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Full Length Research Paper

Light spectra affect the morphoanatomical and chemical features of clonal *Phyllanthus tenellus* Roxb. grown *in vitro*

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***Phyllanthus tenellus* Roxb. is a widely distributed tropical medicinal plant and highly valued for its therapeutic properties. Since variable light conditions can significantly alter phenolic compounds that are the main therapeutic constituents of *P. tenellus,* including tannins and flavonoids, the development of this plant and its chemical metabolism in response to different light spectra were investigated. To accomplish this, *P. tenellus* was cultured in modified Murashige and Skoog (MS) medium for 60 days, and its development, leaf anatomy and phytochemistry were analyzed after exposure to white, red, green, yellow and blue light, as well as darkness. Compared to white light, the best *in vitro* morphogenic responses, including rooting percentage, shoot height, number of leaves, and number and length of branches, occurred under exposure to blue and yellow light. Plantlets developed under white and blue lights presented the greatest thickness of palisade and spongy parenchymas. Under dark condition, plantlets showed fragile aspect and the lowest thickness of leaf tissues. In contrast to other light treatments, chlorophyll and carotenoid contents were significantly lower in plants maintained under green light, whereas yellow light improved the production of phenolic compounds. These results highlight the influence of different light spectra on morphoanatomical features and suggest how different light spectra affect secondary metabolite production in the context of preserving this plant's therapeutic integrity.**

Key words: *In vitro* culture, light spectra, phenolic compounds, photomorphogenesis, *Phyllanthus* sp., plant development.

INTRODUCTION

Phyllanthus tenellus Roxb. (Phyllanthaceae), a tropical medicinal plant, is widely dispersed in Brazil. In particular,

this species, also known as quebra-pedra or erva-pombinha, is used in folk medicine to treat kidney disease, urinary bladder disturbances and hepatitis (Lorenzi and Matos, 2008). The therapeutic value of *P. tenellus* arises from its phenolic compounds, including tannins and flavonoids (Huang et al., 2003). Environmental, physical and biotic factors all interact to affect plant development, resulting in morpho-anatomical and physiological phenotypic plasticity (Catoni et al., 2015).

Of all these factors, light quality is preeminent by its ability to influence all phases of plant development, from germination to reproduction, as well as primary and secondary metabolism (Franklin, 2009). Different light qualities may also represent an abiotic stress for the plant, affecting its level of plastidial proteins by the degradation of rubisco enzyme, finally changing its metabolism (Feller et al., 2008). The light-dependent development of plants is a complex process that involves photoreceptor families of red-absorbing phytochromes and UV-A/blue light-sensing cryptochromes (Chen et al., 2004; Franklin, 2009). Some red light responses include adventitious shoot formation, primary leaf development, stimulation of seed germination, inhibition of internode elongation, induction of flowering by action on photoperiod, changes in leaf anatomy layers and synthesis of anthocyanin and phenolic compounds (Chen et al., 2004; Macedo et al., 2011; Victório et al., 2011).

Cryptochromes are flavoproteins involved in photorepair of UV-damaged DNA, and they regulate a wide range of responses in plants, such as inhibition of hypocotyl elongation and leaf expansion, pigment biosynthesis, growth of stems and internode elongation, stomatal opening, chloroplast migration, control of flowering time and phototropism (Franklin, 2009). Additionally, different light spectra interfere with secondary plant metabolism in the acetate-malonate and shikimate pathways to either promote or inhibit the production of phenolic compounds. However, by using tissue culture techniques, it is possible to evaluate how some previously selected conditions may influence plant development responses. Based on this hypothesis, the present study aimed to evaluate the development, leaf anatomy and production of phenolic compounds of *P. tenellus* under specific light qualities and darkness.

MATERIALS AND METHODS

Plant

Samples of the mother plant of *P. tenellus* Roxb. were obtained from the medicinal plant garden at the Biophysics Institute Carlos Chagas Filho, Universidade Federal do Rio de Janeiro (Rio de Janeiro State, Brazil). The voucher specimen is deposited at the Herbarium of the National Museum of Rio de Janeiro under number R 200872.

Tissue cultures

Cultures were established by Victório et al. (2010). Nodal segments (0.8-1.0 cm) were excised from *in vitro* culture seedlings and transferred to flasks (141 × 72 mm) containing 60 ml of basic MS (Murashige and Skoog, 1962) medium reduced to half of NH_4NO_3 and KNO_3 concentrations (MS½N). Plantlets were subcultured every 60 days and maintained at 25 ± 2°C with a photoperiod of 16 h. Light-quality experiments were performed in growth chambers (controlled environments) equipped with Sylvania® fluorescent tubes (F20 W T-12) [approximately 20 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR)] to provide different light spectra: white, blue, green, red, and yellow (Figure 1). White light and darkness conditions were used as control treatments to assess the effect of light on plantlets in the same medium formulation. Light intensities were measured by a quantameter (Biospherical Instruments Inc., QSL-100). Each treatment was completely randomized and consisted of four sets with at least 10 plantlets. Plantlet development was evaluated for 60 days according to the following parameters: number of shoots, number of branches; shoot height (main vegetative axis/main stalk); length of branches; rooting percentage; fresh and dry weights; and senescence percentage. For dry weight determination, groups of 10 plantlets from each treatment were dehydrated at 60°C for 24 h.

Anatomical analysis

Sixty-day-old clonal plantlets of *P. tenellus* exposed to different light qualities were used in histological studies. Branches were fixed in ethanol (70%) during 48 h. Then, leaf samples from the third node of branches of each group of three plantlets were dehydrated in an ethanol series (80, 90 and 95% each hour), infiltrated and embedded in basic Historesin (Leica Microsystems, Germany), then sectioned with a rotary microtome (820 Spencer Microtome, American Optical Corporation, USA). Sample sections 8 µm in thickness were stained in toluidine blue (0.05%) and prepared on permanent slides. The stained sections were examined, and drawings were performed using a Carl Zeiss optical microscope (model 4746.20-990) with a camera lucida attachment. Anatomical measurements were made using an optical Zeiss Axioskop 2 microscope (model DEI-750D, CE) equipped with computer and video and digital cameras (Optronics). About 30 measurements per treatment were carried out for each anatomical parameter. The thickness of adaxial and abaxial epidermis, including papillae and palisade and spongy parenchymas, was evaluated.

Photosynthetic pigments content

To determine total chlorophylls (a and b) and carotenoids, fresh leaves (50 mg) were macerated in dimethylsulfoxide (DMSO) and incubated in hot water at 60°C for 16 h, in semi-darkness. Determination of chlorophyll and carotenoid concentrations was obtained using a spectrophotometer (Spectronic Genesys 2 SERL 3N270093004). After filtering, absorbance of the extracted solution was measured at 649 nm (a chlorophyll), 665 nm (b chlorophyll) and 480 nm (carotenoids). Total concentrations of chlorophyll (chlorophyll a and b) and carotenoids on a fresh weight basis (µg mg^{-1}) were calculated according to the following equations

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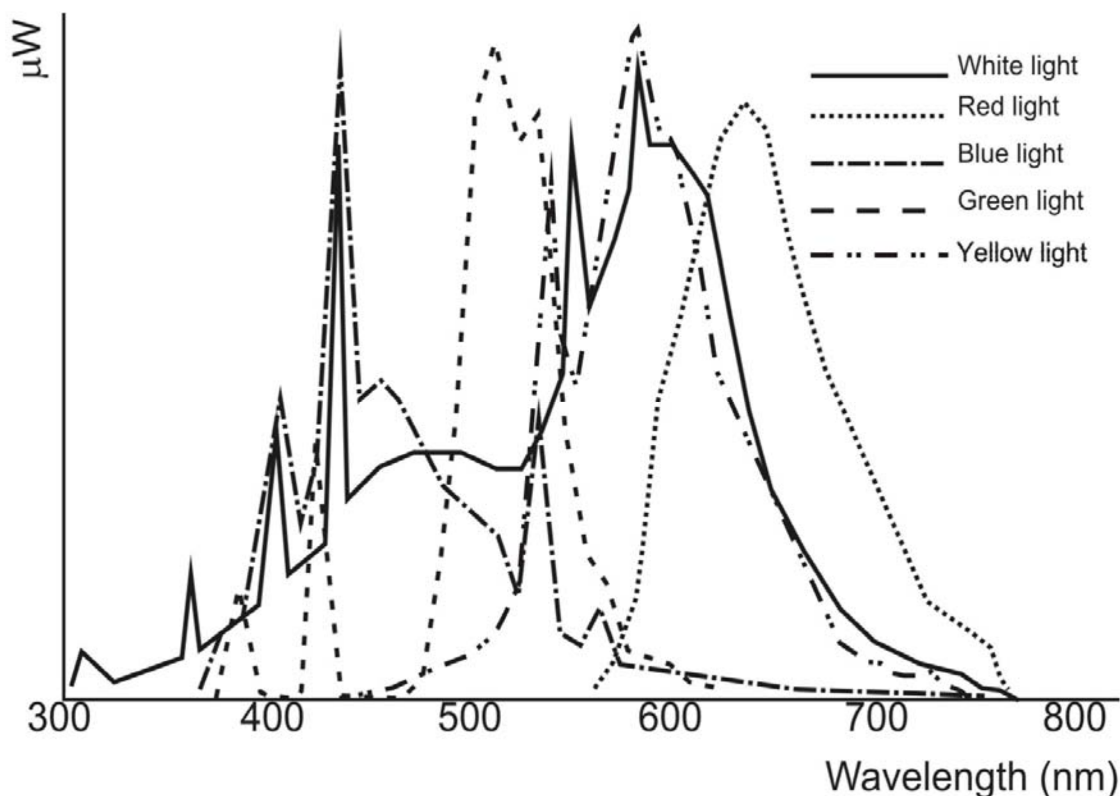


Figure 1. Emission spectra of the light sources used in the different treatments.

(Wellburn, 1994):

Ca = 12.19 A665 – 3.45 A649; Cb = 21.99 A649 – 5.32 A665; Cx+c = (1000 A480 – 2.14 Ca – 70.16 Cb) / 220

Where Ca and Cb are chlorophyll a and b concentration, respectively, and Cx+c is the total carotenoid content.

Chemical analysis

P. tenellus aqueous extract was obtained from dried leaves from about ten plants. Tubes containing a distilled water mixture with dried leaves at 1 g/20 ml were immersed in boiling water for 30 min. Crude extracts were filtered, dried using a lyophilizator, and then dissolved in MilliQ water at 10 mg/ml. High performance liquid chromatography-ultraviolet (HPLC-UV) analyses were performed on a Shimadzu setup equipped with a SPD-M10A diode array detector, LC-10AD pump and CBM-10 interface. Data were acquired and processed by a reversed phase column (Rexchrom®, 25 cm × 5 mm, 5 µm). Analytical separation was done in the following mobile phase: A – H_2O (MilliQ); B - KH_2PO_4 0.1 M plus H_3PO_4 0.1 M plus CH_3CN and C – MeOH with a gradient of solvent B where B = 50% (10 min), B = 50% - 100% (30 min) and B = 50% plus C = 50% (40 min) at a flow rate of 1 ml/min under ambient temperature. The injection volume was set at 20 µl. Detection was accomplished with a diode array detector, and chromatograms were recorded at 280 nm. Qualitative determinations were obtained after two separated extractions from plantlets of each light treatment, and samples were injected in duplicate. Methanol, phosphoric acid, acetonitrile, and potassium dihydrogen phosphate were analytical grade. Geraniin was used as a standard of tannin.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and statistical average comparisons were made through Tukey`s test at 5% significance level. A test of difference between two percentages was conducted, considering $P \leq 0.05$ by t-test.

RESULTS

Figures 2 and 3 show the morphogenic changes of 60-day-old *P. tenellus* plantlets under different light spectra and darkness. Under white light, plantlets presented a healthy aspect, showing a continuous growth that reached 2.6 cm and a rooting percentage of 73.2% within 60 days. Although no other light spectrum improved the number of shoots within 60 days, a greater number of nodes per shoot were obtained under blue light (4.9). The number of nodes may be used as an indicator of proliferation rate. In contrast to white light, shoots elongated more under yellow and blue light, but no change in inter-node elongation was observed among the different light spectra. The highest number of leaves was recorded under yellow light treatment (Figure 3E),

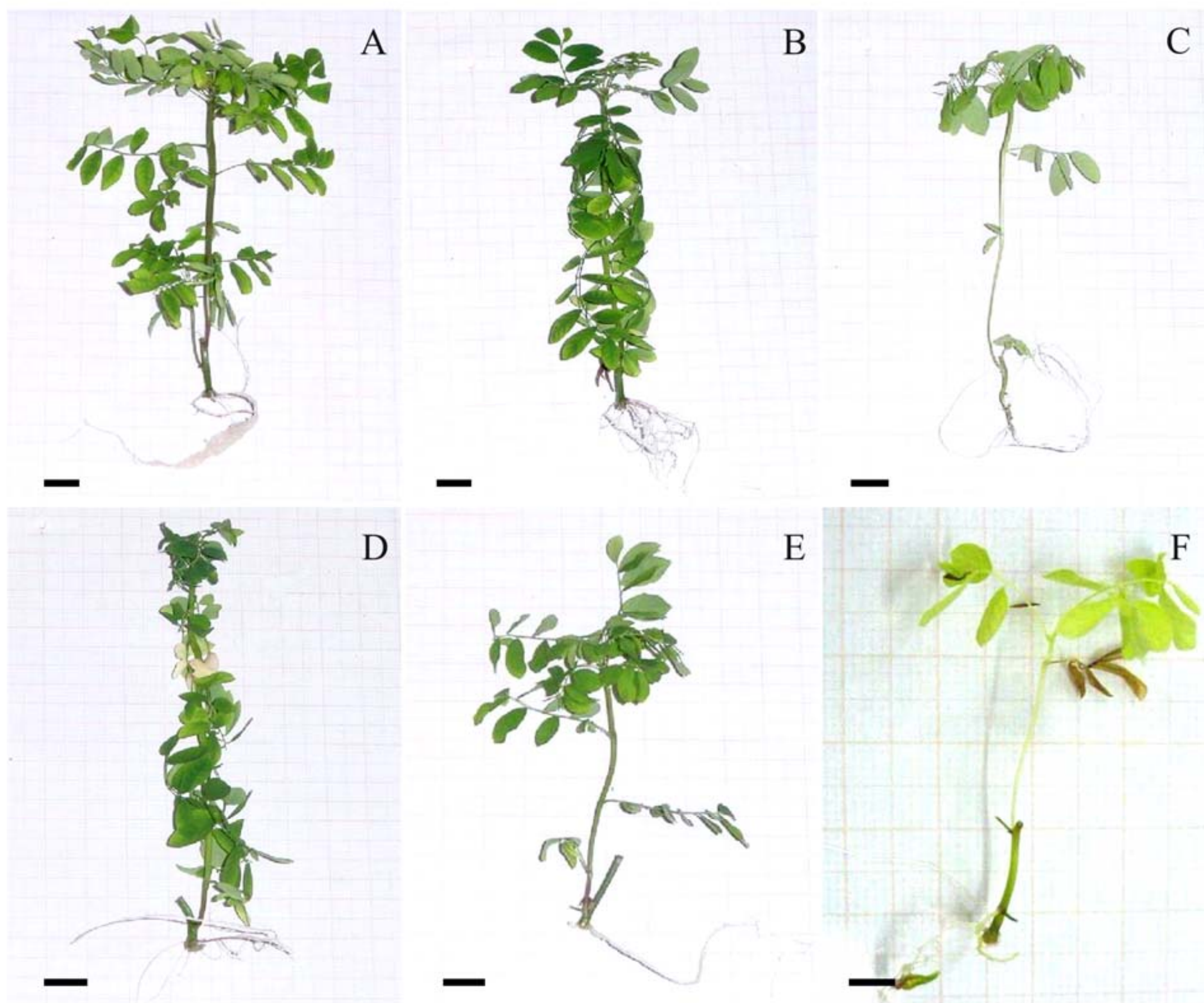


Figure 2. Sixty-day-old plantlets of *P. tenellus* cultured under different light spectra: (A) white, (B) blue, (C) red, (D) green, (E) yellow and (F) darkness. Scale bar=1 cm (A - E) and 20 cm (F).

showing significant differences compared with white light and darkness. Plantlets maintained under red light presented a green homogeneous color for the full width of their leaves, and the length of their branches was higher than that of plantlets under white light treatment. Cultures maintained under green light showed this spectral range as active in the morphogenesis process, resulting in an increased number of shoots and roots. Blue and green lights induced the greatest rooting (Figure 3F). Roots grew thicker under red light when compared with other light treatments. Rooting of plantlets cultured in darkness was 88.2% greater than the percentage of rooting cultured under white light (Figure 3F), thus confirming the ability of *P. tenellus* to establish rooting in darkness, as well as light.

Over the course of 60 days, it was verified that red light delayed leaf senescence of *P. tenellus* plantlets. Plantlets maintained under yellow light and in darkness presented senescence rates greater than 50%, whereas the rates under green light were lower (15.4%) (Figure 3H). No correlation was confirmed between senescence and the decrease of chlorophyll and carotenoid contents (Figure 4). The statistical difference between chlorophyll and carotenoid contents was achieved by the effects of green light, which showed a reduction in carotenoid contents, as well as a and b chlorophylls (Figure 4). Fresh and dry weights of *P. tenellus* plantlets increased by the effects of blue light; these data are in agreement with the highest number of branches (Figure 3G). Transverse sections showed that leaves are amphistomatic with unistratified epidermis. Epidermal cells have an irregular shape and sinuous anticlinal walls. Papillae can be observed on both sides of the leaf blades. Under white, red and green light, the greatest variations in papillae were found on the abaxial side. In transverse sections of leaves of plantlets maintained under darkness, papillae were not observed

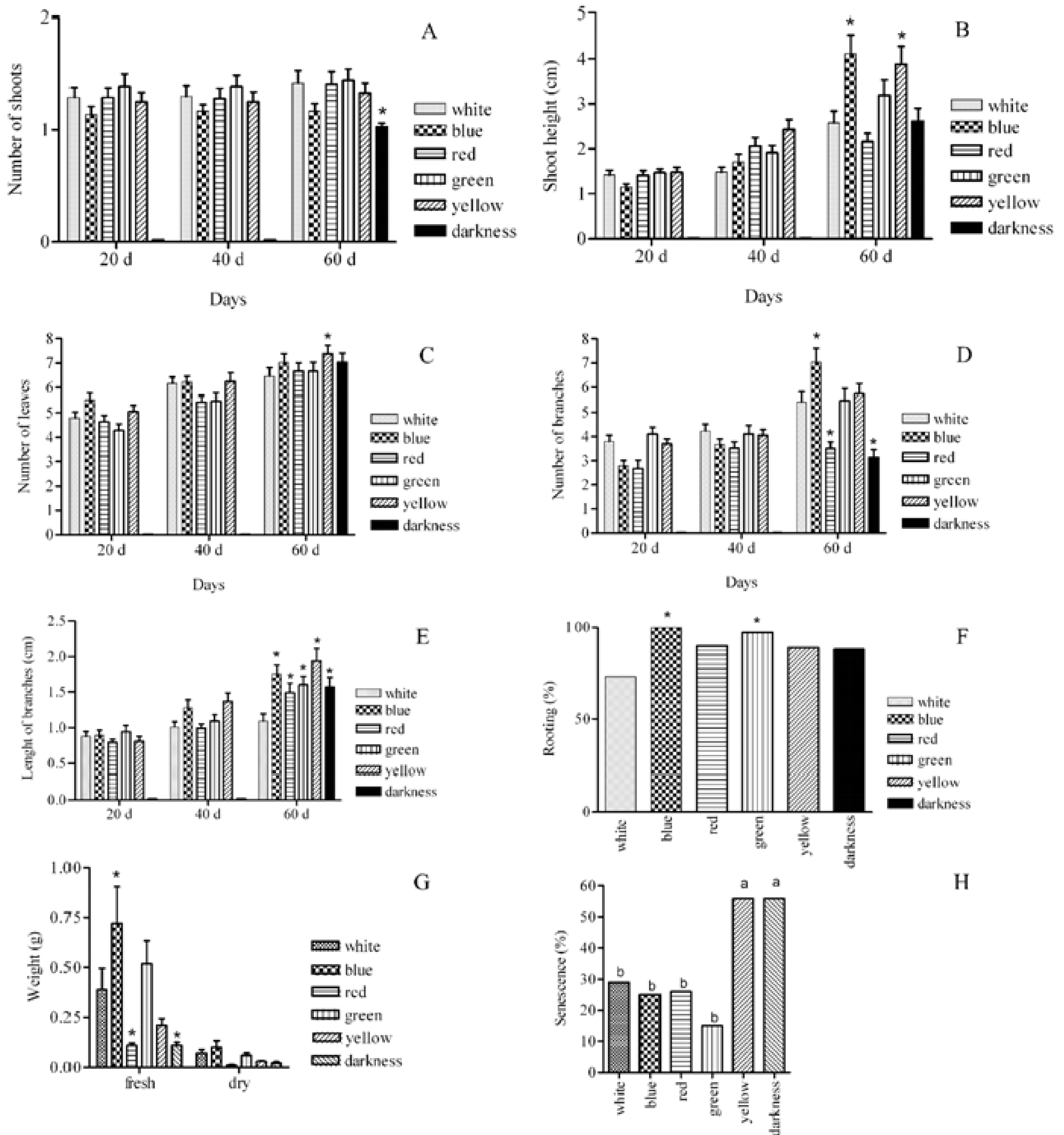


Figure 3. In vitro development of *P. tenellus* under different light spectra. Parameters were evaluated at 20, 40 and 60 days (n≥30). Rooting and senescence (F and H, n≥30); fresh and dry weight (G, n=10). *P<0.05: statistical differences in comparison with white light. Different letters denote statistical differences among treatments. Average ± SD.

on either side (Figure 5). The mesophyll is dorsiventral. The palisade parenchyma is unistratified, and the spongy parenchyma consists of two layers of cells (Figure 5). Thickness measured in the leaves of plantlets cultured under white light resulted in 8.8 µm (adaxial epidermis), 5.0 µm (abaxial epidermis), 19 µm (palisade parenchyma)

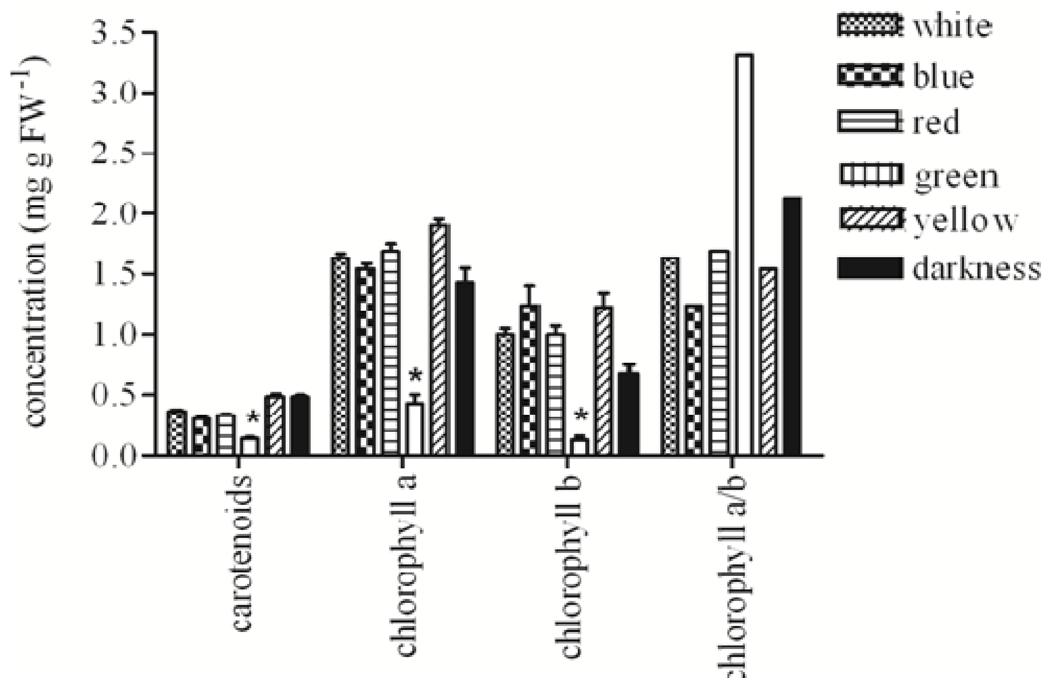


Figure 4. Photosynthetic pigment contents of *P. tenellus* cultured under different light spectra. *P<0.05: statistical differences in comparison with white light (Average ± SD, n=10).

and 29 µm (spongy parenchyma) (Figure 6). By comparing anatomical features of plantlets cultured under different light qualities, it was found that the greatest thickness of adaxial epidermis was verified under red light, showing statistical differences compared to green light (Figure 6). Plantlets maintained under darkness presented lower measurements for all evaluated anatomical parameters compared with light treatments (Figure 6). Palisade parenchyma showed statistical difference when comparing white and other light qualities. The greatest palisade parenchyma thickness was verified under blue light (Figure 6).

In comparison with control treatment (white light), our analysis showed an improvement in the relative amount of compounds with phenolic features in aqueous extracts obtained from plantlets cultured under yellow light (Figure 7A). Geraniin, which is a hydrolysable tannin, was detected in *P. tenellus* extracts at 26 min (RT), a result obtained by comparison with the absorbance maximum of the geraniin spectrum (221 and 277 nm) (Figure 7B). Geraniin was found in plantlets cultured in all light treatments, but not in darkness. Also, under UV spectra flavonoid was found in abundance (Figure 7C).

DISCUSSION

Light spectra have been shown to be an important environmental factor influencing morpho-anatomical and phytochemical features of *P. tenellus* plantlets. With respect to plant development, the number of nodes may be used as an indicator of proliferation rate such that each node marks the origin of a new plant. Accordingly, exposure to blue light, in contrast to control white light, resulted in plantlets with a greater number of nodes and induced the greatest elongation of *P. tenellus* shoots. However, this response is not in agreement with the results of previous researchers who have verified that blue light may either inhibit or increase stem elongation (Islam et al., 1999; Shimizu et al., 2005). We found that exposure to yellow light increased stem elongation, a finding which agrees with studies using *Cattleya walkeriana* (Islam et al., 1999). Also, according to Maas et al. (1995), changes in shoot elongation in response to blue and red light result primarily from cell wall extension, confirming the effects of yellow light, as shown in our study. The effects of yellow light were also shown to improve different morphological features in plantlets of *P. tenellus*, such as high production in the number of leaves together with high production of phenolic compounds, suggesting that phenolic content may improve morphological features. The effects of phenolic com-pound concentrations in either inhibiting or stimulating plant development have been reported by Ozyigit (2008). Plant phenolics are modulators of indole acetic acid (IAA) catabolism, and its concentration may either inhibit or stimulate enzymatic oxidation of auxin hormone, in turn affecting cell elongation and cell division, as well as subsequent plant growth and development. Moreover, plant phenolics increase the rigidity of plant cell walls, since

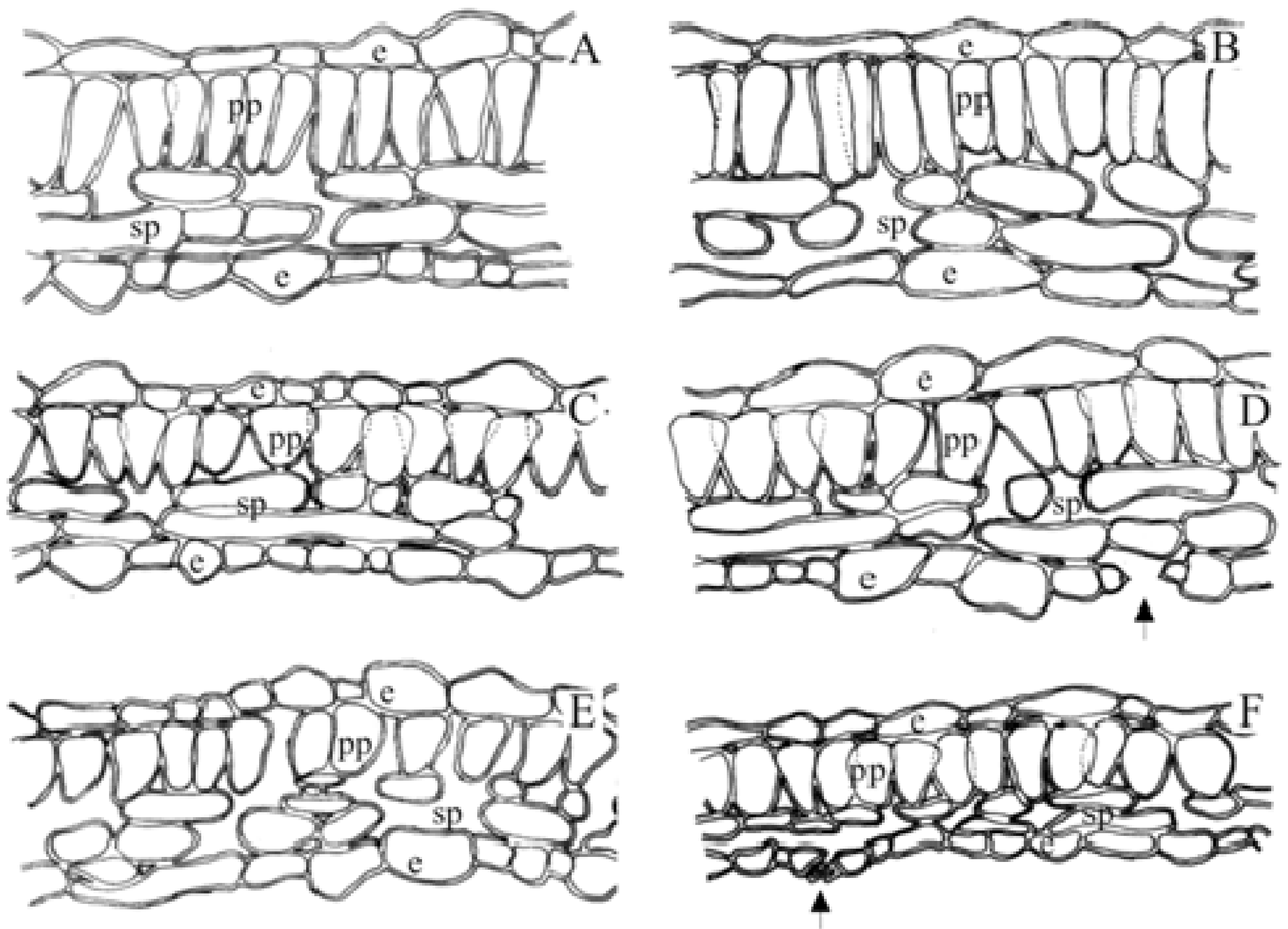


Figure 5. Leaf transverse sections of 60-day-old plantlets of *P. tenellus*. (A) white, (B) blue, (C) red, (D) green, (D) yellow, (F) darkness. e – epidermis, pp- palisade parenchyma, sp- spongy parenchyma. Arrows indicate stomata. Scale bar=50 μm.

since they are precursors of lignin (Arnaldos et al., 2001; Ozyigit, 2008).

A higher number of leaves may result in increasing photosynthetic area and dry weight as a consequence of accumulating sucrose, which is an important energy source, but also a structural component of plant physiology and metabolite production. However, increase in the number of leaves under yellow light did not result in any statistical difference in either dry weight or chlorophyll content among the light treatments, except in relation to the green spectrum, as noted above.

The low production of carotenoid content and chlorophylls a and b under green light indicates that this light band is less efficient in photosynthesis. In support of this conclusion, Klein (1992) suggested that a monochromatic green light was not significantly absorbed by chlorophyll a. However, this author also states that this light band continuously reflects a light wave from chloroplast to chloroplast, acting as an electron carrier.

Thus, after several cycles of reflection, the green light spectrum is still minimally effective in photosynthesis physiology.

Changes in morphogenesis by the effects of spectral green light showed that it is an important factor affecting plant development, contrary to some studies that reported green light to be innocuous to growth (Reichler et al., 2001). According to Folta and Maruhnich (2007), phytochromes and cryptochromes are responsible for the absorption of green light. Therefore, it is clear that studies reporting the effects of green light have drawn

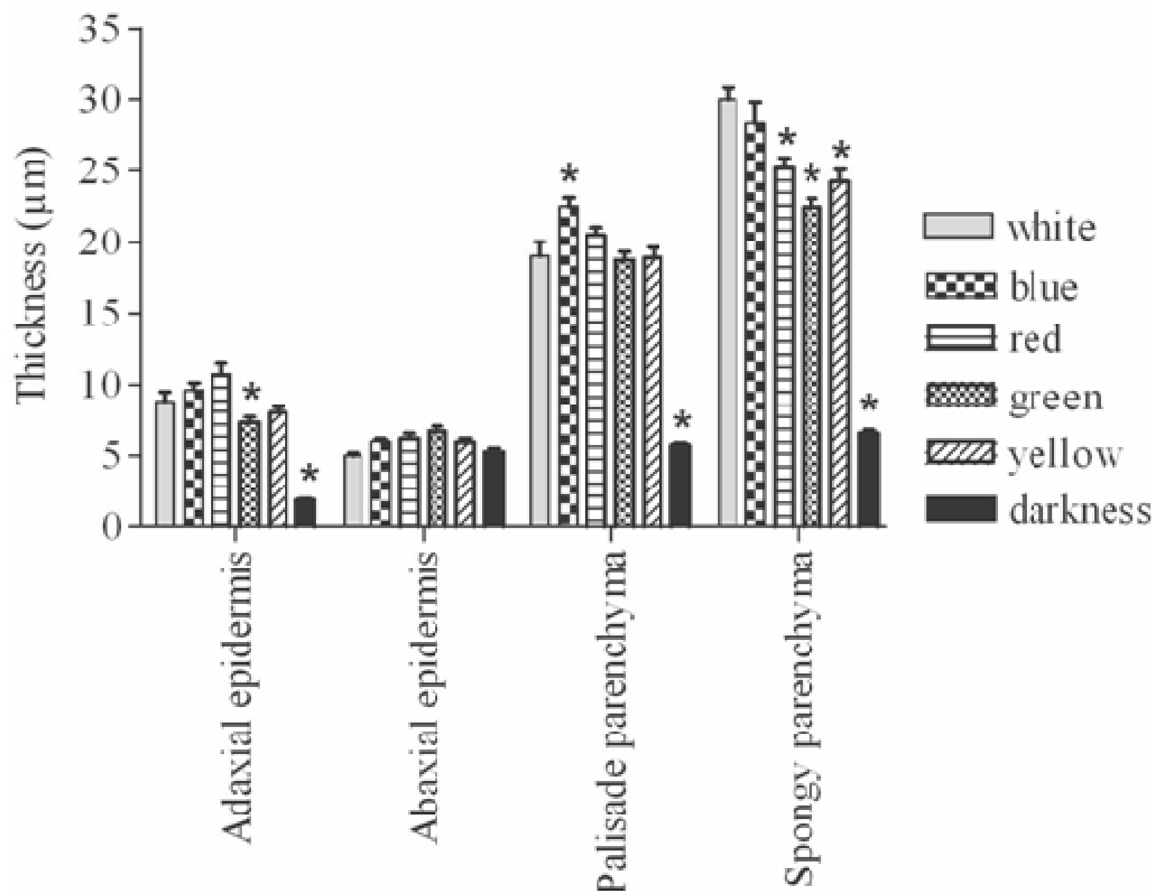


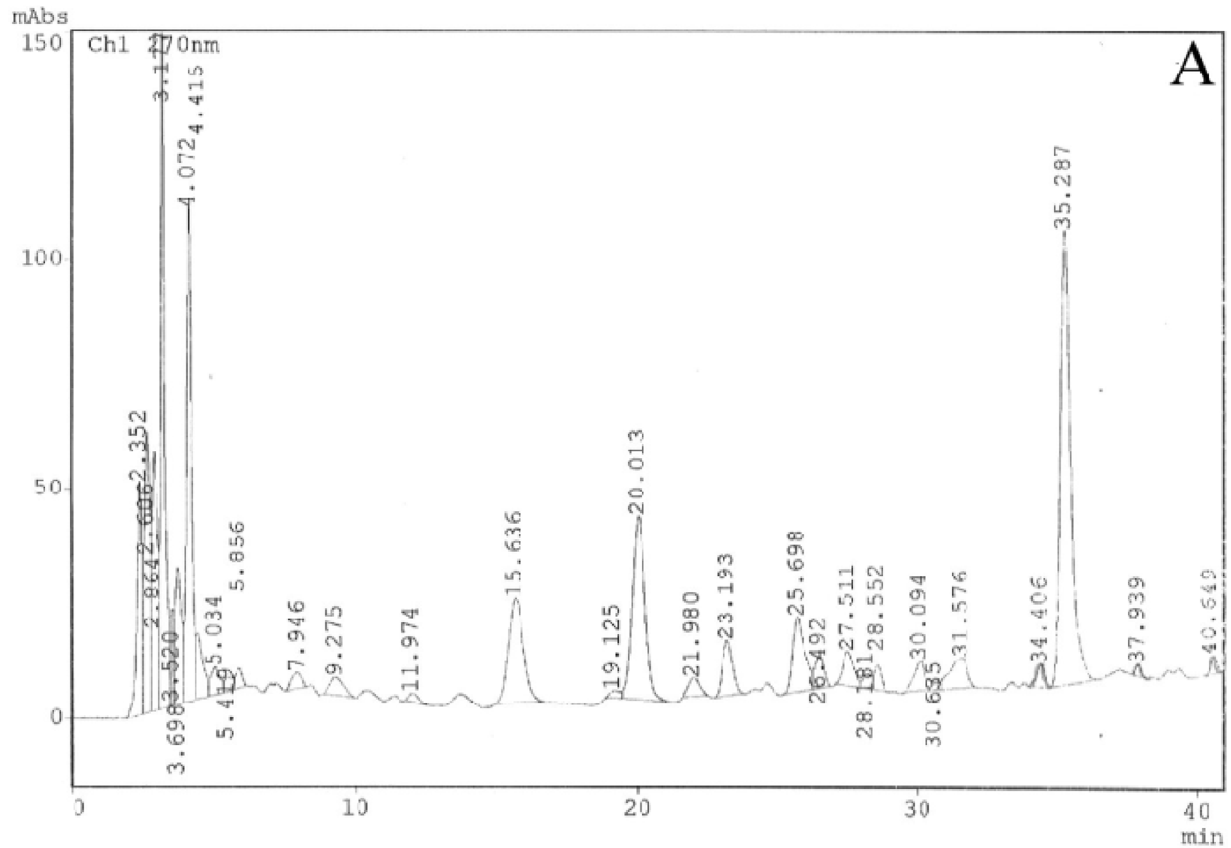
Figure 6. Leaf tissue thickness (μm) of *P. tenellus* cultured under different light spectra for 60 days. *P<0.05: statistical differences in comparison with white light (Average ± SD, n=30)

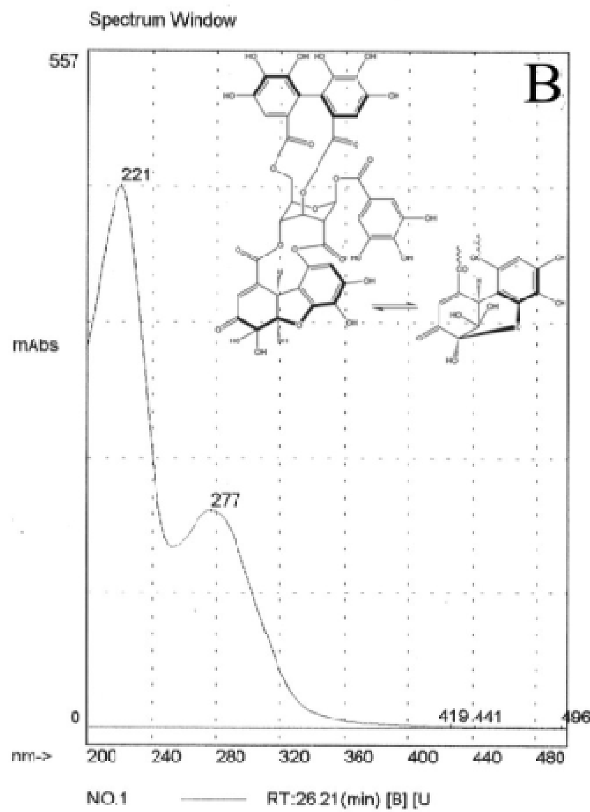
controversial conclusions. That is, this spectrum is sometimes found to be the least effective in promoting plant development, while, at other times, the opposite effect has been found, improving, for example, plant height (Dougher and Bugbee, 2001).

According to Economou and Read (1987), light may influence the rooting process, including the inhibition of root development. Our data showed the positive effects of blue light in improving rooting, while under red light, the roots presented a fragile aspect, results strongly suggesting the importance of light in the formation of plant structures. In contrast to our data, Hunter and Burritt (2004) verified that blue light reduced explant competence for organogenesis. It is important to underscore the fact that plant genotype is essential to fully evaluate the different responses obtained from the different effects of light spectra.

Senescence is controlled by internal signals, such as gene expression and plant hormones, and external factors, including light, that can delay or accelerate the death of cells, specific organs and whole plant (Lim et al., 2007). Initial stages of senescence involve the chloroplast, responsible for photosynthesis, with gradual loss of chlorophyll, proteins and lipids associated with chloroplast (Hodges and Forney, 2000). Many different natural phenolic compounds in plants appear to function primarily in plant defense or as attractants of pollinators. They may also be involved in stages of plant development, and they are widely applied in therapeutic preparations.

In the current study, the correlation between senescence and decreasing chlorophyll and carotenoid contents was not verified, although decline in chlorophyll and progressive yellowing of the leaves from carotenoids became visible during senescence. Leaf senescence of the *P. tenellus* plantlets did not impair acclimatization. Several anatomical parameters were investigated in order to evaluate changes that might ultimately establish a developmental pattern in leaf tissues of *P. tenellus* exposed to different light spectra. For example, we found papillae in *P. tenellus*, as well as *P. urinaria* and *P. amarus*. The presence of epidermal papillae may, in fact, intensify light uptake reaching leaf tissues, thus improving photosynthesis (Vogelmann et al., 1996). When exposed





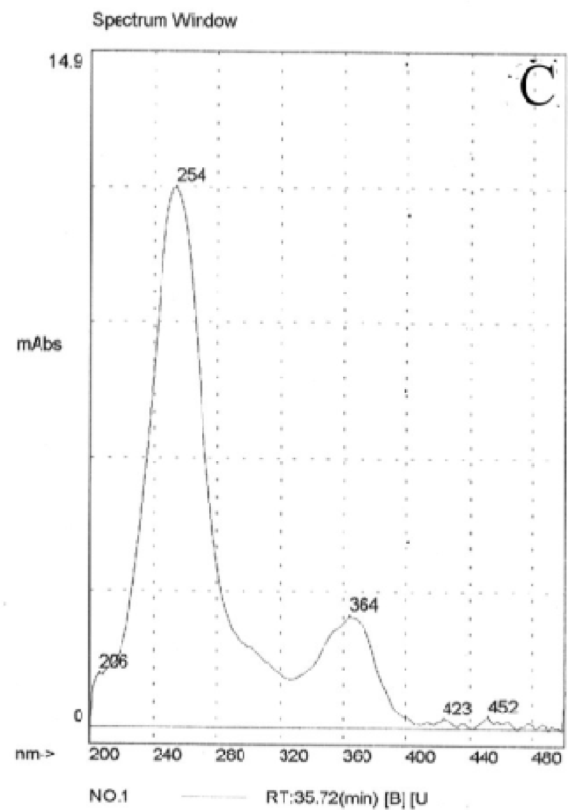


Figure 7. (A) Chromatogram profile of *Phyllanthus tenellus* cultured under yellow spectrum, (B) Geraniin detected in *P. tenellus* extracts at 26 min (RT) (221 and 277 nm), (C) Flavonoid detected at 35 min (254 and 354 nm).

to radiation, leaf tissues may alter structures involved in light absorption. For example, changes in leaf thickness are commonly associated with the number of epidermal, hypodermal and parenchymal layers, particularly palisade parenchyma thickness. Red light increases the thickness of the adaxial epidermis, which, in turn, acts as a defense mechanism that reduces the penetration of radiation in the leaf mesophyll tissues, as observed by Yang et al. (2008). The effects of light qualities on epidermal thickness have also been described by Saebo et al. (1995) who found that blue light increased the area of epidermal cells and palisade parenchyma of *Betula pendula* cultured *in vitro*, resulting in greater leaf area. This finding confirms that light quality is an important factor in cell expansion. Plantlets maintained under darkness presented fragility also visualized by microscopic analysis showing reduction in thickness of leaf tissues and absence of papillae.

Environmental factors are decisive in controlling genetic regulation of plant development and production of secondary metabolites. Thus, the application of light qualities in tissue cultures could be a useful strategy for improving therapeutic metabolites under standardized conditions, and it is certainly useful in plantlet production. In our study, we showed that yellow light stimulated the production of phenolic compounds that are the main therapeutic constituents of *P. tenellus*, including tannins and flavonoids (Huang et al., 2003), suggesting that yellow light plays an important role in the biosynthesis of phenolic compounds in *P. tenellus*. The use of HPLC coupled to a diode array detector revealed a higher amount of phenolic compounds in crude aqueous extracts. Although yellow light has not been commonly used, this study revealed the unequivocal influence of this spectrum in plant development and the production of phenolic compounds.

Conclusion

This study adds important findings related to plant development of *P. tenellus* in response to different light spectra. The best *in vitro* development of *P. tenellus* was found under blue and yellow light. However, in the context of anatomical features, both white and blue light induced the greatest thickness of palisade and spongy parenchymas. Sixty-day-old plantlets of *P. tenellus* revealed a high production of phenolic compounds after yellow light exposure, which seems to be consistent with the better plantlet development observed under this light. This culture system study indicated the importance of light spectra as a simple means of inducing the production of phenolic compounds so important to the therapeutic properties of this medicinal plant.

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Conflict of interest

All authors declare that they have not conflict of interest.

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