	Leaf area/plant (cm^2)	MDA (nmol g^{-1} DW)
LQ	2.454 *	100.0 ns	7.958 ns	21.406 ns	21.407 ns	15.415 ns
Cv	0.771 **	100.0 ns	0.822 **	10.448 ns	10.449 ns	55.057 ns
LQ x VA	100.0 ns	100.0 ns	26.556 ns	27.241 ns	27.242 ns	100.0 ns
CV (%)	35.746	24.645	32.78	32.388	32.388	37.054
	Chl *a* (µg g^{-1} DW)	Chl *b* (µg g^{-1} DW)	Chl *a/b* (µg g^{-1} DW)	Total Chl (µg g^{-1} DW)	Total carotenoids (µg g^{-1} DW)	Bixin (µg g^{-1} DW)
LQ	100.0 ns	100.0 ns	100.0 ns	100.0 ns	100.0 ns	100.0 ns
Cv	100.0 ns	100.0 ns	100.0 ns	100.0 ns	100.0 ns	3.463*
LQ x VA	3.005 *	4.308 *	13.435 ns	2.584 *	2.412*	4.397*
CV (%)	18.159	9.963	11.81	14.297	21.118	17.356

*$P \leq 0,05$ and **$P \leq 0,01$ ns = not significant by Tukey's test $P \leq 0,05$ by F test. Abbreviations: *LQ* = Light quality; *Cv* = Cultivat of *Bixa orellana* L.; *CV* = coefficient of variation; *Chl* = Chlorophyll; *DW* = Dry weight; *MDA* = malonodialdehyde.

GENERAL CONCLUSIONS

The temporary exposure of shoots apexes cultivated *in vitro* to the demethylating compound 5-AZA did not affect the genome global methylation, chlorophylls and total carotenoid content in leaves of *B. orellana.* However, plants submitted to 5-AZA induced the downregulation of the SABBATH family genes and increased the bixin content in the leaves, suggesting that this methyltransferase inhibitor can direct regulate the biosynthesis pathway and the production of bixin in *B. orellana*. Due to the complexity the bixin biosynthetic pathway, the relation among DNA methylation, gene regulation and bixin production needs to be further explored. In addition, this work opens new perspectives on the influence of 5-AZA, and hence the levels of methylation and demethylation, on bixin biosynthesis and its accumulation during the plant development, knowledge that can be a useful tool for the production of *B. orellana* plants with higher bixin content.

The cultivar Piave Vermelha produces 1.6-fold more bixin in leaves as compared to UESB74, and visibly higher bixin content in the seeds. The stomata cells in the leaves of both cultivars followed paracytic arrangement whith peltate trichomes predominant along the leaf adaxial and abaxial surfaces. The irradiance and light quality influenced growth, chlorophylls contents, carotenoids and MDA in the leaves. For the *in vitro* culture of the cultivar Piave Vermelha, the blue/red LEDs are indicated due inducing less stress, increase chlorophyll, total carotenoids and bixin content in leaves. On the other hand, for UESB74 is indicated the culture under fluorescent lights (50 μmol m^{-2} s^{-1}), since it increases the dry mass and total carotenoids in the leaves. This work opens new perspectives on the regulation of the bixin biosynthesis pathway.