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Original Article

Impact of light quality on flavonoid production and growth of *Hyptis marrubioides* seedlings cultivated *in vitro*

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ABSTRACT

Hyptis marrubioides Epling, Lamiaceae, a species from Brazilian Cerrado, has been used against gastrointestinal infections, skin infections, pain, and cramps. Herein, *H. marrubioides* seedlings were cultured *in vitro* under different wavelengths (white, blue, green, red, and yellow) with 50 μmol m^{-2} s^{-1} irradiance and a 16-h photoperiod. After 20 and 30 days of cultivation, shoot length, leaf number, fresh mass, and dry mass were evaluated. The flavonoid rutin content was determined by the HPLC-DAD method. The shoots were longer in plants cultivated under yellow (16.603 ± 0.790 cm, 1.8-fold), red (15.465 ± 0.461 cm, 1.7-fold), and green (14.677 ± 0.737 cm, 1.6-fold) lights than in control plants exposed to white light (9.203 ± 0.388 cm). The number of leaves increased in plants exposed to red (23.425 ± 1.138, 1.1-fold) and green (22.725 ± 1.814, 1.1-fold) lights, compared to control plants (20.133 ± 0.827). Fresh (0.665 ± 0.048 g, 1.2-fold) and dry (0.066 ± 0.005 g, 1.3-fold) mass of seedlings were the highest in seedlings grown under red light, compared to seedlings grown under white light (0.553 ± 0.048 and 0.028 ± 0.004, respectively). However, rutin production was higher under white (0.308 mg g^{-1} of dry weight) and blue lights (0.298 mg g^{-1} of dry weight). Thus, red light induces plant growth and increases leaf number and dry weight in *in vitro*-cultivated *H. marrubioides*, whereas blue and white lights promote the greatest rutin accumulation.

Introduction

The genus *Hyptis*, Lamiaceae, comprises about 300 species, widely distributed, occurring mainly in tropical regions of the Americas and Africa. *Hyptis marrubioides* Epling, commonly known as mint-of-field, is a species of the Brazilian Cerrado traditionally used to treat gastrointestinal and skin infections, pain, and cramps (McNeil et al., 2011). The pharmacological potential of *H. marrubioides* has been previously investigated, and most studies have focused on examination of the chemical composition of essential oils, the main components of which are the sesquiterpenes caryophylla-4(14),8(15)-dien-5β-ol, eudesma-4(15),7-dien-1β-ol, caryophyllene oxide, and β-caryophyllene (Sales et al., 2007; McNeil et al., 2011). Essential oils of *H. marrubioides* have also been studied to investigate their potential for pest control in agriculture, such as the prevention and control of Asian soybean rust (Silva et al., 2012b) and treatment of *Colletotrichum truncatum*-infected soybean seeds (Silva et al., 2012a).

The use of micropropagation techniques has great advantages in the production of high-quality seedlings, as they improve their pharmacological potential (Rao and Ravishankar, 2002) by ensuring the reproduction of identical, high-quality plants (Serafini et al., 2001), which in turn enhances the biosynthesis of special metabolites (Bhuiyan and Adachi, 2003; Zhao et al., 2005). However, the accumulation of secondary metabolites in plants is influenced by various environmental factors such as light quality, UV irradiation, temperature, irrigation, nutrient deficiency, pathogen attack, and heavy metal stress (Dixon and Paiva, 1995; Winkel-Shirley, 2002; Kopsell and Kopsell, 2008).

Light is a key abiotic elicitor in plants that affects, directly or indirectly, growth and development of plants, mainly of those distributed in high-latitude areas (Morini and Muleo, 2003; OuYang et al., 2015). Plant responses do not depend only on the absence or presence of light but also on the variation in light quality (Felippe, 1986). The action of light on plants occurs mainly in two aspects: first, the light source provides the energy required by the plant for

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photosynthesis, especially red and blue light, and second, as a signal received by photoreceptors it regulates growth, differentiation, and plant metabolism (Wang et al., 2001).

Light is one of the most significant environmental factors affecting the accumulation of flavonoids in plants (Huché-Thélier et al., 2016). However, the biosynthesis of flavonoids in response to light quality need to be better understood, especially in *in vitro* culture.

Studies involving the influence of light on the production of secondary metabolites and growth parameters in *H. marrubioides* seedlings cultivated *in vitro* are non-existent. In a previous study, we have evaluated the phytochemical profile of *H. marrubioides* microplants inoculated with isolates of bacteria and endophytic fungi (Vitorino et al., 2013). Photosynthetic studies in *H. marrubioides in vitro* culture involving measuring gas exchange were also conducted by our research group and the irradiance effects, flow rate, and air humidity parameters were investigated. In addition, the chlorophyll a fluorescence and chloroplastidic pigment content were also assessed (Costa et al., 2014). Hence, the aim of the present study was to assess the effect of light quality on growth of *H. marrubioides* seedlings *in vitro* and their flavonoid production. This study furthers our understanding of the factors that promote the growth and increase the resistance of this medicinal species, through the production of flavonoids.

Materials and methods

Plant material and in vitro *cultivation*

Seedlings previously established *via in vitro* germination of *Hyptis marrubioides* Epling, Lamiaceae, seeds suitable for inoculation obtained on the experimental field of the Laboratory of Plant Tissue Culture of the Instituto Federal de Educação, Ciência e Tecnologia Goiano, Campus Rio Verde. A voucher specimen (HRV71) has been deposited at the Herbarium of the Institute (Herbarium HRV).

The seeds were disinfected with 0.2% Bendazol (carbendazim) and 0.2% Alterno (tebuconazole) for 1 h, followed by treatment with 1% sodium hypochlorite for 30 min, and then rinsed thrice with sterile distilled water. Seeds were germinated and maintained on Murashige & Skoog medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} of sucrose and solidified with agar at 3.5 g l^{-1}; the pH was adjusted to 5.8 before autoclaving. The cultures were incubated in a growth chamber for 30 days at 50 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), at an average temperature of 23 ± 1 °C, and a photoperiod of 16 h. Thereafter, the seedlings were sub-cultured on the same medium and incubated in a growth cabinet for 10 days at 50 μmol m^{-2} s^{-1} PAR, 23 ± 1 °C, and 16-h photoperiod. All experiments were carried out in glass flasks containing 50 ml of semisolid medium with five seedlings per flask with a total of forty seedlings.

Light conditions

After 10 days, the cultures were transferred to a cabinet and exposed to continuing irradiation of different light spectra: white (300–750 nm), blue (400–490 nm), green (490–560 nm), red (600–700 nm), and yellow (560–590 nm). The specific light conditions were maintained using TP 40 W lamps (Taschibra® Indaial, Santa Catarina, Brazil), at a light intensity of 50 μmol m^2 s^{-1} and a photoperiod of 16 h. The cultures were evaluated after 20 and 30 days of light exposure. Cultures grown under white light were used as a control. For weight measurements, the whole seedlings without roots was considered.

Chemical analysis

The seedlings were dried at 35 °C in a forced air circulation oven and the dry biomass (200 mg) from the *in vitro* culture of *H. marrubioides* was extracted with 4 ml methanol of high performance liquid chromatography (HPLC) grade using ultrasound bath (30 min). The samples had been previously filtered through a 0.2-μm syringe filter with hydrophilic PTFE membrane (Advantec, Dublin, CA, USA) and transferred into 1-ml HPLC vials. These procedures were conducted in triplicate.

For the quantitative analysis, solutions were done in HPLC grade methanol to obtain solutions containing 0.500, 0.250, 0.125, and 0.063 mg ml^{-1} rutin. Each standard solution was injected in triplicate. The calibration curve was constructed to determine the linearity of the method by plotting the peak area *versus* the concentration of the substance in mg ml^{-1}. The quantitative analysis by HPLC with diode-array detection (DAD) was carried out on a Shimadzu Prominence LC-20AD binary system equipped with a DGU-20A5 degasser, an SPD-20A series diode array detector, a CBM-20A communication bus module, an SIL-20A HT autosampler, and a CTO-20A column oven (Shimadzu, Kyoto, Japan). The analyses were conducted on a Gemini ODS column (250 × 4.6 mm, 5 μm; Phenomenex, Aschaffenburg, Germany) equipped with a pre-column with the same material under the following conditions: injection volume set at 20 μl, flow rate of 1.0 ml min^{-1}, mobile phase was CH_3OH/H_2O/HOAc (5:94.9:0.1, v/v/v) delivered in a linear gradient until 100% CH_3OH in 30 min, 10 min at 100% CH_3OH, and 20 min to return to the initial conditions. The UV detection was set at 254 nm and 40 °C.

Methanol used in the experiments was HPLC grade and it was obtained from J. T. Baker (Avantor Performance Materials, Center Valley, PA, USA). Ultrapure water was obtained by passing redistilled water through a Direct-Q UV3 system from Millipore (Billerica, MA, USA). The flavonoid rutin used as external standard was acquired from the standard bank of the Natural Products Group of the Universidade de Franca.

Statistical analyses

The statistical analysis was performed in Sisvar 5.3 software (Ferreira, 2011). The experiment was conducted in a completely randomized design with four replications. The multi-factorial analysis of variance followed by Tukey multiple comparison tests were used for statistical comparisons ($p \leq 0.05$).

Results

Seedlings growth

The measured growth parameters of *H. marrubioides* seedlings cultivated *in vitro* under different wavelengths showed significant difference (Table 1). During the 20-day cultivation, the seedlings exposed to red light showed the highest shoot lengths (10.175 ± 0.669 cm), which was 1.69-fold higher than the corresponding lengths of control seedlings (6.035 ± 0.511 cm). In addition, after 30 days of irradiation with green, red, and yellow lights, the length of the shoots increased 1.8-fold compared to seedlings exposed to white light. The number of expanded leaves was not affected by light conditions after 20 days of treatment; however, after 30 days of culture, green and red light promoted the most the increase in the number of leaves (22.725 ± 1.814 and 23.425 ± 1.138, respectively).

Shoot dry and fresh weights did not differ among the seedlings irradiated with different lights for 20 days. In contrast, after 30 days of illumination with red light, dry biomass of *H. marrubioides*

Table 1
Shoot length and average number of expanded leaves of *Hyptis marrubioides* seedlings submitted to 20 and 30 days of light treatment.

Light treatments	Evaluation time (days)		Evaluation time (days)	
	20	30	20	30
	Average shoot length (cm)		Average leaf number	
White	6.035[a] Bb ± 0.511[b]	9.203 Ca ± 0.388	12.475 Ab ± 0.927	20.133 Ba ± 0.827
Blue	8.400 Ab ± 0.453	13.030 Ba ± 0.865	13.143 Ab ± 0.667	17.750 Ba ± 1.293
Green	9.900 Ab ± 0.823	14.677 Aa ± 0.737	13.514 Ab ± 0.676	22.725 Aa ± 1.814
Red	10.175 Ab ± 0.669	15.465 Aa ± 0.461	12.600 Ab ± 1.223	23.425 Aa ± 1.138
Yellow	9.871 Ab ± 0.462	16.603 Aa ± 0.790	11.143 Ab ± 1.166	20.067 Ba ± 0.935
CV (%)	15.46		18.87	
	Fresh weight (g)		Dry weight (g)	
White	0.311 Ab ± 0.059	0.553 Aa ± 0.048	0.028 Ab ± 0.003	0.052 Ba ± 0.004
Blue	0.306 Ab ± 0.024	0.549 Aa ± 0.042	0.033 Ab ± 0.002	0.057 Ba ± 0.004
Green	0.334 Ab ± 0.046	0.534 Aa ± 0.053	0.034 Ab ± 0.003	0.053 Ba ± 0.004
Red	0.404 Ab ± 0.067	0.665 Aa ± 0.048	0.035 Ab ± 0.004	0.066 Aa ± 0.005
Yellow	0.246 Ab ± 0.018	0.426 Aa ± 0.025	0.025 Ab ± 0.001	0.045 Ba ± 0.002
CV (%)	29.77		22.14	

[a] Means followed by the same uppercase letter in the column and lowercase letter in the row do not differ significantly according to the Scott-Knott test at 5% probability.
[b] Mean ± standard error.
CV, coefficient of variation.

Table 2
Accumulation of rutin (mg g^{-1} dry weight) in *Hyptis marrubioides* seedlings irradiated with white, blue, green, red, and yellow lights for 20 and 30 days.

Light treatment	Evaluation time	
	Rutin content (20 days) (mg g^{-1} dry weight)	Rutin content (30 days) (mg g^{-1} dry weight)
White	0.243[a] A ± 0.012[b]	0.308 A ± 0.012
Blue	0.084 D ± 0.004	0.298 A ± 0.007
Green	0.101 C ± 0.002	0.198 C ± 0.002
Red	0.105 C ± 0.006	0.192 C ± 0.002
Yellow	0.126 B ± 0.002	0.262 B ± 0.006

[a] Means followed by the same letter do not differ significantly according to the Tukey test at 5% probability.
[b] Mean ± standard error.

seedlings increased 1.27-fold (0.066 ± 0.005 g) compared to dry weight of the control seedlings (0.052 ± 0.004 g).

Accumulation of rutin flavonoid

Methanol extracts of rutin obtained from seedlings cultured under different lights for 20 and 30 days was quantitatively assessed using HPLC-DAD analysis (Table 2). The response of the UV detector at 254 nm was linear from 0.063 a 0.500 mg ml^{-1} for rutin. The obtained regression equation was $y = 4.0 \times 10^7 x - 69{,}442$ with a correlation coefficient (R^2) of 0.9998. After 20 days of culture, the accumulation of rutin in all samples was lower than that in the control (0.243 mg g^{-1} dry weight (DW)); the lowest amount of rutin was detected in plants exposed to blue light (0.084 mg g^{-1} DW). However, after 30 days of treatment, the amount of rutin in seedlings exposed to blue light (0.298 mg g^{-1} DW) was not significantly different to that in control seedlings (0.308 mg g^{-1} DW), whereas its content in seedlings exposed to green (0.198 mg g^{-1} DW), red (0.192 mg g^{-1} DW), and yellow (0.262 mg g^{-1} DW) lights was reduced significantly compared to that in control plants. Overall the results demonstrated that the rutin content in micropropagated plants was lower than that found in wild plants of *H. marrubioides* (1.259 mg g^{-1} DW) obtained in previous studies conducted by our research group.

Discussion

Light quality has important effects on plant growth and development, especially for photosynthesis and photomorphogenesis, the two processes that, through signals received by the photoreceptors, regulate plant growth, differentiation, and metabolism (Guo et al., 2007). Plant growth can be affected by endogenous hormone levels interactions with light quality. The interaction of light quality with plant hormone pathways in regulation growth of vegetal species includes gibberellins (GAs), auxins being the most common is the indoleacetic acid (IAA), cytokinins (CKs), and abscisic acid (ABA) (OuYang et al., 2015).

Another study found that the levels of endogenous CKs, IAA, and GAs are markedly increased in response to low-intensity red to far-red light at low or normal PAR, promoting the growth of hypocotyls in *Helianthus annuus* (Kurepin et al., 2007).

Transcriptome analysis of Norway spruce (*Picea abies*) by OuYang and collaborators revealed that red and blue light are associated with plant growth and development and phytohormone metabolism (OuYang et al., 2015). In addition, they reported that blue light promotes IAA accumulation and phenylpropanoid biosynthesis. In contrast, red light affects stem growth by regulating biosynthesis of GAs. In line with these results, Thwe et al. (2014) reported that irradiation with blue light increases polyphenol content in lettuce and induces the production of rutin and cyanidin 3-*O*-rutinoside in *Fagopyrum tataricum*.

The evaluation of growth parameters in *H. marrubioides* seedlings cultured *in vitro* in our study show that red light almost doubled the length of shoots and increased the number of leaves and dry biomass compared to the control. Concerning the production of secondary metabolites, we found that blue and white light favored the biosynthesis of the flavonoid rutin.

Phenylalanine ammonia-lyase (PAL) and chalcone synthase are important regulatory enzymes of the biosynthesis of phenylpropanoids (C_6C_3 compounds), including flavonoids. PAL is involved in plant response to a variety of biotic and abiotic stresses (Wang et al., 2010; Yousefzadi et al., 2012). According to Awad et al. (2001), light is one of the most important factors in the control of flavonoid synthesis, acting upon a complex system involving several photoreceptors, including UV, blue, phytochrome and the photosynthetic system.

Jeong et al. (2012) studied the influences of four different light-emitting diode lights on flowering and polyphenol variations in the leaves of chrysanthemum (*Chrysanthemum morifolium*). The luteolin-7-*O*-glucoside, luteolin-7-*O*-glucuronide and quercetagetin-trimethyl ether showed highest yield under green light. On the other hand, dicaeoylquinic acid isomer, dicaeoylquinic acid isomer, naringenin, and apigenin-7-*O*-glucuronide were greatest under red light. Taulavuori et al. (2016) evaluated the differences in synthesis of flavonoids and phenolic acids under increasing periods of enhanced blue light in leaves red lettuce and basil. Flavonoids and phenolic acids were found to be species dependent. Dicaffeoyltartaric acid was the major compound in lettuce while rosmaric acid was the prodominant compound in basil.

These examples suggest that the polyphenol production is influenced by light quality composition.

Our results on the growth and metabolism of *in vitro*-cultured seedlings of *H. marrubioides* are in accordance with the data described by OuYang et al. (2015) and Thwe et al. (2014) as well the data reported for other plant species (Vreugdenhil et al., 1998; Kvaalen and Appelgren, 1999; Leal-Costa et al., 2010; Ouzounis et al., 2015).

In summary, we conclude that for the *in vitro* culture of *H. marrubioides* light quality is an important parameter that can be utilized to optimize seedlings growth and accumulation of rutin, in particular the red and blue lights, which are beneficial to growth and rutin metabolism, respectively. Therefore, our results may be useful as theoretical basis for studies related to quality control of growth and production of secondary metabolism of *H. marrubioides*.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

RCNP and NAAB performed the *in vitro* experiments, ACBAMH contributed to the supervision of laboratory work, and critical reading of the manuscript, RCNP and LPP contributed to the chromatographic analysis, ACC, FGS, MLAS, WRC, PMP and AHJ contributed to the analysis of experimental data and critical revision. All authors helped in preparing the paper and approved the submission of the final version.

Conflicts of interest

The authors declare no conflicts of interest.

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