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Led enhance the growth characteristics and affects the *ex vitro* aclimatization of sugarcane

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ABSTRACT

The proper selection and optimization of light and gas-permeable membranes in the culture flasks, can benefit plant quality and improve *ex vitro* acclimatization capacity. The objective was to compare the morpho-physiological responses of *in vitro* sugarcane plants in different LEDs and sealing flask types and their impact in *ex vitro* acclimatization. Plants previously established *in vitro* were used and inoculated in flasks sealed with rigid caps with or without gas-porous membranes, and maintained in growth room (25 ± 2 ºC, 16 h photoperiod, and 72 µmol $m^{-2}s^{-1}$ irradiance from LEDs (white, blue/red, blue or red). At 62 days of *in vitro* cultivation growth and physiological parameters were evaluated. Blue/red LEDs and flasks without gas-porous membranes provided a higher number of shoots and blue LEDs a higher plant height. We observed greater number of leaves per clump in blue/red LED, and length of the largest root in flasks without gas-porous membranes. Red LEDs provided higher shoot fresh mass and the plants under blue and white LEDs had higher root fresh mass, being the same performance observed in shoot and root dry mass. The highest content of chlorophyll *a* and carotenoids was observed in plants kept in flasks without membranes and blue and red LEDs. The highest percentage of plant acclimatization were cultured under white LEDs (85%), and blue/red LEDs (70%).For this, to produce sugarcane *in vitro*, we suggest the use of culture flasks whitout membranes and blue/red or white LED lamps, as they provide more hardening plants standing out in excellent acclimatization of cultures.

Index terms: Light-emitting diodes; *Saccharum officinarum*; gas-permeable membranes; natural ventilation.

INTRODUCTION

Sugarcane (*Sacharum officinarum* L.) is a commercially important crop for tropical regions with a warm temperate climate (Kaur; Sandhu, 2015). Sugarcane is a clonal propagation crop, that is, the plants are obtained from multiple annual stem cuttings at each planting, which can lead to the spread of diseases (Redae; Ambaye, 2018). Still, the main deficiency of this technique is the slow propagation rate of new cultivars, requiring fast multiplication procedures for commercialization in a short period of time (Kaur; Sandhu, 2015; Redae; Ambaye, 2018). To solve these problems, micropropagation technology has been used, characterized by rapid multiplication, and obtaining new varieties of disease-free sugarcane (Redae; Ambaye, 2018).

In *in vitro* cultivation by the conventional micropropagation method, plants are produced in sealed flasks with minimal gas exchange, which can produce plants with hyperhydric leaves and / or with peculiar characteristics capable of limiting acclimatization to environmental conditions. Under these conditions, CO_2 concentrations in cultivation flasks are generally low, and together with other factors, they can cause physiological stress to plants which directly affects morphogenesis and growth (Xiao; Niu; Kozai, 2011). To overcome these restrictions and improve the *in vitro* culture environment, gas-porous membranes have been used (Gris et al., 2021), and are capable of allowing

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gas exchange between the *in vitro* and *ex vitro* environments, providing a reduction in relative humidity inside the culture flasks, an increase in transpiration and absorption of nutrients and water by plants, which favors photosynthesis and improves rates growth (Kozai, 2010; Xiao; Niu; Kozai, 2011).

Several studies report the advantage of using natural ventilation and forced ventilation with CO_2-enrichment. For natural ventilation, studies with *Pfaffia glomerata* (Iarema et al., 2012; Saldanha et al., 2012; Corrêa et al., 2015a; Corrêa et al., 2015b), *Capsicum annuum* (Batista et al., 2017), *Physalis angulata* (Santos et al., 2020), *Acca sellowiana* (Caetano et al., 2021), *Capsicum frutescens* (Gris et al., 2021), *Epidendrum fulgens* (Fritsche; Pinheiro; Guerra, 2022), *Guazuma ulmifolia* (Jesus-Santana et al., 2022), for forced ventilation, studies with *Pfaffia glomerata* (Saldanha et al., 2013; Saldanha et al., 2014; Ferreira et al., 2019), *Lippia alba* (Batista et al., 2017), *Etlingera elatior* (Pinheiro et al., 2021), among others. These studies brought the benefits of the use of gas-porous membranes, which provides a reduction in relative humidity and ethylene gas inside the flasks, which can eliminate or drastically reduce morphophysiological disorders and increase survival during acclimatization.

Among numerous factors, such as composition of the culture medium, gas exchange in the culture flasks, ambient temperature and characteristics of the explant, the light source remains an important regulator of plant development (Pawłowska et al., 2018; Miler et al., 2019). As light sources, fluorescent lamps are normally used, however, in recent years, a recently developed light technology; light-emitting diodes (LEDs) can directly convert electricity into light energy, with advantages such as specific wavelengths, high light efficiency, low energy consumption, cold light source, small volume, long life, and energy saving (Lazzarini et al., 2017; Batista et al., 2018; Pawłowska et al., 2018; Xu; Liang; Yang, 2019).

The use of LEDs offers great possibilities to modify light characteristics (Fiutak; Michalczyk, 2020), and allow precise control of the spectrum and the use of monochromatic light, which is valuable for research and industry purposes, but impossible to obtain with traditional fluorescent lamps (Miler et al., 2019). The LED lamps produced have been adopted with specific wavelengths in blue (450–470 nm) and red (650–665 nm), as they promote photosynthetic metabolism (Bantis; Ouzounis; Radoglou, 2016), with photosynthesis being the metabolic pathway for plant growth and development (Batista et al., 2018).

Light quality is particularly efficient in improving the developmental characteristics associated with autotrophic growth habits (Yeh; Chung, 2009) as it provides a higher concentration of photons and leads to better photosynthetic productivity (Miler et al., 2019). Thus, LEDs allow researchers to eliminate other wavelengths found within normal white light (Yeh; Chung, 2009), and so, compare them, because the quality of light influences the morphogenesis of plants. Therefore, the proper selection and optimization of light parameters are crucial in all plant tissue culture systems (Miler et al., 2019).

The objective of the present study was to compare the morpho-physiological responses of sugarcane *in vitro* plants in different light qualities and sealing flask types and their impact in *ex vitro* acclimatization. In addition, knowledge about the cultivation of sugarcane in different light qualities may help to optimize micropropagation systems of this species.

MATERIAL AND METHODS

Plant material and cultivation conditions

The experiment was conducted in the Tissue Culture and Aromatic Extracts Laboratory of the Federal University of Santa Maria (UFSM, *Campus* Frederico Westphalen. Sugarcane tolets (*Saccharum officinarum*), registered by UFSM XIKA FW (20160021) (Knapp et al., 2019), were excised, washed in running water for 1 hour and kept in plastic trays containing two sheets of Germitest® paper.

The trays were kept in biochemical oxygen demand (BOD), with a temperature of 28 °C and a photoperiod of 16 hours of light for 15 days, for the development of the buds. After this period, the selection of more developed lateral buds (about 4 cm long) was carried out and taken to a laminar flow chamber, in which they were immersed in 70% alcohol for 1 minute, followed by disinfestation in sodium hypochlorite (2.5% active chlorine) for 15 minutes, followed by three rinses of autoclaved distilled water.

Four explants were inoculated per culture flask containing 30 mL of MS medium (Murashige;

Skoog, 1962) containing 6-benzylaminopurine (BAP, 1.11µM), myo-inositol (100 mg L^{-1}), sucrose (30 g L^{-1}), and solidified with 6 g L^{-1} agar (Agargel®, João Pessoa, PB, Brazil), and the pH adjusted to 5.8, previously autoclaved at 121 ºC and 1.5 atm., for 15 minutes. The material was kept in a growth room in the dark for seven days, and then taken to the condition of a photoperiod of 16 hours of light, with a temperature of 25 ± 2 °C, light irradiance of 72 µmol $m^{-2}s^{-1}$ from two tubular white LED lamps, being subcultured every 30 days and maintained in the same conditions. For the experiment, sugarcane buds of approximately 2.7 cm in length were previously subcultured in the same culture medium mentioned above, up to the required amount of adventitious shoots (totaling eight subcultures of 30 days each).

For the conduct of the experiment, adventitious shoots were inoculated into 550 mL glass flasks containing 40 mL of the previously mentioned semi-solid MS culture medium, without the addition of plant growth regulators. The cultures were maintained in growing room conditions, with a temperature of 25 ± 2 ºC, a photoperiod of 16 hours of light, with light irradiance of 72 µmol $m^{-2}s^{-1}$ from four sets of two LEDs tube lamps (TECNAL, TEC LAMP®, Piracicaba, Brazil) each: white LEDs, red LEDs, blue LEDs, or blue/red LEDs (in the proportion of 60 and 40%, respectively). The flasks were sealed with rigid metal lids without holes or with lids with two 8 mm diameter holes each, being covered by gas-porous membranes, following the Saldanha et al. (2012) methodology.

Variables analyzed

At 62 days of *in vitro* cultivation, the following were evaluated: number of shoots, number of leaves per clump, plant height (cm), length of the largest root (cm), shoot fresh mass (g), shoot dry mass (g), root fresh mass (g), root dry mass (g), chlorophyll *a* (µg cm^{-2}), carotenoids (µg cm^{-2}).

To determine the dry masses, the material was taken to a drying oven with forced ventilation of air and temperature of 45 ºC until reaching constant mass.

Quantification of chlorophyll *a*, *b* and carotenoids

For determination of chlorophyll *a*, *b* and carotenoids this, five leaf discs of 3 mm in diameter each were taken from the second and third leaf of the apex towards the base and incubated in 3 mL of dimethyl sulfoxide (DMSO) saturated with $CaCO_3$. The discs remained in glass tubes covered with aluminum and kept in the dark for 48 hours, at room temperature, following the methodology proposed by Santos et al. (2008). After this period, the absorbance of the samples was determined in a Bel Photonics® SP 1105 spectrophotometer using a 10 mm optical path quartz cuvette. The chlorophyll *a*, *b* and carotenoids concentrations (µg mL^{-1}) were determined with the equations methodology proposed by Wellburn (1994):

Chlorophyll $a = 12.47A_{665.1} - 3.62A_{649.1}$

Chlorophyll $b = 25.06A_{649.1} - 6.5A_{665.1}$

Carotenoids = $(1000A_{480} - 1.29$Chlorophyll *a* $- 53.78$Chlorophyll *b*$) / 220$

Experimental design

The experiment was conducted in a completely randomized design, 4x2 factorial scheme, with four light qualities (white LEDs, red LEDs, blue LEDs, and blue/red LEDs) and two sealing types (rigid metal covers without hole) and with two holes covered by gas-porous membranes), totaling eight treatments, with ten repetitions each, and the experimental unit composed of five sugarcane buds per flasks. Half of these repetitions were used for the destructive variables and the other half to be acclimatized in greenhouse conditions.

For the variables chlorophyll *a, b* and carotenoids, the same design was previously reported, however, the experimental unit was composed of a test tube containing five leaf discs each.

Acclimatization

At 62 days of *in vitro* cultivation, the plants were acclimatized in transparent plastic pots (200 mL) containing Carolina® substrate, remaining for seven days in laboratory conditions with an average temperature of 22 ºC, with a subsequent stay in the greenhouse for another 30 days located in 27º23'44"S, 53º25'39"W, and average temperature between 20 and 30 ºC, and, remaining in sombrite® 70% and irrigated according to the need of the culture, in which the percentage of survival was accomplished at the end of the process.

For the variable percentage of acclimatization *ex vitro*, the same design was previously reported,

with ten repetitions and the experimental unit composed of one plant per pot, being evaluated after 30 days of acclimatization under *ex vitro* conditions.

Statistical analysis

All variables were subjected to analysis of variance and when significant, the means compared by the Tukey test, at 5% significance, using the SISVAR statistical program (Ferreira, 2011).

RESULTS AND DISCUSSION

Different light qualities and sealing types affected *in vitro* growth of sugarcane (Figure 1 and 2). After analysis of variance, our results indicated a significant interaction between light qualities and sealing types factors for the variables number of shoots, plant height, shoot fresh and dry mass, root fresh and dry mass, chlorophyll *a* and carotenoids. Separately, for the light qualities factor, there were significant differences between the number of leaves per clump and percentage of acclimatization, and for the sealing types factor, there was a significant difference for the variables number of leaves per clump and length of the largest root ($P < 0.05$). For chlorophyll *b*, there was no significant difference ($P > 0.05$).

For number of shoots, there was no significant difference for flasks with gas-porous membranes, however, in flasks without membranes, there was a greater multiplication of shoots when subjected to blue/red LEDs, not differing only from red LEDs (9.43 e 8.12, respectively, Figure 2A). When sealing types were compared, a significant difference was observed for the wavelengths of blue/red and red LEDs, in which the plants that remained in flasks with rigid caps showed an increase in the number of shoots when compared to those kept in flasks with membranes (Figure 2A). Huan and Tanaka (2004) suggest that LEDs are effective for *Cymbidium* and blue/red LEDs (25 and 75%, respectively) promote greater protocorm formation. Liu et al. (2014) observed a greater number of leaves of *Platycodon grandiflorum* when the plants were kept under blue LEDs. One of the determining factors for the photosynthetic efficiency of plants is the wavelength and maximum absorption by chlorophylls, occurring in the red/blue light band (Victório; Kuster; Lage, 2007), which influenced the number of sugarcane sprouts in the present study.

Plants under blue/red LED conditions (30.06) produced a greater number of leaves per clump, when compared to all other light qualities. For the sealing types, flasks without membranes (28.00) increased this number of leaves (Figure 2B). Similar results were observed in *Capsicum frutescens* cultivars that is, plants under red LEDs and blue/red LEDs and flasks without membranes produced more green leaves (Gris et al., 2021).

Plants submitted to flasks without membranes and blue LEDs (7.95 cm) had a greater increase in the length of the aerial part when compared to other light qualities. When we analyzed the sealing types, a significant difference was observed in plants under blue/red LEDs and flasks with membranes (6.43 cm), when compared to those without membranes, the same being observed for plants under blue LEDs (7.95 cm) (Figures 1 and 2C). When exposed to blue LEDs, Liu et al. (2014) report that photosynthetic capacity is increased, which reflects an increase in soluble sugar and carbohydrates in plant metabolism, consequently increasing in explant length. For the variable length of the largest root, it was observed that when the plants remained in flasks with rigid lids (5.93 cm) there was a greater increase, when compared to those with membranes (Figure 1, 2D).

Plants in flasks with membrane and red LEDs had an increase in shoot fresh mass (SFM, 2.24 g), differing only from those under white LEDs. Furthermore, plants under blue LEDs and white LEDs showed the greatest increase in root fresh mass (RFM, 1.44 and 1.51 g, respectively) in flasks without membranes (Figure 3A). The same SFM trend, we observed for root fresh mass (RFM), in plants under blue/red LEDs in flasks without membranes (0.27 g), with lower RFM. Also, plants under blue LEDs and white LEDs showed a greater increase in RFM (1.44 and 1.51 g, respectively) also in flasks without membranes (Figure 3A).

We observed the same behavior for SDM, with increments in flasks without membranes and blue LEDs and white LEDs (0.331 and 0.301 g, respectively) (Figure 3B). For root dry mass (RDM), plants in flasks without membranes associated with blue LEDs, red LEDs, and white LEDs showed a significant increase in RDM (0.102, 0.106, and 0.114, respectively) (Figure 3B). In general, the use of flasks without membrane provides an increase in fresh and dry mass of shoots and roots (SFM, RFM, SDM, and RDM), with the exception of those under blue/red LEDs, which showed the opposite response.

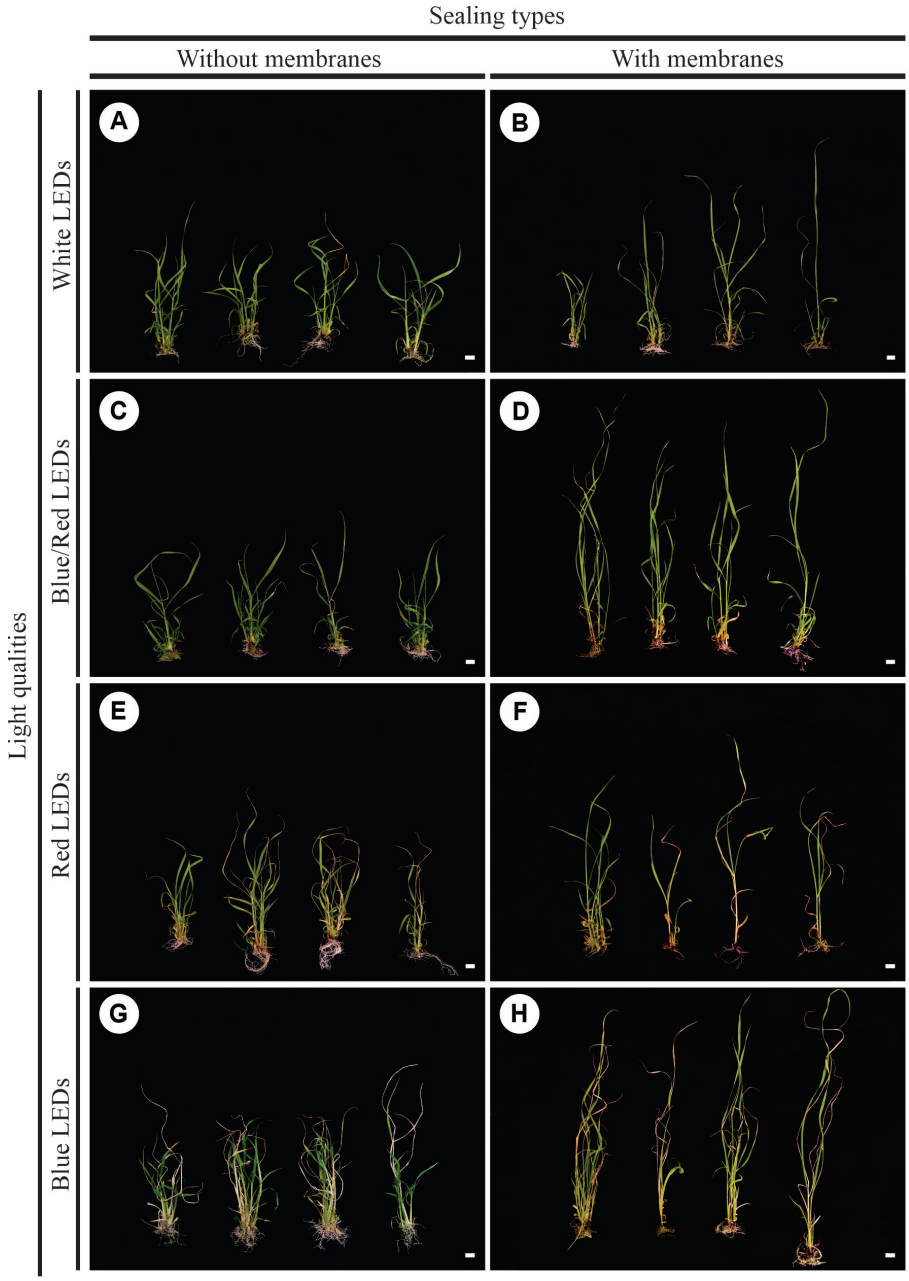

Figure 1: Sugarcane plants at 62 days of *in vitro* cultivation kept in a growth room and submitted to light qualities and in flasks with different sealing types. (A-B) Plants grown under white LEDs and flasks without and with gas-porous membranes, respectively; (C-D) blue/red LEDs and flasks without and with gas-porous membranes, respectively; (E-F) red LEDs and flasks without and with gas-porous membranes, respectively; (G-H) blue LEDs and flasks without and with gas-porous membranes, respectively. Bars: 1 cm.

As in the present study, *Achille amillefolium* plants, grown under red LEDs, obtained greater accumulation of dry matter of the shoots (Alvarenga et al., 2015). However, this response may be different for each species, for example, in *Panax vietnamensis*, when the plants were grown under blue/red LEDs (40/60%, respectively), they obtained an increase in fresh and dry mass, height of plant, leaf diameter and leaf length (Nhut et al., 2015), and for *Heliconia orthotricha*, higher fresh

mass was also observed in proportions of blue/red LEDs and in white fluorescent (Takeui et al., 2016), proving that there is no general rule for using the ideal proportion and lighting conditions for specific crops (Nhut et al., 2015).

Plants grown under blue LEDs and red LEDs and flasks without membrane produced the greatest increase in chlorophyll *a* (23.92 and 23.10 µg cm^{-2}, respectively). For the flasks with membranes, there was no difference between

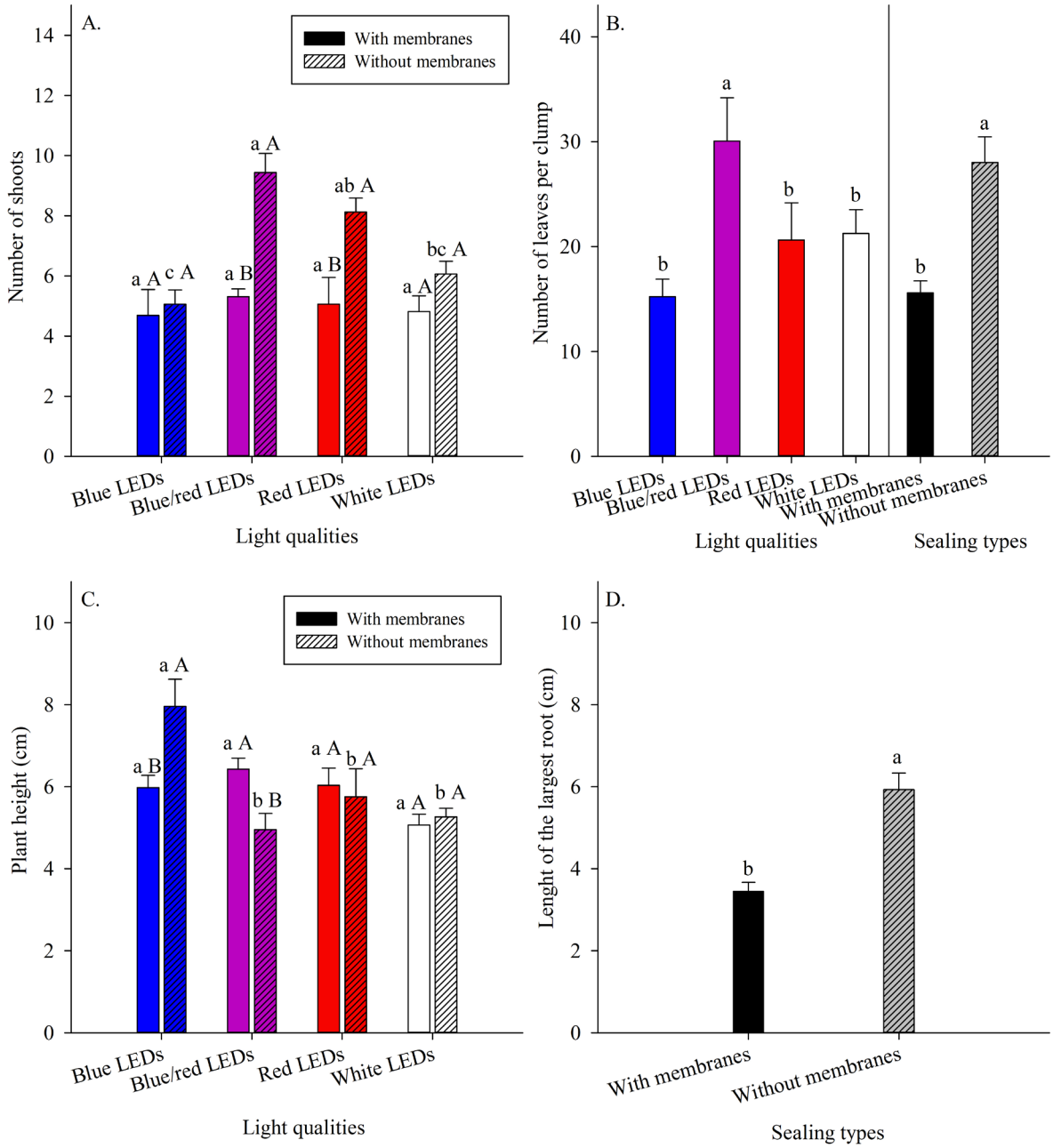

Figure 2: (A) Number of shoots; (B) Number of leaves per clump in the factor light qualities and sealing types; (C) Plant height; (D) Length of the largest root of sugarcane (*Saccharum officinarum*) *in vitro* subjected to different light qualities and sealing types. *(A, C) Same lowercase letters for light quality and equal uppercase letters for sealing types, do not differ from each other, (B, D) equal letters do not differ by Tukey's test, at 5% significance; values represent the mean ± standard error.

the light qualities (Figure 4A). For the carotenoid variable, plants kept in flasks without membrane produced higher levels when kept under red LEDs (5.52 µg cm^{-2}), being superior only to those produced under blue/red LEDs (4.67 µg cm^{-2}) (Figure 4B). Plants in flasks with membranes and red LEDs had an increase of 5.06 µg cm^{-2} and was superior only to those kept in blue LEDs (3.89 µg cm^{-2}) (Figure 4B). Photosynthetic metabolism and consequently autotrophic growth in plants are improved as chlorophyll molecules absorb blue and red wavelengths more efficiently, as the

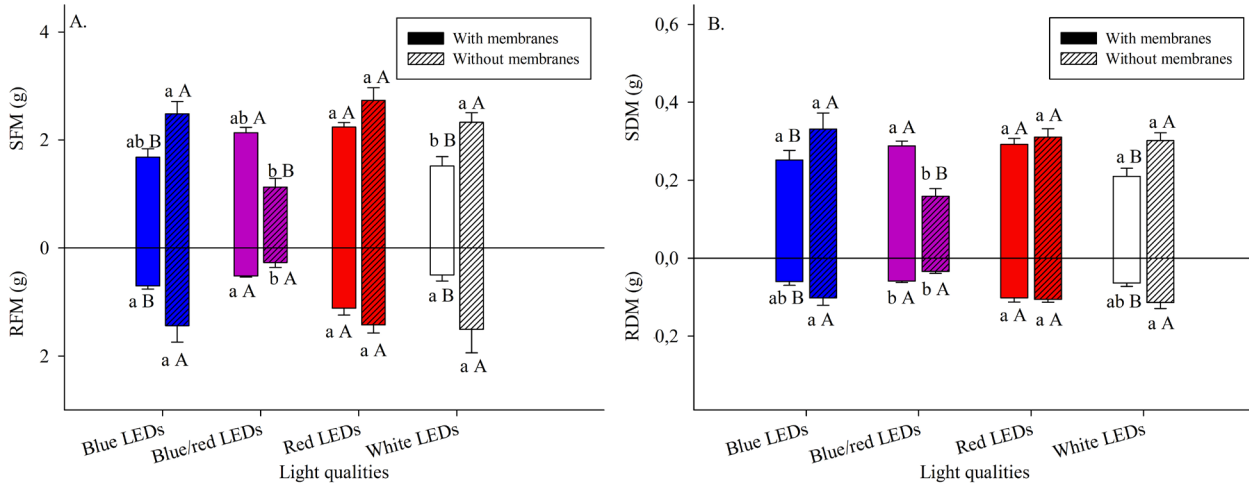

Figure 3: (A) Shoot fresh mass and root fresh mass (SFM, RFM, respectively); (B) and shoot dry mass and root dry mass (SDM, RDM, respectively) of sugarcane plants (*Saccharum officinarum*) submitted to different light qualities and sealing types in *in vitro* cultivation. *Equal lower-case letters for light quality and equal upper-case letters for sealing types, do not differ by Tukey's test, at 5% significance; values represent the mean ± standard error.

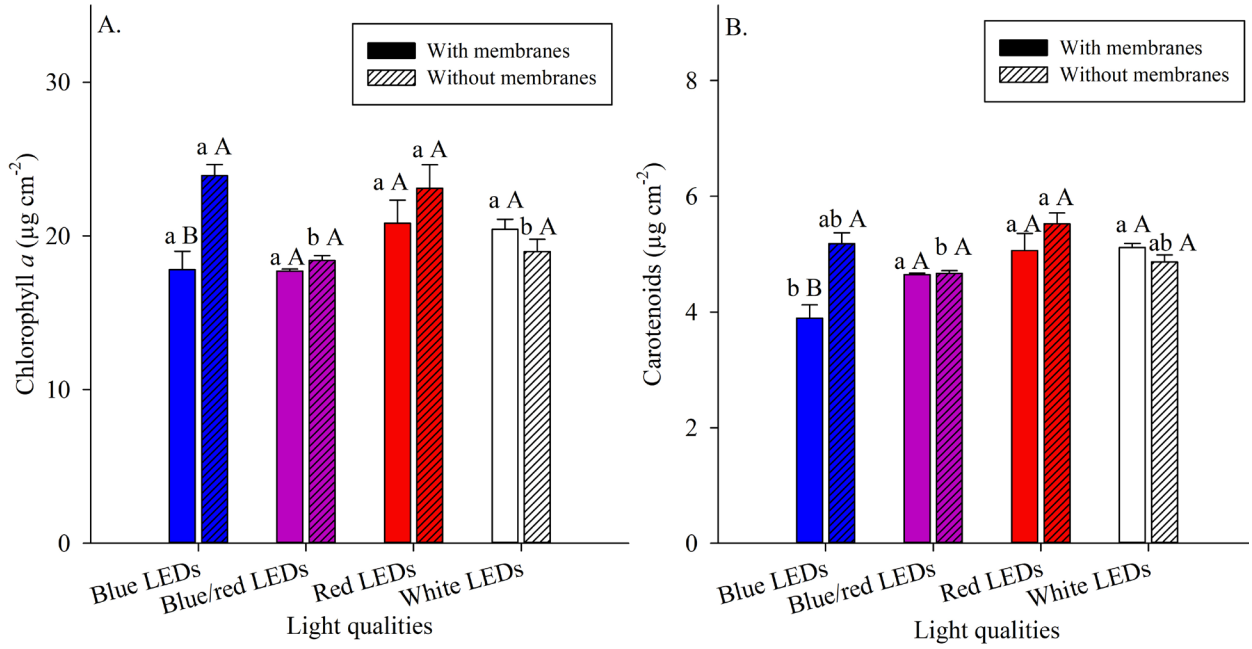

Figure 4: Chlorophyll *a* (A) and carotenoids (B) from sugarcane plants (*Saccharum officinarum*) submitted to different light qualities and sealing types in *in vitro* conditions. *Equal lower-case letters for light quality and equal upper-case letters for sealing types, do not differ by Tukey's test, at 5% significance; values represent the mean ± standard error.

superimposed pattern of luminescence spectrum of the blue LED (450-470 nm) and the red LED (650-665 nm) corresponds well to the light absorption spectrum of carotenoids and chlorophyll (Yeh; Chung, 2009).

When *Heliconia orthotricha* plants were grown under a monochromatic blue LED, an increase in chlorophyll content was observed, being higher when compared to other light sources (Takeui et al., 2016). The same authors observed an increase in the number of shoots and a decrease in plant height when they were kept under blue/red LEDs (30/70%, respectively). Often, the use of light qualities (LEDs) can provide an increase in the amount of chlorophyll and carotenoids in plant tissues, which can be converted into greater leaf area or even benefit the shoot multiplication rate (Liu et al., 2014; Nhut et al., 2015). Thus, changes in chlorophyll synthesis due to changes in spectral quality may allow advantages in plant growth (Larcher, 2003), as plant development and physiology are highly influenced by specific wavelengths, varying according to species (Lazzarini et al., 2017).

When plants of *Heliconia Orthotricha* were grown under a monochromatic blue LED, an increase in chlorophyll content was observed, being greater when compared to other light sources (Takeui et al., 2016). The same authors observed an increase in the number of shoots and a decrease in plant height when they were kept under blue/red LEDs (30/70%, respectively).

For the percentage of acclimatization, we observed a difference only for the light qualities factor, and there was no significant difference for the sealing types. After 30 days of *ex vitro* cultivation, plants grown under white LEDs had better acclimatization (85%), followed by blue/red LEDs (70%) and red LEDs (65%), and did not differ only from blue LEDs (30%) (Figure 5). During the *in vitro* multiplication of sugarcane, Ferreira et al. (2017) reported that the use of white LEDs increased the content of carotenoids, which may confer advantages to plants during the *ex vitro* acclimatization phase. However, these authors did not observe the use of white, red, blue, and blue/red LEDs during the sugarcane elongation/rooting, and their reflection during *ex vitro* conditions, which is proven in the present work, with benefits to increase the hardness of plants.

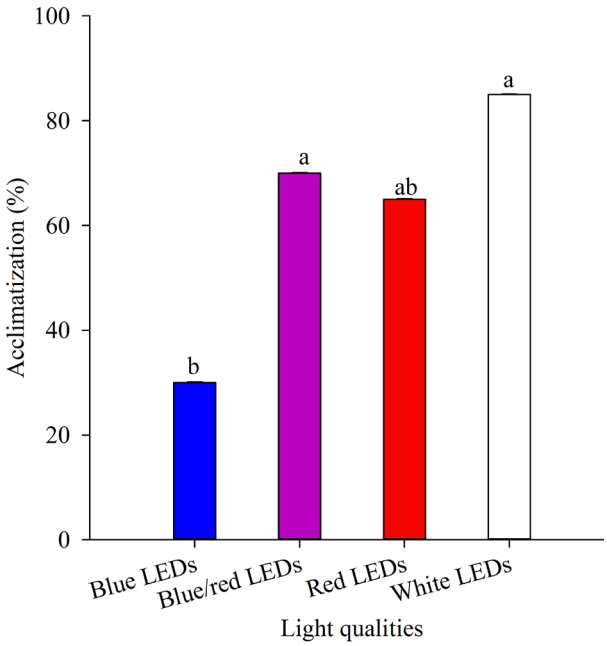

Figure 5: Percentage of acclimatization after 30 days of *ex vitro* cultivation of sugarcane plants (*Saccharum officinarum*) submitted to different light qualities in *in vitro* conditions. *Same lower-case letters do not differ by Tukey's test, at 5% significance; values represent the mean ± standard error.

CONCLUSIONS

Sugarcane plants may present varied morphophysiological responses to light quality, so that the use of this factor alone *in vitro* may be able to guarantee high rates of plant acclimatization. Finally, we suggest that the use of culture flasks whitout membranes and blue/red or white LED lamps, as they provide more hardening plants standing out in an excellent acclimatization of cultures.

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