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Effects of light quality on rutin production and growth of *Physalis angulata* (Linn.) seedlings cultured *in vitro*

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Abstract

Physalis angulata Linn. is a plant with great importance in folk medicine for its various therapeutic properties and the production of active compounds. It is known as *camapú* in Brazil. The *P. angulata* seedlings were cultured *in vitro* under different light qualities such as white (control), blue, green, red, and yellow at 16 h photoperiod. After 30 days of culture, the shoot length, number of leaves, fresh and dry matter and rutin content were evaluated in triplicate in methanol extracts of seedlings exposed to the different lights by High-Performance Liquid Chromatography- Diode Array (HPLC-DAD). The mean shoot length was longer in seedlings cultured under yellow light (22.83 ± 0.65 cm, 1.62-fold), red light (22.58 ± 0.44 cm, 1.6-fold), or green light (20.57 ± 0.72 cm, 1.46-fold) than seedlings exposed to white light (14.13 ± 0.26 cm). There were no differences in the mean number of leaves between seedlings grown under the remaining lights and white light. Fresh (1,152 ± 0.16 g) and dry weight (0.078 ± 0.01 g) were higher in seedlings grown under white light. However, rutin production was higher under blue light (2.78 ± 0.05 μg g^{-1} by dry weight) and green light (2.40 ± 0.06 μg g^{-1} by dry weight). Therefore, the various light qualities affected the growth of *P. angulata* seedlings differently under *in vitro* culture condition. The blue and green lights promoted greater accumulation of rutin in this species.

Keywords: Light quality; *Physalis angulata*; Tissue culture; Rutin.
Abbreviations: ANOVA_analysis of variance; CV_coefficient of variation; HPLC-DAD_high-performance liquid chromatography - diode array; LED_light emitting diode; MS_murashige and skoog; PAR_photosynthetically active radiation; pH_potential of hydrogen; UNESP_são paulo state university; UV_ultraviolet; UV-VIS_ultraviolet-visible spectrophotometry.

Introduction

Known popularly in Brazil as *camapú*, *Physalis angulata* Linn. is a fruit-bearing species of the Solanaceae family with wide ecological adaptation and distribution in tropical and temperate regions of the world (Bastos et al., 2006; AM and Nidavani, 2014). *P. angulata* is used for medicinal purposes as an analgesic, sedative, antirheumatic, antidiuretic, anti-inflammatory, antimalarial, and antiasthmatic, as well as for bladder and prostate problems and liver disorders (Bastos et al., 2006, 2008; Zhang and Tong, 2016). The medicinal effects may be related to the chemical constituents that already characterized in the leaves, which include diterpenes, esters, flavonoids, ceramides, and withasteroids, among others (Tomassini et al., 2000; Zhang and Tong, 2016). The flavonoids are of particular relevance in the genus *Physalis* due to their antioxidant effects, free radical-scavenging properties, and prevention of degenerative diseases (Pérez-Castorena et al., 2013; Medina-Medrano et al., 2015). Due to its medicinal importance, species of the genus *Physalis* have been the subject of research in studies on propagation and bioactive compounds (Ramadan, 2011).

The culture of *Physalis* presents some barriers to its commercial production. *In vitro* tissue culture techniques have shown promise in large-scale production, with the goal of isolating compounds of interest. In tissue culture, it is possible to use elicitors that stimulate plant defense

mechanisms, promoting metabolism to protect the cell and/or whole plant (Ramirez-Estrada et al., 2016).

Light is considered a fundamental abiotic elicitor for plants. It acts directly and/or indirectly in the growth and development of the plant, promoting modifications in metabolite production (Zavala and Ravetta, 2001; Zoratti et al., 2014). Plants respond specifically to the intensity and quality of light (Wang et al., 2009; Casierra-posada and Peña-olmos, 2015). Thus, it is necessary to optimize the light quality in the spectral range corresponding to the action of different photoreceptors, such as cryptochromes and phototropins. The absorption of light through these photoreceptors induces photomorphogenic responses in plants (Macedo et al., 2011; De Lucas and Prat, 2014; Casierra-posada and Peña-olmos, 2015). Blue light (400-500 nm) and red light (600-700 nm) promote greater plant growth because the action spectra have maximum absorption at these wavelengths (Li and Kubota, 2009; Hernández and Kubota, 2014).

The use of different light sources at *in vitro* condition opens up new perspectives for micropropagation and the study of light on explants (Takeui et al., 2017). As an example, the use of blue light may promote higher levels of carotenoids, total polyphenols and antioxidants in *Lactuca sativa* (Johkan et al., 2010); higher chlorophyll content in *Vaccinium myrtillus* and *Doritaenopsis* (Shin et al., 2008; Hung et al., 2016); and increased biosynthesis of flavonoids, flavonoids, flavonols, and jasmonic acid in *Picea abies* (Ouyang et al., 2015). *Pisum sativum* irradiated with blue light obtained higher levels of chlorophyll, and those with red light showed higher β-carotene content and antioxidant activity (Wu et al., 2007).

Although some studies show the effects of light quality, works using this tool in plant micropropagation are still scarce, and the effects of light spectrum and irradiance levels on the production of chemical compounds of interest in seedlings grown *in vitro* are not clear. The objective of this work was to evaluate the influence of light quality on the *in vitro* growth of *P. angulata*, as well as to quantify the flavonoid rutin in this micropropagated species, aiming to use it in future studies on its production in cell suspension cultures, for commercialization and exploitation of the biotechnological potential of the species.

Results

Seedling growth and Shoot lengths

During the 30 days of culture of *P. angulata* seedlings in the different light environments, greater shoot lengths were observed in the seedlings grown under green (1.45-fold), red (1.59-fold), and yellow light (1.61-fold) light compared to white light (control).

On the other hand, the blue light did not alter the growth parameter in comparison to light control.

Number of leaves

The number of expanded leaves per seedling was not affected by light conditions after 30 days of treatment compared to the control treatment (6.43 ± 0.25 leaves) (Table 1).

Fresh and dry biomass

Seedlings grown under blue, green, and red light did not differ from each other in accumulation of fresh biomass (0.932 ± 1.13 g, 0.951 ± 1.14 g, and 0.998 ± 1.14 g, respectively) or dry biomass (0.073 ± 0.01 g, 0.065 ± 0.01 g, and 0.067 ± 0.01 g, respectively). However, the weights for these treatments were lower than for the control treatment (1.152 g fresh weight and 0.078 g dry weight). The treatment of seedlings grown under yellow light had the lowest weights among all the light treatments (0.803 ± 1.11 g fresh weight and 0.053 ± 0.01 g dry weight) (Fig. 1).

Analyzing the three growth factors together, it is observed that red, green and yellow lights intensify the shoot lengths compared to white light. However, the blue light was not significantly different to white light used as control. On the other hand, the light quality did not interfere in the number of expanded leaves. Concerning the fresh and dry biomass content, the white light promoted the best production of both biomass content, followed by green and blue red lights, and contrasting with the yellow light that provided the lowest the dry and fresh weights.

Accumulation of the flavonoid rutin

HPLC-DAD was used to evaluate the rutin content in the samples. After 30 days of culture, the highest accumulation of rutin per flask was observed in seedlings exposed to blue light (2.78 ± 0.05 μg g^{-1} by dry weight), followed by seedlings cultured under green light (2.40 ± 0.06 μg g^{-1} by dry weight) and white light (2.23 ± 0.06 μg g^{-1} by dry weight). Seedlings exposed to red (1.56 ± 0.02 μg g^{-1} by dry weight) and yellow light (1.66 ± 0.05 μg g^{-1} by dry weight) had the lowest rutin accumulations. The highest mean rutin yields were found in seedlings grown under blue (0.2032 ± 0.004 μg) and white light (0.1739 ± 0.004 μg). The yields of the seedlings grown under red and yellow light did not differ from the control, with mean values of 0.1045 ± 0.002 μg and 0.0882 ± 0.003 μg, respectively (Fig. 2).

Discussion

Light effects can be categorized into photoperiod (duration), intensity (quantity), direction, and quality (wavelength), including UV light (Zoratti et al., 2014).

The light quality regulates the plant growth and development by different mechanisms including the selective activation of light receptors such as phytochrome, cryptochome or phototropin. Thus, different light colors can activate specific plant receptors

In addition, the white light is formed by all other lights, giving it a broader spectrum (300-750 nm). It is expected that its response in plant growth will be different from example red light, whose more delimited spectral region associated with a narrow bandwidth (600-700 nm).

In plant tissue culture, the source and the quality of the light directly affect the multiplication and rooting of explants *in vitro*, since the biological efficiency of the culture media as well as the hormonal balance of the tissues are affected by the light characteristics. In addition, light qualities interfere with the morphogenic and physiological development of seedlings grown *in vitro* (Vasil and Thorpe, 2003; Neves et al., 2016), affecting shoot length, leaf production, dry weight, root formation, and photosynthetic production,

Table 1. Mean shoot length and mean number of expanded leaves of *P. angulata* after 30 days of culture in different light treatments.

Light Treatment	Mean shoot length (cm)	Mean number of leaves
White	14.131 ± 0.26 [2 b]	6.43 ± 0.25 [a]
Blue	15.44 ± 0.58 [b]	6.30 ± 0.20 [a]
Green	20.57 ± 0.72 [a]	6.10 ± 0.20 [a]
Red	22.58 ± 0.44 [a]	6.36 ± 0.31 [a]
Yellow	22.83 ± 0.65 [a]	6.06 ± 0.28 [a]
CV (%)	8.44	9.14

[1]Means followed by the same letter do not differ from each other at 5% probability by Tukey's test. [2]± Standard error of the mean. CV (%) = coefficient of variation.

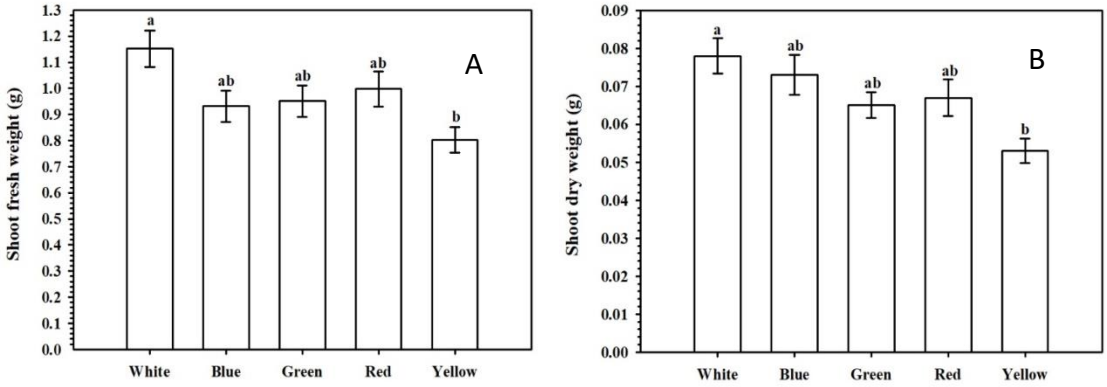

Fig 1. Accumulation of fresh (A) and dry weight (B) in *P. angulata* seedlings at 30 days of exposure to different light spectra.

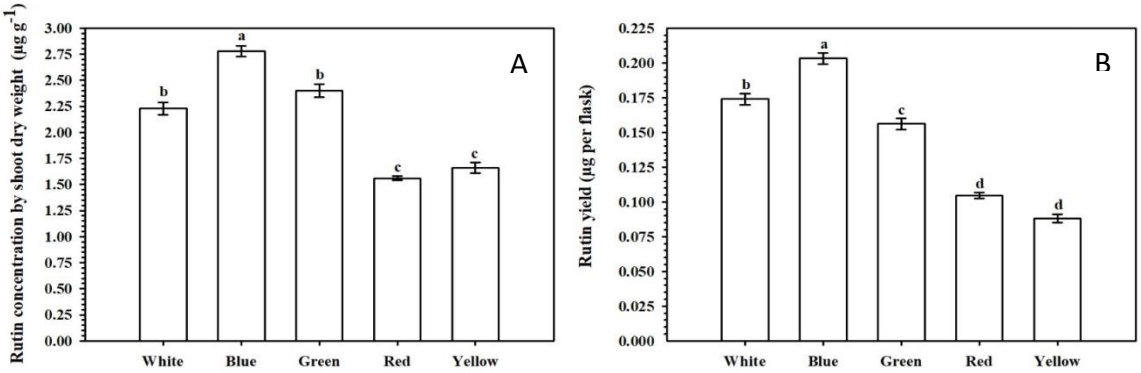

Fig 2. Rutin concentrations and yields (A and B) in *P. angulata* seedlings grown under different light qualities. Identical letters indicate no differences according to Tukey's test at a significance level of 5%. * Vertical bars represent the standard error of the triplicate mean.

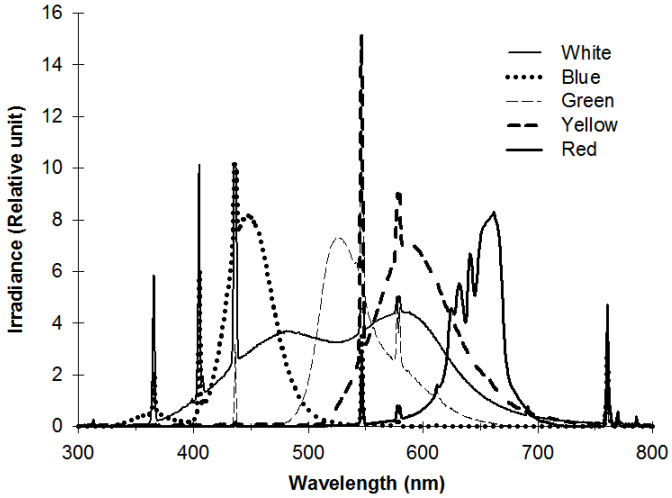

Fig 3. Spectral characteristics of fluorescent lights used in the treatments of *P. angulata* seedlings at 30 days of e xposure to light spectra: white (300-750 nm), blue (400-490 nm), green (490-560 nm), red (600-700 nm), or yellow light (560-590 nm).

among others (Sæbø et al., 1995; Macedo et al., 2011). In the present work, two independent actions were observed, one dependent on the green, red, and yellow lights, which affected shoot length. The other action depended on the white light, which positively affected the fresh and dry weight, possibly evoking the role of specific photoreceptor systems in the plant response. Our results are in concordance with the studies carried out with *Taraxacum japonicum*, in which light directly affected the growth of plants under different light qualities, where 100% rooting was promoted by red light. Moreover, the *in vitro* cultures irradiated with blue light caused an increase in fresh weight and chlorophyll content (Moon et al., 2006). Regarding the growth parameters such as fresh and dry weight, we found that the maximum increase was obtained for white light, followed by red, green and blue lights. In the same way, the fresh weight, dry weight, and leaf area of *Chrysanthemum* increased when irradiated with white, red, and blue light at *in vitro* condition (Kim et al., 2004). Heo et al. (2006) reported that light quality directly affect the *in vitro* growth of a *Vitis* sp. variety, since fresh and dry weight and photosynthetic rates were increased when the seedlings were exposed to white, red, or a mixture of blue and red light but were negatively affected only when irradiated with blue light. However, in *in vitro* cultures of *Brassica napus*, the proliferation rate was higher in seedlings grown under blue light than under white light (Li et al., 2013). In our study, the number or expanded leaves was not affected by light conditions, contrasting with the case of *Anoectochilus formosanus*, where green light promoted higher numbers of branches, leaves, and roots when compared to other light qualities (Haque et al., 2016). In addition to inducing changes in morphological and physiological parameters, light quality influenced the concentration and composition of several primary and secondary metabolites (Gobbo-Neto and Lopes, 2007; Zoratti et al., 2014). In this context, if a plant is able to withstand a low-light environment or a deficit in adequate light, that could be a viable alternative to increase the content of secondary metabolites (Hou et al., 2010). Flavonoids and hydroxycinnamic acids are the main phenolic compounds in fruits (He and Giusti, 2010). Such compounds, especially flavonoids and phenylpropanoids, protect against photodamage provided by these metabolites. They absorb and/or dissipate solar energy, preventing UV radiation from damaging more internal tissues (Koyama et al., 2012; Zoratti et al., 2014). Our studies demonstrate that the biosynthesis of the flavonoid rutin was stimulated by blue light and had its smallest contents under the red and yellow lights. The effects of light on the biosynthesis of flavonoids have been reported in several species, where the light intensity and quality influence the growth and accumulation of total flavonoids. In studies with *Erigeron breviscapus*, biomass and flavonoid production were higher when pants were grown under white film, compared to yellow, red, and purple film (Su et al., 2006). In *Vitis* sp. treated with LED light emitters, anthocyanin concentrations were highest in the bark treated with blue light, followed by red light treatment (Kondo et al., 2014). In *in vitro* cultures of *Rehmannia glutinosa*, blue and red light treatments showed a significant increase in total flavonoid levels in leaf and root extracts (Manivannan et al., 2015). Ghasemzadeh et al. (2010) showed that the synthesis of flavonoids in the Halia Bara variety of *Zingiber officinale* was improved at lower light intensities. Additionally, in experiments on *Ligustrum vulgare* leaves, there was a greater accumulation of flavonoids under red light and little accumulation of this metabolite under green light (Tattini et al., 2004). In *in vitro* cultures of *Ruta graveolens,* the amount of phenolic acids was stimulated by white and blue lights, whereas the total concentration of furanocoumarins was higher when exposed to blue light. Blue light has been recognized as an important regulator that positively controls the germination, photosynthetic capacity, chlorophyll content, leaf expansion, stem elongation, and height of plants, when compared with white and red light (Sæbø et al., 1995; Carvalho and Folta, 2014; Takeui et al., 2017). Blue light strongly induces flavonoid production *in vitro* in species of the genus *Alternanthera* (Reis et al., 2015), bioflavonoid production in *in vitro* cultures of *Cyclopia subternata* calli (Kokotkiewicz et al., 2014), and total flavonoid production in *in vitro* cultures of *Capsicum annuum* (Hoffmann et al., 2015). Several species of the genus *Physalis* are important sources of phenolic compounds with significant antioxidant activity (Tomassini et al., 2000; İzli et al., 2014; Medina-Medrano et al., 2015), such as rutin, quercetin, and kaempferol (Sathyadevi and Subramanian, 2015). Rutin has a wide range of therapeutic properties, including antioxidant activity and a role in improving symptoms of lymphatic insufficiency, often associated with circulation problems and bleeding. This flavonoid is found in fruits of *Physalis patula* Mill., *P. solanaceus* (Schltdl.) Axelius, *P. subulata* Rydb (Tomassini et al., 2000), and *P. peruviana* (Licodiedoff et al., 2013); in the calyx of *P. solanaceus* (Pérez-Castorena et al., 2013); in leaves of *P. angulate* (Ismail and Alam, 2001); in the shoots of *P. orizabae* (Maldonado et al., 2012); and in *in vitro* callus cultures of *P. peruviana* (Lashin and Elhaw, 2016). In the present work, the rutin content in *P. angulata* seedlings cultured *in vitro* show that the light quality directly affected the accumulation of this metabolite. The accumulation of rutin was increased in the presence of blue light and inhibited in the presence of the other light qualities, when compared to the control light. These results indicate that rutin can be used as a specific chemical marker of *P. angulata* and is therefore a relevant indicator of food traceability and drug authenticity. Light quality is an important environmental factor in plant tissue culture *in vitro*, influencing the development of the plant, in terms of both its morphology and the production of compounds of interest. This study suggests that the light intensity and quality significantly affect *in vitro* growth as well as rutin accumulation of *P. angulata* seedlings. In addition, the production of high-quality plants of this species with biotechnological potential is possible through *in vitro* culture, under an adequate mixture of light qualities.

Materials and methods

Plant material collection and in vitro culture conditions

The *in vitro* culture of *P. angulata* was carried out at the Instituto Federal de Educação, Ciência e Tecnologia Goiano (Goiano Federal Institute of Education, Science and Technology) - Rio Verde Campus (GO), and the samples of the plant material were identified by Prof. Dr. Júlio A. Lombardi, and deposited in the Herbarium of the Institute of Biosciences of Rio Claro (SP) of the Universidade Estadual Paulista (São Paulo State University, UNESP), under

accession number 65899. The plant material used was obtained by *in vitro* germination of seeds of mature fruits collected from seedlings grown in a nursery (geographical location: 17°48'15.9"S, 50°54'22"W, 752 m altitude). The fruits of *P. angulata* were washed in running water for 20 minutes, then immersed in 70% alcohol for 30 seconds and placed in a solution of sodium hypochlorite plus water (1:2) for 15 minutes. In a laminar flow cabinet, fruits were washed three to four times with distilled and autoclaved water. With the aid of tweezers and a scalpel, the fruits were cut in half to extract the seeds. The seeds were then placed in flasks with 40 mL of MS medium (Murashige and Skoog, 1962), containing half of the salt concentration (50% MS), plus 30 g L^{-1} of sucrose and 3.5 g L^{-1} of agar, adjusted to pH 5.8. The cultures were maintained in a growth room at 25 ± 2°C under daylight-white fluorescence lamps (Taschibra® 40 W, Indaial, Santa Catarina, Brazil), with an irradiance of 40 to 55 µmol m^2 s^{-1} and a photoperiod of 16 hours.

After 30 days, the seedlings were transplanted using explants of the species originated from *in vitro* multiplication. For the transplantation, nodal segments approximately 2 cm long, containing one or two axillary buds, were added to new MS culture medium with the original concentrations, supplemented with 30 g L^{-1} of sucrose, 4.0 g L^{-1} of agar, and pH adjusted to 5.8. The flasks with 40 mL of medium were sealed with PVC film. The seedlings were standardized to 2 cm in length, two expanded leaves, and five seedlings per flask.

Experimental conditions

The seedlings were subcultured and grown under identical culture conditions for seven days and then transferred to white (300-750 nm), blue (400-490 nm), green (490-560 nm), red (600-700 nm), or yellow light (560-590 nm) environments, using 40-W Taschibra® fluorescent lamps (Indaial, Santa Catarina, Brazil) with an irradiance of 50 ± 5 µmol m^2 s^{-1} under a photoperiod of 16 h. Spectral quality was determined using al USB2000 spectroradiometer (Ocean Optics, Dunedin, FL, USA) (Fig. 3), and the light intensity was adjusted using a PAR sensor (QSO-S model, Decagon Devices, Pullman, WA, USA). The seedlings were kept under these conditions for 30 days. The light chambers were sealed with a black cloth to prevent light interference.

Biometric analyses

After 30 days of growth in the light chamber, the plants were evaluated for fresh weight, dry weight, shoot length, and number of leaves per *P. angulata* seedling. After this time, the seedlings were removed from the flasks and immediately weighed to obtain the fresh weight of each one. They were then dried in a forced-air oven at 35°C until reaching a constant weight and then weighed to obtain the dry weight.

Quantitative analysis by high-performance liquid chromatography - diode array (HPLC-DAD)

Rutin accumulation was quantitatively assessed by HPLC-DAD in the methanol extracts of seedlings exposed to the different lights. After the dry material had been stored in a dry and cool environment, the chemical analyses were carried out. In this procedure, the dry samples evaluated were prepared using 200 mg of seedlings powder in 5 mL of HPLC-grade methanol, then extracted for 20 min in an ultrasonic bath. The samples were analyzed in triplicate.

To obtain the calibration curve, 2 mg of the flavonoid rutin was used as an external standard, and 2000 µL of HPLC-grade methanol was added to obtain a stock solution of 1.0 mg mL^{-1}. Successive dilutions of the stock solution were then performed to obtain the following concentrations: 0.5, 0.25, 0.125, 0.0625, and 0.00625 mg mL^{-1}. Each solution was injected in triplicate into a SHIMADZU Prominence-LC-20AD high-efficiency liquid chromatograph, equipped with an automatic injector (SIL-20A HT), coupled to a UV-VIS model SPD-M20A detector with a diode array. The analytical column used was Phenomenex Phase GEMINI, (250 x 4.6 mm, 5 µm), C18, equipped with a pre-column of the same material. The oven was model CTO-20[a] which maintained at 40°C. The volume injected was 20 µL, and the flow rate was 1 mL min^{-1}. The analysis was performed over 60 min using as eluents in a linear gradient $CH_3OH/H_2O/CH_3COOH$ (5:94, 9:0, 1 v/v/v), 100% CH_3OH for 30 min, 100% MeOH for 10 min, and finally 20 min to return to the initial condition.

Rutin was quantified based on the peak area at wavelength of 254 nm, using the calibration curve generated, as well as its inherent parameters, namely, the equation of the line y = 42,888,414.5140x – 41,424.6938 and the linear, angular, and correlation coefficients (R^2), which were obtained using Excel® 2016 software. The linearity of the curves obtained between the concentration and the peak area of rutin presented R^2 = 0.9996. The flavonoid rutin, used as an external standard, was acquired from the standards bank of the Natural Products Group of the University of Franca (SP).

Experimental design and statistical analysis

For the growth variables (shoot length and number of leaves) and for the calculation of fresh and dry weight, a completely randomized experimental design was used, which consisted of five treatments, each composed of one type of light and five replicates. Each replicate consisted of one flask with five explants. The numerical data were statistically evaluated using ANOVA followed by Tukey's test (5%) for comparison of means in SISVAR® software (Ferreira, 2011).

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