

Impact of MS concentration and light qualities on alpine red ginger elongation and rooting *in vitro*

Marcos Vinícius Marques Pinheiro^{1*}, Alana Paula Chiavagatti Schmidt², Guilherme Masarro-Araujo², Evandro Holz², Wagner Campos Otoni³, Denise Schmidt²

ABSTRACT

Some factors in *in vitro* cultivation, such as formulation of the culture medium and the quality of the light source, may vary the response of the plants in those conditions, favoring the photosynthetic metabolism obtaining more rustified plants capable of benefiting from the conditions of *ex vitro* cultivation. Thus, the objective was to evaluate *in vitro* elongation/rooting of *Alpinia purpurata* cv. Red Ginger under different light-emitting diodes (LEDs) and saline concentration of the MS medium. For this, previously established *in vitro* plants were maintained for 55 days in 550 mL glass flasks containing Carolina® substrate and MS culture medium with different salt concentrations, under LEDs qualities. The following parameters were evaluated: number of green and senescent leaves, shoot length, length of the largest root, fresh and dry mass of leaves, stem and root, root volume, photosynthetic pigments [chlorophyll *a*, chlorophyll *b*, total chlorophylls (*a+b*) and carotenoids ($\mu\text{g cm}^{-2}$)], stomatal density and acclimatization percentage (after 33 days of *ex vitro* cultivation). After 55 days, plants grown under red LEDs and full-strength MS culture medium is indicated and promote efficient shoot length and photosynthetic pigment content, respectively. These conditions provided plants better adapted for *ex vitro* acclimatization.

Index terms: *Alpinia purpurata*; light-emitting diode (LEDs); culture medium.

INTRODUCTION

The Zingiberaceae family comprises perennial monocotyledonous plants distributed in tropical regions around the world (Raju et al., 2019). The species *Alpinia purpurata* is marked by the beauty of the inflorescences, with lush green leaves and large and attractive bracts, which justifies the commercialization of plants and their flowers besides the use in landscaping (Chan; Wong, 2015; Sanches et al., 2016). These plants have important medicinal properties present in essential oils, popularly used in the treatment of various diseases, such as hypertension, inflammatory processes, and other therapeutic potentials, as well as in cooking such as spices and condiments in many regions of Asia (Victorio, 2011 ; Ghosh; Rangan, 2013; Chan; Wong, 2015).

The largest production of plants of this species occurs by vegetative propagation via rhizomes; however, the main disadvantage is the spread of diseases that can occur in successive plantings. Thus, *in vitro* cultivation is an important technique to produce plants from tissues, organs or cells grown in culture medium with necessary nutrients, energy and water

(Phillips; Garda, 2019) and that promotes the massive propagation of several plants with physiological and phytosanitary quality (Araruna et al., 2017). MS medium (Murashige; Skoog, 1962), initially formulated for the cultivation of *Nicotiana* sp., has been used for various herbaceous species and ornamental plants, including alpinas. However, the response of plants may vary depending on the culture medium used and the formulation of salts, as well as phytohormones, since the concentrations of these elements and other osmotically active compounds may culminate in the activation or inactivation of the metabolic pathways that proceed *in vitro* plant development (Araruna et al., 2017).

The quantity and quality of light influence the absorption of nutrients by the roots, directly affecting plant growth and development (Sakuraba; Yanagisawa, 2018; Ferreira et al., 2016). Light-emitting diodes (LEDs) are low-cost light sources used in plant production and have specific wavelengths and intensities, being found in blue (450-470 nm) and red LEDs (650-665 nm), capable of triggering photoreceptors to induce morphological and physiologically responses in plants such as rooting

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¹Universidade Estadual do Maranhão/UEMA, São Luís, MA, Brasil

²Universidade Federal de Santa Maria/UFSM, Frederico Westphalen, RS, Brasil

³Universidade Federal de Viçosa/UFV, Departamento de Biologia Vegetal, Viçosa, MG, Brasil

*Corresponding author: macvini@gmail.com

or branch elongation (Yeh, 2009; Agarwal; Gupta, 2016; Huché-Thélier et al., 2016; Senol; Kilic; Tasdelen, 2016; Batista et al., 2018).

In conventional *in vitro* cultivation, there may be restriction of the photosynthetically photon flux, reduced gas exchange and decreased rates of transpiration and photosynthesis, reducing the production of photoassimilates, which may lead to reduced survival rate during culture acclimatization (Kozai, 2010; Xiao; Niu; Kozai, 2011). Some factors in *in vitro* cultivation, such as changing the culture medium, imposing gas exchange, reducing relative humidity and the quality and intensity of light can benefit the photosynthetic metabolism of plants, obtaining more rustified plants and better able to adapt to *ex vitro* conditions (Couto et al., 2014; Xiao; Niu; Kozai, 2011; Rodrigues et al., 2011).

In this way, the present study aimed to evaluate the *in vitro* elongation and rooting of *Alpinia purpurata* cv. Red Ginger under different light qualities LEDs and MS salt concentrations and the impact on the vitroplants acclimatization.

MATERIAL AND METHODS

Plant material and growing conditions

Alpinia purpurata cv. Red Ginger were previously successively subcultured (six times) until there are enough adventitious shoots necessary, in MS culture medium supplemented with 1.5 mg L⁻¹ 6-benzyladenine (6-BA), 0.1 mg L⁻¹ α -naphthalenoacetic acid (ANA), 100 mg L⁻¹ myo-inositol, 30 g L⁻¹ sucrose, solidified with 6 g L⁻¹ agar (AgarGel®, João Pessoa, PB, Brazil), and pH adjusted to 5.8, previously autoclaved at 121 °C and 1.5 atm for 15 minutes. The plants were maintained under controlled growth room conditions, with a temperature of 25 \pm 2 °C, 16 hours light photoperiod, under 72 μ mol m⁻² s⁻¹ irradiance provided by white LEDs lamps.

To conduct the experiment, two adventitious shoots (approximately 5 cm with three to five expanded leaves) were used per 550 mL volume flask, sealed with a metal lid with two 8 mm holes, covered by porous membranes, made according to the methodology of Saldanha et al. (2012). In each flasks, 40 mL of culture medium was used [100% salt MS (MS) or 50% salt MS

(MS1/2)], added 0.1 mg L⁻¹ ANA, 100 mg L⁻¹ myo-inositol, 30 g L⁻¹ sucrose, with 20 g of Carolina® substrate per flask. The pH was adjusted to 5.8 \pm 0.1 prior to autoclaving. After inoculation, the vegetative materials were kept for 55 days in a growth room with 16 hours photoperiod, 72 μ mol m⁻² s⁻¹ irradiance, from two TECNAL TEC LAMP® tubular lamps for each color [white LEDs; blue LEDs; red LEDs or red/blue LEDs (at 60 and 40%, respectively)], and temperature of 25 \pm 2 °C.

Experimental design and analyzed variables

The experiment was conducted in a completely randomized design, in a 4x2 bifactorial scheme, with four light qualities (white LEDs, blue LEDs, red LEDs and red/blue LEDs) and two MS salts concentrations (full strength (100%) – MS; and half strength - MS1/2), totaling eight treatments, varying the number of repetitions for the variables analyzed and the experimental unit. All variables were evaluated at 55 days of *in vitro* culture, and acclimatization percentage variable after 33 days of *ex vitro* cultivation.

Growth measurements

For the variables number of shoots, number of green leaves, number of senescent leaves, shoot length (cm), largest root length (cm), fresh and dry leaf mass (g), fresh and dry stem mass (g), fresh and dry root mass (g), root volume (mL), five replicates were used, and the experimental unit was composed of two plants per flask.

Photosynthetic pigments

To determine the photosynthetic pigments, chlorophylls *a* (Chl*a*) and *b* (Chl*b*), total chlorophylls (*a+b*; ChlT) and carotenoids, five discs, 5 mm in diameter each, were obtained from second leaf green of plants (from apex to base) and incubated in glass tubes containing 5 mL of dimethyl sulfoxide (DMSO) saturated with calcium carbonate (CaCO₃), kept in glass tubes and in the dark, according to the adjusted methodology of Santos et al. (2008), totaling four repetitions for each treatment. After 48 hours in the dark, absorbance was determined on a SPECORD 50 / PLUS® spectrophotometer (Analytikjena, Germany) using a 10 mm quartz cuvette. Wellburn (1994) methodology was followed to establish wavelengths and equations to calculate the concentrations of chlorophylls

[*a*, *b*, totals (*a+b*)] and carotenoids (CAR) and the unit of these variables being in $\mu\text{g cm}^{-2}$.

For the photosynthetic pigments variables [chlorophyll *a*, chlorophyll *b*, total chlorophylls (*a+b*) and carotenoids ($\mu\text{g cm}^{-2}$)] four replications were used and the experimental unit consisted of a glass tube).

Stomatal density

The stomatal density (stomata mm^{-2}) was performed using the epidermis printing technique from the methodology described by Weyers and Johansen (1985), with a drop of cyanoacrylate ester under the glass slide. The abaxial or adaxial face of the first fully-expanded and green leaf from the apex to the base of the plant was pressed for approximately 30 seconds, leaving the impression of the epidermis on the blade. From the prepared slides, images were captured with the aid of a LEICA® (DM 1000 – Leica) optical microscope coupled to a camera (DFC 295 – Leica), using a 20x magnification (area of $596 \times 447 \mu\text{m}$). The determination of stomatal density was performed using the Anati Quant 2 software, from the stomatal cell count.

Because it is an amphistomatic species with greater amounts of stomata on the abaxial face, stomatal density was performed on both sides, adaxial and abaxial faces of the leaf, four repetitions and the experimental unit composed by a blade with paradermic impression of both sides of the leaf.

Acclimatization

After 33 days of *ex vitro* cultivation, the acclimatization percentage was evaluated, using three replications, in which each experimental unit was composed of two plants. Plants with 55 days of *in vitro* cultivation in MS salts concentrations and light qualities, as described above, were transferred to plastic bottles containing Carolina® substrate in greenhouse for more 33 days, with daily average temperature of 20 to 30 °C, kept in sombrite® 60% and regularly irrigated.

Statistical analysis

The variables were subjected to analysis of variance and the means were compared by the Scott-Knott 5% probability test using the SISVAR software (Ferreira, 2011).

RESULTS AND DISCUSSION

By the analysis of variance F test at 5% of significance, there was no significant difference for the light quality factor for the variables shoot length (cm), number of shoots, fresh leaf mass (g) and chlorophyll *b* (chl*b*, $\mu\text{g cm}^{-2}$). Considering chlorophyll *a* (Chl*a*, $\mu\text{g cm}^{-2}$), total chlorophyll (ChlT, $\mu\text{g cm}^{-2}$), carotenoids (CAR, $\mu\text{g cm}^{-2}$) and abaxial stomatal density there was significant difference only for the MS concentrations factor, and adaxial stomatal density there was no significant difference for any factor. There was a significant difference for the interaction between the factors for acclimatization percentage variable ($p < 0.05$). For the other variables analyzed (number of green leaves, number of senescent leaves, length of the largest root, root volume, leaf dry mass, root fresh and dry mass and stem fresh and dry mass) there were no significant differences.

Plants grown under white LEDs and red/blue LEDs induced higher number of shoots (0.85 and 0.67, respectively) than the other light qualities (Figure 1A). Similar results were observed in *Saccharum officinarum*, which in white LEDs promoted a higher number of shoots (Ferreira et al., 2016) and under red/blue LEDs in *Corymbia* (Souza et al., 2019).

The red LEDs promoted higher shoot length (7.91 cm), according to Figure 1B. Similar results were observed in gerbera plantlets besides stimulated adventitious root formation (Pawłowska et al. 2018). Light quality, among other factors of occurrence in *in vitro* condition, can influence the shoots number, providing different effects on plant growth and morphogenesis (Li; Tang; Xu, 2013).

Blue and red/blue LEDs provided higher fresh leaf mass accumulation (0.17 and 0.14 g, respectively) (Figure 1C), probably the blue light increased the leaf area and the fresh leaf mass accumulation of alpinia plants. This higher fresh leaf mass is related to the higher photosynthetic activities induced by those light qualities. Red/blue LEDs provided higher fresh mass accumulation in *Doritaenopsis* (Shin et al., 2008); which is correlated with higher photosynthetic efficiency due to wavelength peaks close to chlorophyll absorption and carbohydrate accumulation increased by red light spectrum (Rocha et al., 2013).

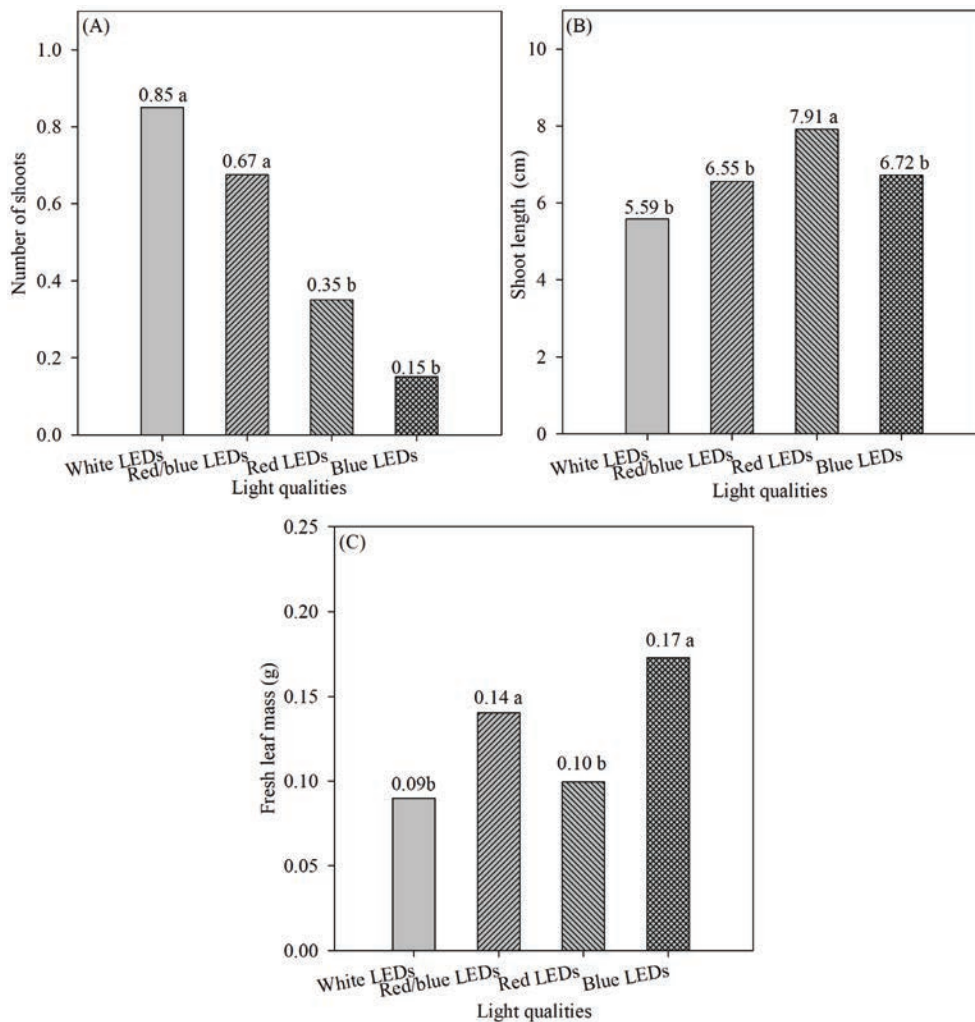


Figure 1 – Number of shoots (A), shoot length (cm) (B) and fresh leaf mass (g) (C) of *Alpinea purpurata* in different light qualities. *Equal letters do not differ from each other by the Scott-Knott test at 5% probability.

Exposure to red LEDs increased stem elongation and to blue LEDs reduced plant height (Figure 2E - H). Daud, Faizal and Geelen (2013) report the red LEDs benefits to shoot growth in *Jatropha curcas*. This fact can be attributed to the shortening of internodes and reduction of cell lengthening caused by blue light (Huché-Thélier et al., 2016; Metallo et al., 2018). As a result, alpine plants submitted to this light spectrum presented shoot length increase.

The Chl a of the plants submitted to the MS medium was superior (20.09 $\mu\text{g cm}^{-2}$) when compared to MS 1/2 (Figure 3A), being also superior for carotenoids (4.85 $\mu\text{g cm}^{-2}$) (Figure 3B) and for total chlorophyll (22.89 $\mu\text{g cm}^{-2}$) (Figure 3C). There is a relationship between chlorophyll

contents and nutrients nitrogen and magnesium are known to be constituents of the chlorophyll molecule. Considering that in the MS1/2 culture medium these nutrients are half their concentrations, this fact explaining the relationship of nutrients to the amount of chlorophylls. When properly supplemented with nutrient doses such as nitrogen, magnesium, potassium and sulfur, present higher levels of photosynthetic pigments, chlorophyll and carotenoids (Freire et al., 2013). In our work, the complete MS medium provided higher accumulation of Chl a , Chl T and CAR due to the greater availability of nutrients that are primordial in the constitution of photosynthetic pigments. However, this response is species dependent,

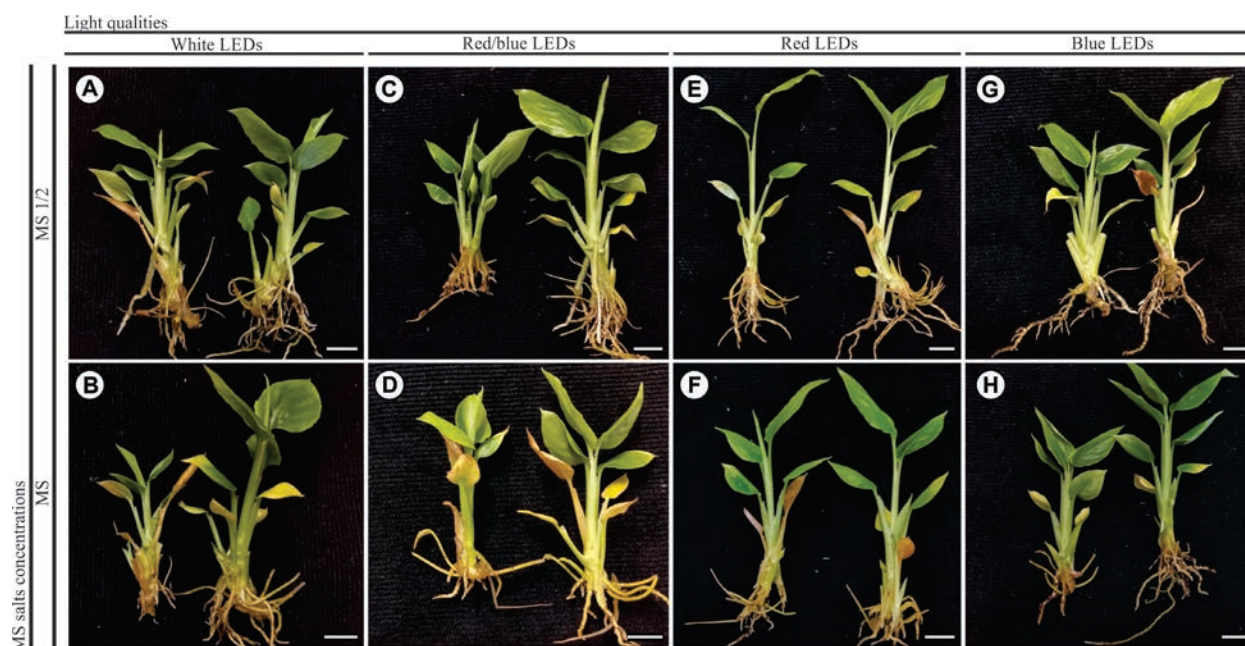


Figure 2 – *Alpinia purpurata* at 55 days *in vitro* maintained at light qualities and MS salts concentrations [white LEDs (WL) + MS 1/2 (A), white LEDs + MS (B), red/blue LEDs + MS 1/2 (C), red/blue LEDs + MS (D), red LEDs + MS 1/2 (E) and Red LEDs + MS (F) LEDs, Blue LEDs + MS 1/2 (G) Blue LEDs + MS (H)]. Bars: 1 cm.

for example, the use of half of macro and micronutrient concentrations benefited the elongation and rooting *in vitro* of banana cv. Pacovan (*Musa sp.*) (Pinheiro; Carvalho; Martins, 2018).

Plants kept under white LEDs produced a higher concentration of Chlb ($3.13 \mu\text{g cm}^{-2}$) when compared to other LEDs (Figure 3D). Similar results were obtained by Ferreira et al. (2016) with *Saccharum officinarum*. Thus, white LEDs provided greater accumulation of Chlb, since the composition of the pigments that are part of the photosynthesis process is influenced by the light quality, which also has the effect of delaying chlorophyll degradation (Darko et al., 2014; Streit et al., 2005). Thus, white LEDs favored the biosynthesis/conservation of Chlb, and for the other LEDs provided the degradation of this pigment.

For the stomatal density in the leaf abaxial epidermis, the significant difference was observed for the MS salt concentrations, in this case there was higher stomatal density in MS medium ($78.36 \text{ stomata mm}^{-2}$) when compared to MS 1/2 (Figure 3E). The reduction of stomatal density in the presence of smaller salt

concentrations in the culture medium may be a way of adapting to nutrient restriction conditions.

For acclimatization percentage, although presenting interaction between light qualities and MS salt concentrations, the significant difference was only observed for plants grown under white LEDs, in the different MS salt concentrations. MS1/2 was the one that provided the highest percentage of plant acclimatization (90%), after remaining 33 days in a protected environment (Figure 4 and 5). For the others light qualities, there were no difference between MS and MS1/2. The reduction of salts from MS medium to MS1/2 in a study with *Schomburgkia gloriosa* provided higher average growth variables, being more efficient and less expensive (Dezan et al., 2012), due to the reduction in half of the ingredients that are part of the MS medium. This may explain the greater percentage in the acclimatization of the present study, in which, even without significant difference (except for white LEDs) between MS and MS1/2, treatments with MS1/2 had a higher survival percentage (Figure 4). The success of tissue culture is influenced by the nature of culture medium used and the ability of plants to adapt to

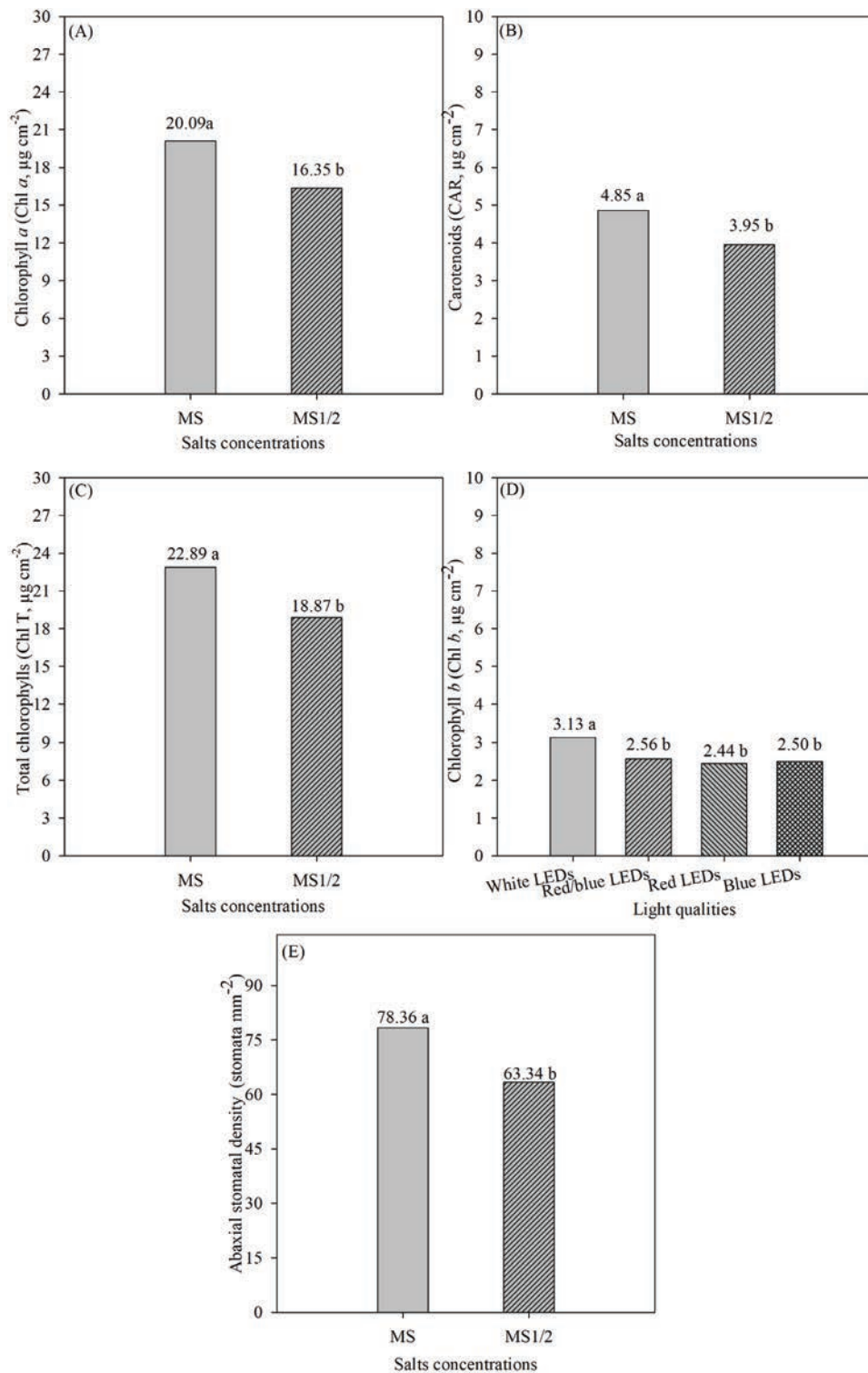


Figure 3 – Chlorophyll *a* (Chl *a*, $\mu\text{g cm}^{-2}$) (A), carotenoids (CAR, $\mu\text{g cm}^{-2}$) (B), Total chlorophyll (Chl T, $\mu\text{g cm}^{-2}$) (C), as a function of MS concentrations of the culture medium; chlorophyll *b* (Chl *b*, $\mu\text{g cm}^{-2}$) (D) as a function of light qualities; and abaxial stomatal density (stomata mm^{-2}) (E) of *Alpina purpurata*. *Equal letters do not differ from each other by the Scott-Knott test at 5% probability.

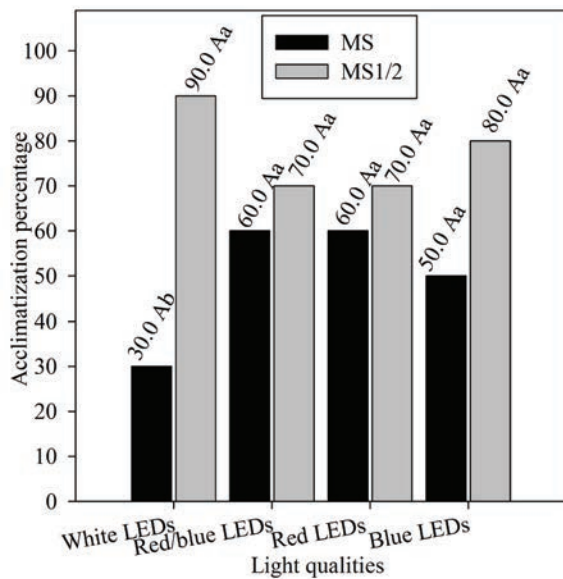


Figure 4— Acclimatization percentage (%) of *Alpinia purpurata* submitted to different MS salts concentrations (MS and MS 1/2) and different light qualities. *Lower case letters in the same culture medium do not differ in light qualities; and capital letters in the same light quality do not differ the MS salts concentrations by the Scott-Knott test at 5% probability.

natural environmental condition after the completion of *in vitro* production that is acclimatization *ex vitro* (Zahara; Datta; Boonkorkaew, 2016).

We observed that the increase of shoots length favors rooting and acclimatization, ie, when we observe the growth variables under the white LEDs, the number of shoots presented higher average.

In general, the high percentage of acclimatization observed for this species may be related to the gas exchange provided during the *in vitro* cultivation. Plant acclimatization occurs from morphophysiological modifications that can be observed in plants exposed to atmospheric-like CO₂ concentrations still *in vitro* cultivation (Batista et al., 2017); This cultivation characteristic can be provided by the presence of porous membranes to gases, which allows better exposure to CO₂ (Batista et al., 2017); providing in turn, higher shoot quality and survival, lower chlorosis, and senescence during *ex vitro* acclimatization (Nguyen; Xiao; Kozai, 2016; Pérez-Jiménez et al., 2015; Rodrigues et al., 2011; Walter; Rosa; Streeck, 2015).



Figure 5 – *Alpinia purpurata* plants from different MS salts concentrations and light qualities, maintained for 33 days under greenhouse (A and B).

CONCLUSIONS

In vitro cultivation of *Alpinia purpurata* with red LEDs and full-strength MS culture medium is indicated, as they promote efficient shoot length and photosynthetic pigment content, respectively. These conditions provided plants better morphophysiological adapted for *ex vitro* acclimatization.

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