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Milech, Cristini; Ribeiro Lucho, Simone; Moraes Kleinowski,, Alícia; Berwaldt Dutra, Débora; Mühlenberg Soares, Mariana; Jacira Bolacel Braga, Eugenia
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Production of pigments in *Alternanthera sessilis* calli mediated by plant growth regulators and light

Cristini Milech*, Simone Ribeiro Lucho, Alírcia Moraes Kleinowski, Débora Berwaldt Dutra, Mariana Mühlenberg Soares and Eugenia Jacira Bolacel Braga

*Laboratório de Cultura de Tecidos, Programa de Pós-Graduação em Fisiologia Vegetal, Departamento de Botânica, Instituto de Biologia, Universidade Federal de Pelotas, Capão do Leão, 96010-900, Pelotas, Rio Grande do Sul, Brazil. *Author for correspondence. E-mail: crismilech.cm@gmail.com

ABSTRACT. Among the compounds produced by plants, pigments such as betalains have received attention from both food and pharmaceuticals industries. The *Alternanthera sessilis* species produces these pigments, though in small quantities, and so it is necessary to increase production. Thus, many studies use elicitors that are capable of triggering physiological or morphological responses in plants. The objective was to establish callus production in *A. sessilis* grown under different combinations of growth regulators and light qualities and to assess whether these factors can increase betalain and flavonoid production. Leaf and internodal explants in MS medium with different growth regulators were used to obtain calli, which were subsequently transferred to a betacyanin induction medium remaining for 40 days under different light qualities (white, blue, red, and dark). The most suitable treatment for callus formation and subsequent betalain and flavonoid induction was to combine a medium containing $6.7 \mu\text{mol L}^{-1}$ 2,4-D and $9.0 \mu\text{mol L}^{-1}$ BAP and blue light. Physical elicitation by light combined with appropriate concentration of growth regulators on calli can increase production of commercially important metabolites.

Keywords: betalain, flavonoids, secondary metabolites, medicinal plants.

Produção de pigmentos em calos de *Alternanthera sessilis* mediados por reguladores de crescimento e luz

RESUMO. Dentre os compostos produzidos pelas plantas, os pigmentos, como as betalaínas, vêm recebendo destaque tanto pela indústria alimentícia como farmacêutica. A espécie *Alternanthera sessilis* produz esses pigmentos, porém em pequenas quantidades, sendo necessário incrementar a produção. Para isso, muitos estudos utilizam elicitores que são capazes de desencadear respostas fisiológicas ou morfológicas nas plantas. O objetivo do trabalho foi estabelecer a produção de calos de *A. sessilis* crescidos quando submetidos a diferentes combinações de reguladores de crescimento e qualidades de luz, e avaliar se esses fatores são capazes de incrementar a produção de betalaínas e flavonoides. Foram utilizados explantes foliares e internodais em meio MS com diferentes reguladores de crescimento para obtenção dos calos que, posteriormente, foram transferidos para meio de indução de betacianina, onde permaneceram por 30 dias sob diferentes qualidades de luz (branca, azul, vermelha e escuro). O tratamento mais propício para formação de calos e consequente indução de betalaínas e flavonoides foi a combinação do meio contendo $6,7 \mu\text{mol L}^{-1}$ 2,4-D e $9,0 \mu\text{mol L}^{-1}$ BAP e a luz azul. Conclui-se que a elicitação física pela luz em conjunto com a concentração adequada de reguladores de crescimento em calos é capaz de incrementar a produção de metabólitos de interesse comercial.

Palavras-chave: betalaína, flavonoides, metabólitos secundários, plantas medicinais.

Introduction

Light is one of the most important environmental factors for plants, it is a source of energy affecting their growth and development. However, excessive light can result in the accumulation of reactive oxygen species (ROS) and create disorders in plants, leading to death. In the evolutionary process, in order to protect themselves against the harmful ROS effects, plants develop

protection mechanisms, including alterations in the production of secondary metabolites, such as pigments (anthocyanins, carotenoids, betalains) and antioxidants (Tariq, Ali, & Abbasi, 2014).

Tissue culture techniques - including callus formation - are widely used as alternative method for producing and accumulating secondary metabolites (Al-Jibouri, Abed, Ali, & Majeed, 2016). Calli can be induced in response to organogenetic

stimuli using different growth regulators and environmental conditions and, in general, they present varied forms and sizes and a certain degree of cellular wall thickening (Carvalho et al., 2011).

One example of natural products receiving attention in the pharmaceutical market is betalains. A class of water soluble nitrogen containing plant pigments of the order Caryophyllales which consists of the yellow betaxanthins and the violet betacyanins (Gandia-Herrero, Escribano, & Garcia-Carmona, 2005; Yusuf, Shabbir, & Mohammad, 2017).

Within the Caryophyllales order, the *Alternanthera* genus stands out in terms of betalain production and *Alternanthera sessilis* species is quite well known for its medicinal properties, thus being of interest to associate its possible pharmacological effects with the optimization of pigment production (Hossain, Faisal, Rahiman, Jahan, & Rahmatullah, 2014).

Due to environmental fluctuations in nature, these plants cannot provide sufficient amount of such compounds. Therefore, the use of biotechnological tools is common to meet the demand to try producing and increasing the quantities of these metabolites, such as practicing *in vitro* cultivation associated with using elicitors (Pérez-Alonso et al., 2014).

Elicitors are chemical, physical, or biological products or stimuli that are able to induce morphological or physiological alterations in organisms (Vasconsuelo & Boodland, 2007). As a physical factor, light is especially important for betalain production, as seen in studies carried out on beetroot (Shin, Park, & Paek, 2013) and *Suaeda salsa* (Wang & Liu, 2006), as it acts in reprogramming plant metabolism. According to Zhao, Sun, Gao, Sui, and Wang (2011), the quality and quantity of light affect betacyanin synthesis.

In this context, the purpose of this paper was to use *in vitro* cultivation to produce calli in *A. sessilis* grown under different combinations of growth regulators and light qualities, aiming at evaluating whether these factors can increase betalain and flavonoid production.

Material and methods

We used three parent plant specimens of *Alternanthera sessilis* (L.) R. Br. Ex DC. (Amaranthaceae) for *in vitro* culture. Plants were collected in the municipalities of Rio Grande, state of Rio Grande dos Sul (2006) grown in the greenhouse identified by Élen Nunes Garcia. The voucher specimen was deposited in the Herbarium

of the Botany Department of the *Universidade Federal de Pelotas* in RS, under 24.534.

Leaf and internodal explants originated from aerial parts of plants cultivated *in vitro* in MS medium (Murashige & Skoog, 1962) were used to induce calli. Therefore, MS medium containing 100 mg L⁻¹ of mioinositol, 30 g L⁻¹ of sucrose, 7 g L⁻¹ of agar, and pH adjusted to 5.8 was used. The media were supplemented with 2,4-D, in 0.0, 4.5, 6.7, and 9 µmol L⁻¹ concentrations, and BAP in 0.0, 4.5, 6.7, and 9 µmol L⁻¹ concentrations, totaling 16 types of cultivation medium. Moreover, combinations of 2,4-D (9 µmol L⁻¹) x IAA (1.4, 2.8, 4.2, and 5.6 µmol L⁻¹) were tested, totaling four types of medium. Petri dishes were randomly distributed with occasional rotation and kept in the dark at 25 ± 2° C for 20 days and then transferred to light for 10 days where they were kept a 16 hours photoperiod at 25° C ± 2 (Reis et al., 2017).

After 30 days, the percentage of calli formed in the callus inductor medium (CIM) was evaluated. The formed calli were transferred to dishes with MS medium with 2.2 µmol L⁻¹ of Thidiazuron (TDZ) and 5.3 µmol L⁻¹ of naphthalene acetic acid (NAA), 0.5 mg L⁻¹ of adenine, and 3 mg L⁻¹ of ascorbic acid (AA), known as BIM medium – betacyanin induction medium according to Zhao, Sun, Chen, and Wang (2010), and kept under white light provided by fluorescent bulbs.

This stage was carried out in order to choose the best media for callogenesis induction and pigment formation. After choosing, the experiment was repeated with the four best callus inductor media (CIM), which were called M1, M2, M3, and M4, and subsequently distributed between the different light qualities (white, blue, red, and dark), in the BIM medium for 40 days and 16 hours photoperiod at 25° C ± 2. The different light qualities were provided by fluorescent tube (Sylvania® - 40W) for white light, blue compact fluorescent lamp for blue light (Taschibra® - 14W - peak emission in 470 nm), and red compact fluorescent for red light (G-light® - 15W - peak emission in 660 nm). The photon flux densities for the white, blue, and red lights, measured with a Hansatech® Quantum Sensor QSRED light meter were 25, 12, and 22 µmol m⁻² s⁻¹, respectively. Dishes exposed to the dark remained in incubator.

In order to extract the flavonoids and bethanidine, fresh mass of calli obtained in each treatment were used, which were macerated in a porcelain mortar using acetate/methanol (70/30%, v/v) extraction buffer, pH 5.0, plus sodium ascorbate of 10 mM. The homogenized product was filtered

in gauze and centrifuged at 10.000 g for 20 minutes, at 4° C (Reis et al., 2017).

The flavonoid concentration was expressed in μmol of quercetin per gram of fresh mass and the readings were carried out in a spectrophotometer at 330 nm. For bethanidine the molar extinction coefficient used for the calculation was $\epsilon = 54000 \text{ M}^{-1} \text{ cm}^{-1}$ at a wavelength of 536 nm.

In order to extract the amaranthine, bethanin, and miraxanthine, the methodology described above was used, substituting the extraction buffer with a 10 mM phosphate buffer, pH 6.0. The amaranthine, bethanin, and miraxanthine concentrations were determined using a molar extinction coefficient of $\epsilon = 56600 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon = 65000 \text{ M}^{-1} \text{ cm}^{-1}$, and $\epsilon = 48000 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. The readings were carried out in a spectrophotometer at 536 nm for amaranthine and bethanin and at a 480 nm wavelength for miraxanthine.

In order to determine the best callus induction medium and type of explant, the experimental design was completely randomized, represented by 20 media types and two explants types. In order to choose the best combination of medium and light quality, the design was in a 4 x 4 factorial scheme (four media and four light qualities). Both experiments were conducted in triplicate, each one containing five explants. The data were subjected to variance analysis ($p \leq 0.05$) and the average compared by Tukey test with a 5% error probability, using the Winstat 1.0 Statistical Program (Machado & Conceição, 2007).

Results and discussion

As the objective of this work was producing pigments in callus culture, we evaluated three main aspects in the cultivation (percentage of callus formation, color and callus appearance) to select the best culture media for experimental sequence as shown in Table 1.

Considering these three aspects, the media showing these three desired characteristics were those containing the 2,4-D growth regulator at concentrations of $6.7 \mu\text{mol L}^{-1}$ and $9.0 \mu\text{mol L}^{-1}$ in association with BAP at these concentrations (M11, M12, M15 and M16). Pigments observed in these media are shown in Figure 1.

Although medium 13 showed favorable results in relation to callus induction, it formed friable calli and, according to Ahmad, Rab, and Ahmad (2015), very friable calli accumulate water and tend to undergo rapid oxidation, compact calli are more desirable. Although exhibiting good callus production rate, Media 10 and 14, were not efficient in pigment production, suggesting that high concentrations of BAP are required to express the pigment.

The use of two auxins combined (2,4-D and IAA) was not efficient for callus formation in internodal explants however, in leaf explants, the calli formation percentage reached 50%, corroborating Kakani, and Peng (2011), who found that for callus formation a combination of cytokinin and auxin is needed.

Table 1. Percentage of callus formation in callus induction medium (CIM) and callus coloring derived from leaf (L) and internodal (I) explants of *Alternanthera sessilis* after 20 days in the dark and 10 days in the light in betacyanin induction medium (BIM).

Induction Medium	% of callus formation		Coloring		Aspect	
	Leaf	Internodal	Leaf	Internodal	Leaf	Internodal
Medium 1 (without regulator)	0	0	-	-	-	-
Medium 2 (0.0 2,4-D + 4.5 BAP)	0	0	-	-	-	-
Medium 3 (0.0 2,4-D + 6.7 BAP)	0	0	-	-	-	-
Medium 4 (0.0 2,4-D + 9.0 BAP)	0	0	-	-	-	-
Medium 5 (4.5 2,4-D + 0.0 BAP)	0	0	-	-	-	-
Medium 6 (4.5 2,4-D + 4.5 BAP)	50	0	Black	-	Compact	-
Medium 7 (4.5 2,4-D + 6.7 BAP)	66.7	0	Black	-	Compact	-
Medium 8 (4.5 2,4-D + 9.0 BAP)	100	91.6	Green	Black	Compact	Compact
Medium 9 (6.7 2,4-D + 0.0 BAP)	91.6	8.6	Green	Green	Friable	Compact
Medium 10 (6.7 2,4-D + 4.5 BAP)	91.6	100	Brown	White	Compact	Compact
Medium 11 (6.7 2,4-D + 6.7 BAP)	58.3	75	Rosy	Rosy	Compact	Compact
Medium 12 (6.7 2,4-D + 9.0 BAP)	91.6	100	Rosy	Rosy	Compact	Compact
Medium 13 (9.0 2,4-D + 0.0 BAP)	100	75	Rosy	Brown	Friable	Compact
Medium 14 (9.0 2,4-D + 4.5 BAP)	100	91.6	Rosy	Brown	Compact	Compact
Medium 15 (9.0 2,4-D + 6.7 BAP)	100	100	Rosy	Rosy	Compact	Compact
Medium 16 (9.0 2,4-D + 9.0 BAP)	100	100	Rosy	Rosy	Compact	Compact
Medium 17 (9.0 2,4-D + 1.4 IAA)	0	0	-	-	-	-
Medium 18 (9.0 2,4-D + 2.8 IAA)	8.3	0	Brown	-	Compact	-
Medium 19 (9.0 2,4-D + 4.2 IAA)	50	0	Brown	-	Compact	-
Medium 20 (9.0 2,4-D + 5.6 IAA)	16.6	0	Brown	-	Compact	-

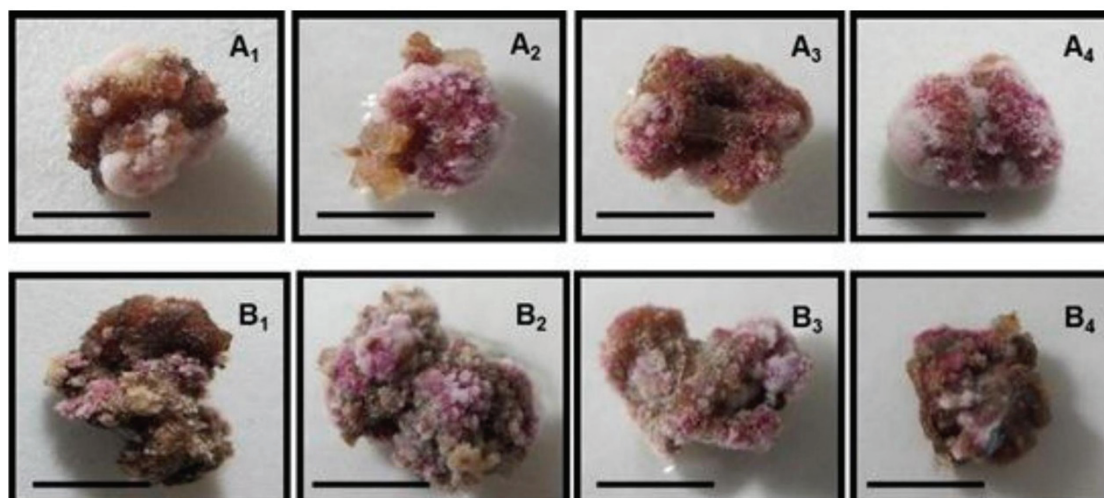


Figure 1. Callus pigmentation of *Alternanthera sessilis* in leaf (A) and internodal (B) explants growth in different callus inductor media and after transferring to a betacyanin inductor medium, after 40 days of cultivation. (A1-B1) – calli originating from medium 11 ($6.7 \mu\text{mol L}^{-1}$ 2,4-D + $6.7 \mu\text{mol L}^{-1}$ BAP), (A2- B2) – calli originating from medium 12 ($6.7 \mu\text{mol L}^{-1}$ 2,4-D + $9.0 \mu\text{mol L}^{-1}$ BAP), (A3-B3) – calli originating from medium 15 ($9.0 \mu\text{mol L}^{-1}$ 2,4-D + $6.7 \mu\text{mol L}^{-1}$ BAP), and (A4-B4) – calli originating from medium 16 ($9.0 \mu\text{mol L}^{-1}$ 2,4-D + $9.0 \mu\text{mol L}^{-1}$ BAP). Bar = 1 cm.

Auxin (2,4-D) alone or combined with cytokinins, such as BAP, is widely used to stimulate callus formation, however, the concentration of these regulators should be defined for each species (Castro, Braga, Souza, Coimbra, & Chagas, 2016). In a study carried out by Trejo-Tapia et al. (2008), a 1 mg L^{-1} of 2,4-D in *Beta vulgaris* combined with 1.0 mg L^{-1} of CIN produced calli in leaf explants with a high betalain concentration. When evaluating callus formation in *Barringtonia racemosa*, Dalila, Jaafar, and Manaf (2013) observed that the best results were obtained with a 1.5 mg L^{-1} concentration of 2,4-D together with cytokinin in 0.5 to 1.0 mg L^{-1} concentrations.

The plant growth regulator 2,4-D has greater mobility and lower oxidation and conjugation rates, and so it would be efficient for promoting calli together with indole acetic acid (IAA) or naphthalene acetic acid (NAA), explaining the results obtained in this paper (Naz & Khatoun, 2014).

The media containing the concentrations of growth regulators that presented the best pigment formation in callus development in white light were repeated and called M1 ($6.7 \mu\text{mol L}^{-1}$ 2,4-D + $6.7 \mu\text{mol L}^{-1}$ BAP), M2 ($6.7 \mu\text{mol L}^{-1}$ 2,4-D + $9.0 \mu\text{mol L}^{-1}$ BAP), M3 ($9.0 \mu\text{mol L}^{-1}$ 2,4-D + $6.7 \mu\text{mol L}^{-1}$ BAP), and M4 ($9.0 \mu\text{mol L}^{-1}$ 2,4-D + $9.0 \mu\text{mol L}^{-1}$ BAP). The material originating from these media was transferred to a BIM medium and exposed to different light qualities (dark, red, blue, and white), where it remained for 40 more days.

The results for the concentration of flavonoids present in *A. sessilis* calli (Figure 2A) revealed that

there was interaction between the light treatments and cultivation medium, with it being possible to observe that the blue light combined with the M2 cultivation presented the most significant results, reaching an average of $9.32 \mu\text{mol}$ of quercetin per 100 g of callus fresh mass, this quantity being greater than that found by Reis et al. (2015) where in the maximum concentration obtained with this species in blue light was $1.6 \mu\text{mol}$ of quercetin per 100 g of callus fresh mass.

The blue light would be associated with increased gene expression at specific points on the flavonoid biosynthesis route, such as the *FaCHS* expression, according to Kadomura-Ishikawa, Miyawaki, Noji, and Takahasni (2013). Studying *Kolonchoe pinnata*, Nascimento et al. (2013) observed accentuated increase in flavonoids when exposed to blue light. This increase was also observed by Xu et al. (2014), total anthocyanin content of strawberry fruits significantly increased after four days of treatment with blue light ($40 \mu\text{mol m}^{-2} \text{ s}^{-1}$) at 5°C compared to the control fruits.

The amaranthine content was also significantly greater in the combination of M2 medium and blue light, reaching a value of 147.6 mg of amaranthine in 100 g of callus fresh mass (Figure 2B). This value was much higher compared with previous studies that used elicitors to increase this pigment in species of the *Alternanthera* genus, in which the maximum concentrations obtained were approximately 40 mg of amaranthine in 100 g of fresh mass (Perotti et al., 2010; Reis et al., 2015).

The bethanidine concentration in M2 combined with blue light was 71.42 mg in 100 g of callus fresh

mass, this result being significantly greater than in the other treatments (Figure 2C). This increase was also observed in the betanin concentration, reaching an average of 59.34 mg of betanin per 100 g of callus fresh mass (Figure 2D). Similar betanin concentrations were obtained by the study from Reis et al. (2015), but when *A. sessilis* and *A. brasiliana* plants were exposed to red light.

For the betaxanthin levels (Figure 2E and 2F), it was possible to observe that the interaction between the callus cultivation medium and blue light was not significant, with only the isolated parameters being significant. The cultivation media known as M1 and M2 were more efficient in the production of betaxanthins. With regards to light quality, blue light was significantly better, having resulted in an increase of up to 10 times the miraxanthin content compared with the white treatment. Bhuiyan,

Murakami, and Adachi (2002), by evaluating cell cultures of the *Portulaca* sp genus, also observed an increase in this parameter when the plants were exposed to blue light.

Analyzing the effect of light on betacyanin production in *Mesembryanthemum crystallinum*, Vogt et al. (1999) verified that an increase in photon flux resulted in an increase in betacyanin quantity, with it being possible to observe with the naked eye that younger leaves had a more intense purple coloring than the control and those that remained under lower flux densities. The increase in betalain production in callus of *Portulaca* exposed to blue light was also observed by Kishima, Shimaya, and Adachi (1995), where they justify that this fact may have occurred through a signaling system that would activate the gene expression given by the blue light photoreceptor.

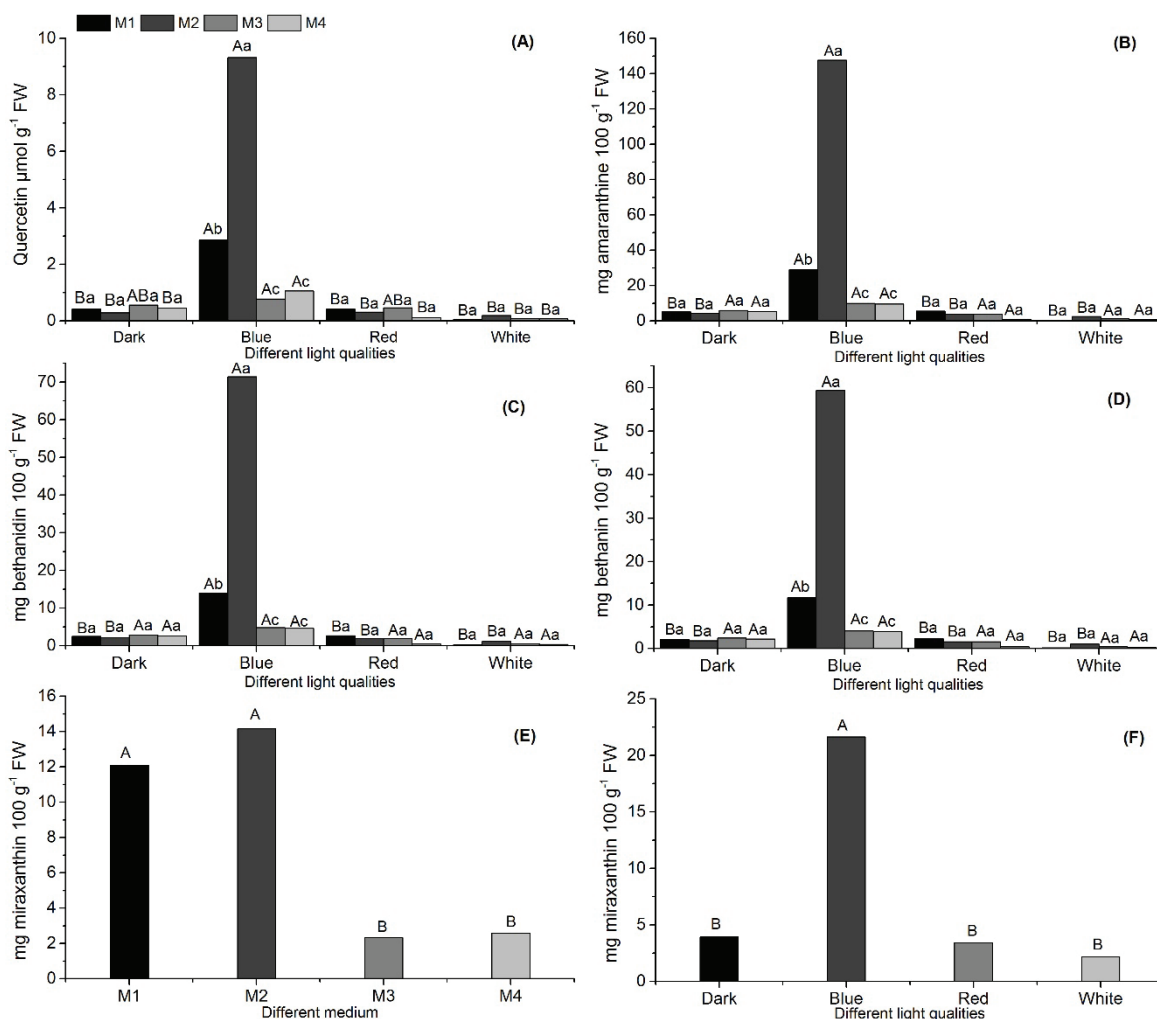


Figure 2. Level of Flavonoids (A), Aمارanthine (B), Bethanidine (C), Bethanin (D), and Miraxanthine (E and F) present in the calli of *Alternanthera sessilis* plants, after elicitation in different light qualities. M1 ($6.7 \mu\text{mol L}^{-1}$ 2,4-D + $6.7 \mu\text{mol L}^{-1}$ BAP), M2 ($6.7 \mu\text{mol L}^{-1}$ 2,4-D + $9.0 \mu\text{mol L}^{-1}$ BAP), M3 ($9.0 \mu\text{mol L}^{-1}$ 2,4-D + $6.7 \mu\text{mol L}^{-1}$ BAP), and M4 ($9.0 \mu\text{mol L}^{-1}$ 2,4-D + $9.0 \mu\text{mol L}^{-1}$ BAP). Averages followed by different uppercase letters differ between each other in relation to the type of light for the media. Averages followed by different lowercase letters compare cultivation media in relation to a particular light using the Tukey test ($p < 0.05$).

According to Heo et al. (2012), blue light acts directly in the signaling of secondary metabolism genes, as well as being important for the formation of chloroplasts and pigments, since red is involved in the development of the photosynthesis apparatus.

Kinoshita et al. (2003) reported that blue light is responsible for maintaining electric potential in the membranes, forcing the stomata to open and allowing more CO₂ to enter, and consequently increasing photo assimilate content, which would stimulate the production of secondary metabolites.

Cryptochromes are blue light receptors linked to a flavin and pterine, and responsible for mediating various plant responses, such as regulation of circadian rhythms, depolarization of membranes, anthocyanins production, and other effects (Yu, Liu, Klejnot, & Lin, 2010).

Since these cryptochromes act in the production of anthocyanin, we believe that these receptors may also be associated with the production of betalains, once they are exclusively present in the order Caryophyllales, replacing anthocyanins (Gandia-Herrero et al., 2005). According to Khan and Giridhar (2015) in prehistory, these pigments coexisted and during evolution, some anthocyanin synthesis enzymes were lost and this order evolved the formation of pigments for their protection, through another metabolic route. They also reinforce that the similarity can be explained by hegemony analyzes, in which L-DOPA dioxygenase, enzyme that leads to the formation of betalamic acid precursor of many betalains, bears similarity to enzymes of the route of anthocyanin biosynthesis.

According to Cao et al. (2012), light acts in the formation of the dihydropyridine portion of betalains, the chromophore, responsible for the formation of color, and therefore the absence of this could impair biosynthesis.

The fact that the calli exposed to white light presented a lower concentration of betalains in this experiment may be explained by the study from Hatlestad et al. (2015), who affirm that this light is responsible for degrading betalains that are already formed, however, it would be fundamental for initiating biosynthesis, since white light acts in the DOPA-dioxygenase enzyme at the beginning of the pathway.

Lee et al. (2014) reported that light is not a limiting factor in betalain synthesis, but that in combination with other factors it can alter the quantity of product formed. This association between different factors supports the results obtained in this paper, in which significant increases in betacyanins were obtained with the M2

cultivation medium with a 6.7 $\mu\text{mol L}^{-1}$ 2,4-D and 9.0 $\mu\text{mol L}^{-1}$ BAP, in combination with blue light.

Other question possible is that different light qualities can generate a plant imbalance and form oxygen reactive species and may have been the cause of this accentuated increase in betacyanins in response to blue light, in combination with the M2 medium, since betacyanins are considered antioxidant.

In a study with *Saueda salsa*, Wang and Liu (2006) reported an increase in betacyanins in the dark and that, probably, the signal for triggering this increase had been the accumulation of oxygen reactive species. Ramakrishna and Ravishankar (2011), in their reviews regarding the effect of abiotic factors in increasing secondary metabolites, cite various papers that have proven that different colors and spectrums are associated with the production of metabolites of interest and they also suggest that the production of oxygen reactive species induce antioxidant pathways such as betalains.

Lage, Tirado, Vanicore, Sabino, and Albarello (2015) emphasize that cell suspensions and calli are considered the most efficient biotechnological techniques for *in vitro* production of special metabolites, favoring the formation of a more homogenous system with high cellular proliferation with potential for large-scale production of these pigments.

Conclusion

The use of natural pigments has been gained a great deal of commercial appeal, but their production is limited, since large mass quantities should be used for extraction and purification. The use of biotechnological techniques used in this work, such as the *in vitro* culture for callus production in *A. sessilis* grown under different combinations of growth regulators and light qualities, were efficient in increasing biosynthesis of betalains with potential for subsequent commercial production.

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