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Light and culture medium formulations for in vitro germination and development of Brassavola perrinii[1]

Luz e formulações de meio de cultivo na germinação e desenvolvimento in vitro de Brassavola perrinii

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ABSTRACT - The improvement of *in vitro* propagation techniques using appropriate artificial light sources and culture medium formulations is important to allow the mass production of individuals with high genetic variability. Thus, this research aimed to study the effect of light sources from fluorescent lamps and light-emitting diodes (LEDs) and culture medium formulations on the germination and initial development of the orchid species *Brassavola perrinii*. The experiment was set up in a completely randomized design in a 5 × 4 factorial scheme, consisting of five light sources – fluorescent lamp (100%), white LED (100%), blue LED (100%), red LED (100%), and blue LED (50%) + red LED (50%) – and four culture medium formulations – MS, ½ MS, VW, and K –, with four replications and a mean of 125 seeds per plot. The percentage of germination, chlorophyll protocorms, and protocorm development through the classes P1, P2, P3, and P4 to calculate the protocorm development index were evaluated at 90 days after sowing. The data were subjected to analysis of variance and the means were compared by the Tukey test ($p < 0.05$). The percentage of germination for *B. perrinii* was not influenced by light spectra but was higher in the ½ MS and VW formulations. The highest protocorm development index of *B. perrinii* was observed when the species was submitted to the light spectrum of the lighting source with fluorescent lamps in the VW culture medium.

Key words: Light sources. Protocorm. Asymbiotic sowing. *In vitro* sowing.

RESUMO - O aperfeiçoamento de técnicas de propagação *in vitro*, com auxílio da escolha adequada da fonte de luz artificial e das formulações do meio de cultivo, são importantes, pois viabilizam a produção em massa de indivíduos com grande variabilidade genética. Assim, a presente pesquisa teve por objetivos estudar fontes de luz a partir de lâmpadas fluorescentes e diodos emissores de luz (LEDs) e formulações de meio de cultivo na germinação e no desenvolvimento inicial da espécie de orquídea *Brassavola perrinii*. O experimento foi instalado em delineamento inteiramente casualizado em esquema fatorial 5 × 4, sendo cinco fontes de luz: lâmpada fluorescente (100%); LED branco (100%); LED azul (100%); LED vermelho (100%) e LED azul (50%) + vermelho (50%), e quatro formulações de meio de cultivo, sendo MS, ½ MS, VW e K, com quatro repetições e média de 125 sementes por parcela. Aos 90 dias após a semeadura foram avaliadas a porcentagem de germinação, protocormos clorofilados e o desenvolvimento dos protocormos através das classes: P1, P2, P3 e P4 para cálculo do índice de desenvolvimento protocormos. Os dados foram submetidos à análise de variância e as médias foram comparadas pelo teste de Tukey $p < 0,05$. Para *Brassavola perrinii*, concluiu-se que o percentual de germinação não foi influenciado pelos espectros de luz, mas foi maior nas formulações ½ MS e VW. O maior índice de desenvolvimento dos protocormos de *B. perrinii* foi observado quando submetidos ao espectro de luz da fonte de iluminação com lâmpadas fluorescentes em meio de cultivo VW.

Palavras-chave: Fontes de luz. Protocormo. Semeadura assimbiótica. Semeio *in vitro*.

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INTRODUCTION

Orchidaceae is the second largest family of flowering plants (CHASE *et al*., 2015). The number of genera is estimated between 559 and 880 and the number of species between 18,814 and 30,000 (GIVNISH *et al*., 2015; HSIAO *et al.*, 2011; ZOTZ, 2013). Orchids are plants with ornamental characteristics and relevant economic importance, as they are used and commercialized worldwide (POPOVA *et al*., 2016).

Brassavola stands out among the main genera found in Brazil (FARIA *et al.*, 2012) due to its ability to intercross with different genera, such as *Cattleya*, *Laelia*, *Sophronitis*, and *Epidendrum*, enabling the production of hybrids with morphological characteristics of interest (SOARES *et al*., 2012).

The asymbiotic sowing of orchids is a very relevant technique from a commercial and ecological point of view, as the seeds of these species have a rudimentary embryo with little nutritional reserve (ARDITTI, 1992). For this reason, the seed depends exclusively on association with specific mycorrhizal fungi to germinate and complete the protocorm development phase in a natural environment (AGUSTINI; SUFAATI; SUHARNO, 2009).

The choice of the culture medium formulation is one of the most important factors in the process for the success of asymbiotic *in vitro* cultivation, as it must provide all the necessary characteristics for seedling germination and growth (SOARES *et al*., 2012). The nutritional requirement varies not only for each orchid species but also for each stage of development (JORGE; ABRÃO; SUZUKI, 2015).

Culture medium formulations used in the germination and development of orchids are composed of macro-and micronutrients, vitamins, and mineral salts (MURASHIGE; SKOOG, 1962). MS (MURASHIGE; SKOOG, 1962), VW (VACIN; WENT, 1949), and K (KNUDSON, 1946) are the most used media for sowing and *in vitro* cultivation of orchids, but there are no reports in the literature about which would be the best medium for *B. perrinii*.

In addition to the cultivation medium, light is an important factor in the regulation of plant growth and development, especially within a production process. The light source spectrum must meet the plant requirements for photosynthesis and photomorphogenic development, varying according to each species (SILVA, 2014).

Although the fluorescent lamp is commonly used in controlled environments for plant growth (BULA *et al*., 1991), an alternative light source has been used in *in vitro* cultivation, the so-called light-emitting diodes (LEDs) (YEH; CHUNG, 2009). LEDs have several advantages over traditional lighting systems, such as durability, lower power consumption, simpler and more reliable electronic circuits, lower heat emission, reduced size, specific wavelength, and adjustable light intensity and quality (YEH; CHUNG, 2009).

The use of LED lighting systems has already been studied and has shown positive results for some orchid species such as *Bletilla ochracea* (GODO *et al*., 2011), *Oreorchis patens* (BAE; OH; KIM, 2014), *Cymbidium* (NHUT; TUNG; TANAKA, 2018), and *Phalaenopsis* (NHUT; TUNG; TANAKA, 2018).

Thus, this research aimed to study the influence of light sources from fluorescent lamps and light-emitting diodes (LEDs) and culture medium formulations on the germination and initial development of the orchid species *B. perrinii*.

MATERIAL AND METHODS

The experiment was carried out between December 2017 and March 2018 in the laboratory of *in vitro* cultivation of the School of Agricultural Sciences (FCA) of the Federal University of Grande Dourados (UFGD). Seeds of *B. perrinii* from artificial pollination were used. The seeds were taken from a mature capsule previously harvested, desiccated for 14 days in a desiccator with silica gel (25 ± 2 °C and 75% RH), packed in aluminum foil, and stored under refrigeration (5 ± 2 °C).

The initial seed viability was evaluated using the tetrazolium (2,3,5-triphenyltetrazolium chloride) test before the experiment was set up, according to the methodology of Hosomi *et al.* (2012). An amount of 0.002 g of seeds, divided into three replications, with each replication composed of about 500 seeds, was used. The seeds were counted using a stereoscope and those with a pinkish-red color were considered viable. The seed size was measured considering the length (mm) x width (mm) in an electron microscope, using the software Spot Basic.

Samples of 0.005 g of seeds were weighed for each treatment. The seeds are disinfested with 70% ethanol for 1 min followed by 0.8% sodium hypochlorite for 5 min in a laminar flow cabinet. After this period, a triple wash was performed with autoclaved distilled water (1 min each) and the volume was completed to 100 mL with sterile distilled water for *in vitro* sowing. Sowing was carried out using an automatic pipette, with which 1 mL of the seed suspension was inoculated per plot, with a mean of 125 seeds each.

Sowing was carried out in four culture medium formulations: MS (MURASHIGE; SKOOG, 1962), ½ MS (MS at half salt concentration), VW (VACIN; WENT, 1949), and K (KNUDSON, 1946). The culture media were solidified before sowing with 6.4 g L^{-1} of agar and supplemented with 30 g L^{-1} of sucrose. The pH was adjusted to 5.8 using

HCl (0.1 M) or KOH (0.1 M), when necessary. The culture media were distributed in 50-mL capacity polypropylene vials. Each vial was filled with 20 mL of culture medium and sealed with three layers of polyvinyl chloride (PVC) plastic film. The vials were autoclaved at 120 °C at 1 atm for 20 min.

The treatments were placed in a growth room with a photoperiod of 16 hours, a temperature of 25 ± 2 °C, and irradiance of 30 µmol m^{-2} s^{-1}, under five light sources: FL – fluorescent lamp (100%) at 440–550 nm, WL – white LED (100%) at 440–550 nm, BL – blue LED (100%) at 460 nm, RL – red LED (100%) at 630 nm, and BRL – blue LED (50%) at 450 nm and red LED (50%) at 630 nm. The light spectrum was measured using an Ocean Optics portable spectrometer (model MMO with fiber optics). The experiment was conducted in duplicate.

A study of seed germination was carried out at 90 days after sowing to evaluate the percentage of germination and percentage of chlorophyll protocorms. The evaluation was performed by removing the material from the vials with distilled water and placing it in checkered (0.5 × 0.5 cm) acrylic plates (2.0 × 2.0 × 0.5 cm), using a binocular stereoscopic microscope with zoom for better visualization of the material.

The development of protocorms was evaluated according to the methodology of Suzuki *et al.* (2009), in which they were classified according to morphological classes: stage P1 – chlorophyll swollen protocorms, stage P2 – protocorms with the formation of the first leaf, stage P3 – protocorms with two leaves, and stage P4 – protocorms with leaves and one or more roots.

The data from the morphological evaluation of protocorms allowed calculating the protocorm development index according to the methodology of Spoerl (1948). Each replication was multiplied by the weights 1, 2, 3, and 4, according to the respective stages. The index of each replication was obtained by the mean of the sum of all stages of development present in them.

A completely randomized design was used, and the treatments were arranged in a 5 × 4 factorial scheme (light sources × culture media), with four replications each. The data were subjected to analysis of variance using the F-test and the percentage values were previously transformed into arcsine $\sqrt{\frac{x}{100}}$ to meet the data normality standards. The original means were kept, with significant results compared using the Tukey test ($p < 0.05$). The analyses were performed using the AgroEstat statistical program (BARBOSA; MALDONADO JÚNIOR, 2015).

RESULTS AND DISCUSSION

The seeds of *B. perrinii* have a mean length of 0.46 mm and width of 0.10 mm, with initial viability of 83.97% (Table 1).

No interaction was observed between the factors light source and type of culture medium in the percentage of germination, showing an isolated result only for the type of culture medium. Similar results were observed in *Bletilla ochracea*, whose means of seed germination showed no significant difference under different LED light sources (GODO *et al.*, 2011). In contrast, Bae, Oh, and Kim (2014) observed a higher percentage of germination (90.9%) when seeds of the terrestrial orchid *Oreorchis patens* were subjected to the red LED light.

The highest percentage of germination of *B. perrinii* seeds was observed for the culture media VW (98.51%) and ½ MS (98.00%), regardless of the light source (Table 2). These results suggest that the artificial light source is not a limiting factor for *B. perrinii* germination.

Melo *et al.* (2015) evaluated the *in vitro* germination of the species *Cattleya labiata*, as well as the hybrids of *Cattleya violacea* estriata pedrinho x *Cattleya violacea* pedrinho and *Cattleya violacea* Pedrinho x *Cattleya violacea*, and observed that the ½ MS medium is more favorable than MS. However, the best response to the percentage of germination was observed in the VW medium for *Cattleya bicolor* (SUZUKI *et al.*, 2010) and *Calopogon tuberosus* var. *tuberosus* (KAUTH *et al.*, 2008).

The variable percentage of chlorophyll protocorms showed an isolated effect of light sources and culture medium formulations. The ½ MS culture medium provided the highest percentage of chlorophyll

Table 1 - Biometrics and initial viability of *Brassavola perrinii* seeds

Seed characteristics	Brassavola perrinii
Seed length (mm)	0.46 ± 0.13 a
Seed width (mm)	0.10 ± 0.01
Initial viability – TTC (%)	83.97 b

[a] Mean of 50 seeds. [b] Mean of 1500 seeds

protocorms (51.23%), that is, live protocorms (Table 2). The same result has been observed in other studies with *Cattleya labiata* (MELO *et al.*, 2015). In these studies, the percentage of germination is directly associated with the percentage of chlorophyll protocorms. Possibly, this higher percentage of survival may be related to the ideal concentration of total nitrogen (30.03 mmol L^{-1}) and potassium (10.30 mmol L^{-1}) in the ½ MS formulation.

A total of 42.39% of chlorophyll protocorms were observed when the seeds were maintained under white LED light, a mean that did not differ significantly from the fluorescent lamp nor the treatments with blue (100%) and red (100%) LED light sources, which showed means of 39.44, 40.63, and 40.61%, respectively (Table 3).

Bae, Oh and Kim (2014) observed different results when comparing light sources in the development of the terrestrial orchid *Oreorchis patens*. The authors found that the highest percentage of protocorm formation (94.1%) occurred under the red LED light.

The highest protocorm development index (284) was found in the treatment with fluorescent lamps in interaction with the VW culture medium formulation. However, the highest protocorm development index (250) among treatments in which LED light sources were used was observed in the treatment with the association of blue (50%) and red (50%) LEDs in interaction with the VW medium (Figure 1). LEDs showed the formation of a bell-shaped curve within their respective spectral bands, that is, 460 nm for blue LED, 630 nm for red LED, and peaks of 450 and 630 nm for the combination of the blue and red LEDs, respectively (Figure 2).

The better protocorm development in the treatment with fluorescent lamps may be related to its characteristics because it emits a broad spectrum of light, with peaks in the spectral range of blue (440 nm) and green (550 nm) (Figure 2). According to recent studies, orchid micropropagation can be significantly improved by using light in the spectral range of green, as occurred in two cultivars of *Cymbidium*, where green light favored PLB production or organogenesis (NAHAR; HAQUE; KAZUHIKO, 2016).

Furthermore, this spectral range of blue (440 nm) and green (550 nm) associated with red LEDs or fluorescent lamps significantly improved the regeneration of *Cymbidium* Waltz 'Idol' (KAEWJAMPA; SHIMASAKI, 2012).

Johkan *et al.* (2012) cultivated lettuce under LEDs with different peaks within the spectral range of green (510, 524, and 532 nm) and controls with fluorescent

Table 2 – Influence of culture medium formulations on the percentage of germination and percentage of chlorophyll protocorms from *in vitro* sowing of *Brassavola perrinii* at 90 days after sowing

Culture medium	Germination (%)1	Chlorophyll protocorms (%)1
MS	96.71 bc	31.00 c
½ MS	98.00 ab	51.23 a
VW	98.51 a	39.78 b
K	96.52 c	38.53 b
CV (%)	3.65	5.10

[1] Data transformed into arcsine $(x/100)^{1/2}$ and original means presented in the table. Means followed by the same lowercase letter in the column do not differ from each other by the Tukey test ($p < 0.05$). MS (Murashige and Skoog); ½ MS (MS at half salt concentration); VW (Vacin and Went), and K (Knudson). CV – Coefficient of variation

Table 3 – Influence of the light source on the percentage of chlorophyll protocorms from *in vitro* sowing of *Brassavola perrinii* at 90 days after sowing

Percentage of chlorophyll protocorms[1]					CV (%)
FL	WL	BL	RL	BRL	
39.44 ab	42.39 a	40.63 ab	40.61 ab	37.63 b	5.10

[1] Data transformed into arcsine $(x/100)^{1/2}$ and original means presented in the table. Means followed by the same lowercase letter in the row do not differ from each other by the Tukey test ($p < 0.05$). FL – fluorescent lamp (100%), WL – white LED (100%), BL – blue LED (100%), RL – red LED (100%), and BRL – blue LED (50%) + red LED (50%). CV – Coefficient of variation

lamps and observed that the plants developed normally and grew faster when cultivated under fluorescent lamps than those grown under LEDs.

The second highest result for the protocorm development index occurred under the treatment with the combination of 50% blue and 50% red LEDs (250), which showed peaks of 450 and 630 nm, without peaks in the green band. These results show that the absence and variation of the amount of light incidence in the green range can influence the development and growth of different species.

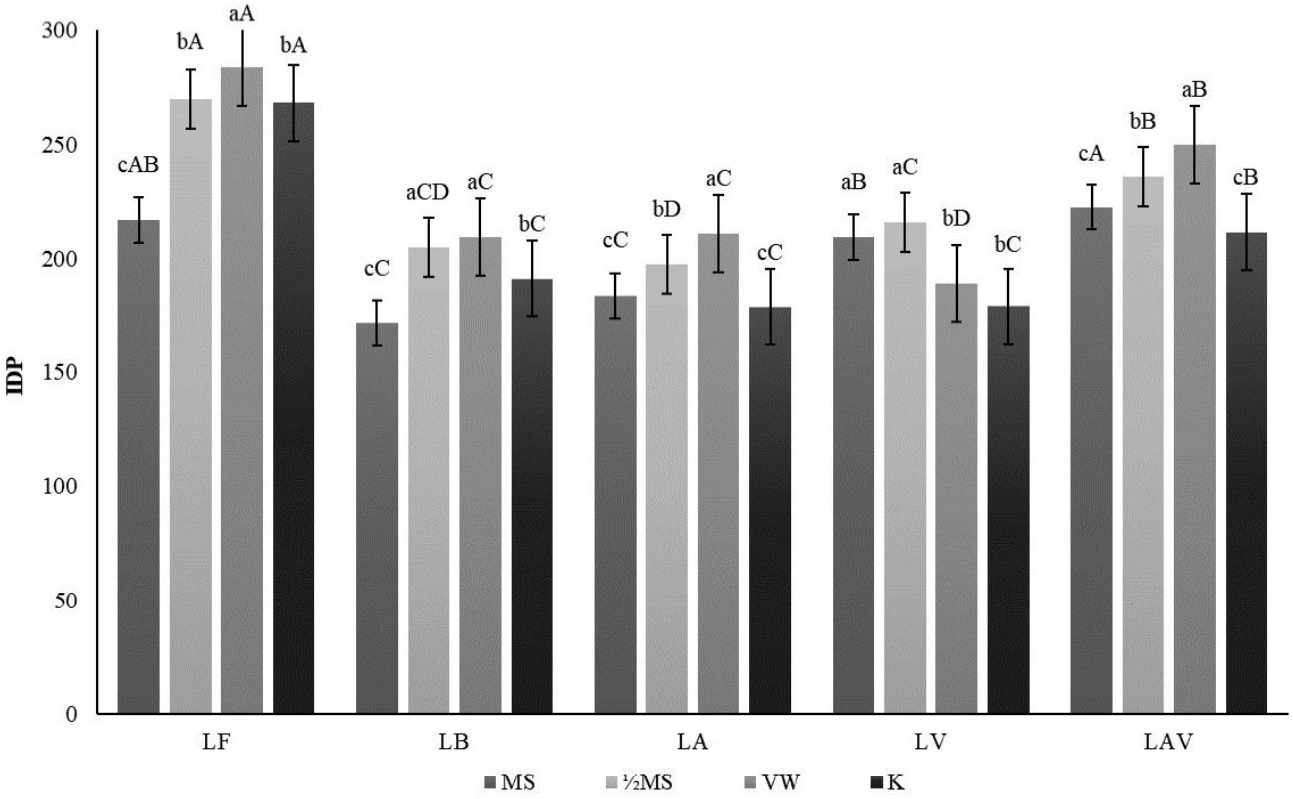
Nhut, Tung and Tanaka (2018) also recommend the combination of blue and red LEDs in the micropropagation of the epiphytic genera *Phalaenopsis* and *Cymbidium*. The authors verified that the use of a lighting system only with LEDs was effective for the photosynthetic process. Moreover, Silva (2014) observed a lower PLB production in nine hybrid *Cymbidium* cultivars when using monochromatic red or blue LED lights, showing the potential for combining lights.

Thus, new studies with different combinations of LEDs (white, blue, and red), covering all bands of the visible spectrum can be carried out, as the use of this light source has advantages such as significantly more efficient radiation for plants and, therefore, fewer lamps and reduced electricity cost.

Furthermore, a better response of the protocorm development index has been observed in VW (Figure 1), which may be attributed to the low nutritional requirement in the protocorm phase (JORGE; ABRÃO; SUZUKI, 2015). Another important point is that this formulation was originally proposed to be used for sowing and asymbiotic development of orchids.

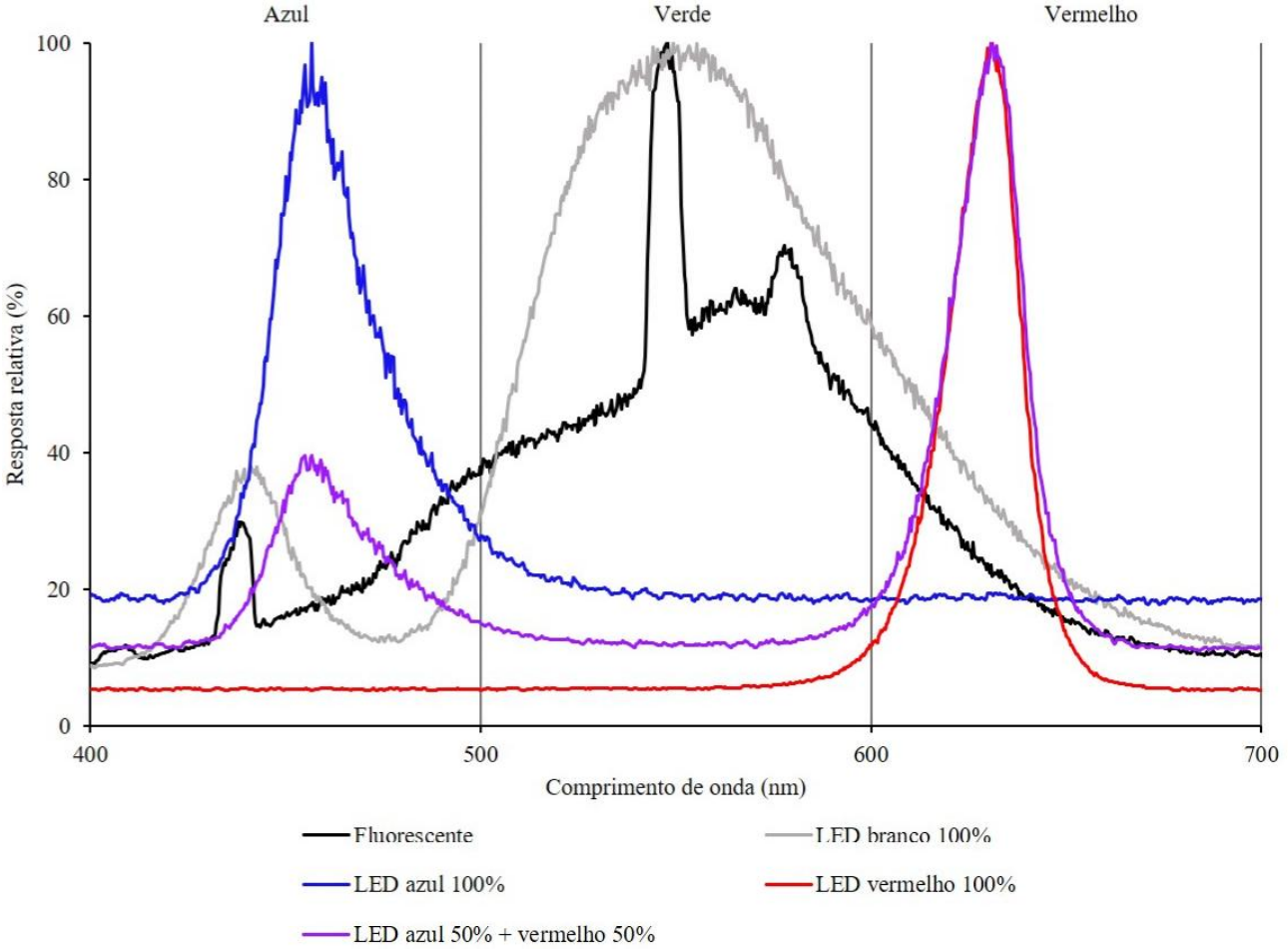
Studies on the protocorm development of the species *Hadrolaelia tenebrosa* (SUZUKI *et al.*, 2009), *Cattleya bicolor* (SUZUKI *et al.*, 2010), *Cattleya warneri* (JORGE; ABRÃO; SUZUKI, 2015), and *Catasetum macrocarpum* (FERREIRA *et al*., 2018) also found that the VW medium allowed obtaining this material at more advanced stages of development when compared to other culture media, as observed in the present study.

Figure 1 - Protocorm development index (PDI) at 90 days after sowing of the species *Brassavola perrinii*. In the bars, lowercase letters compare the culture media and uppercase letters compare the light sources by the Tukey test ($p < 0.05$). FL – fluorescent lamp (100%), WL – white LED (100%), BL – blue LED (100%), RL – red LED (100%), and BRL – blue LED (50%) + red LED (50%). MS (Murashige and Skoog, 1962); ½ MS (MS at half salt concentration); VW (VACIN; WENT, 1949), and K (KNUDSON, 1946)



PDI; FL; WL; BL; RL; BRL

Figure 2 - Distribution of relative spectral energy (%) from fluorescent lamps and light-emitting diodes (LEDs)



Relative response (%); Wavelength (nm); Blue; Green; Red; Fluorescent; White LED (100%); Blue LED (100%); Red LED (100%); Blue LED (50%) + red LED (50%)

CONCLUSION

The percentage of germination for *B. perrinii* was not influenced by light spectra, but it was higher in the ½ MS and VW formulations. The highest protocorm development index was observed when *B. perrinii* was subjected to the light spectrum of the lighting source with fluorescent lamps in the VW culture medium.

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