In vitro culture of heliconia in different light sources

Cultivo *in vitro* de heliconias em diferentes fontes de luz

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ABSTRACT

The use of different light sources on *in vitro* culture, opens new perspectives for the micropropagation and the study of the effect on the explants. The aim of this study was to evaluate the effect of different combinations of LED light sources on the *in vitro* development of *Heliconia orthotricha* cuttings. Explants of the cultivar Total Eclipse were submitted to four subcultures in propagation culture medium (MS) and incubated under treatment with light sources as follows, B100: 100% blue; R100: 100% red; B70R30: 70% blue + 30% red; R70B30: 70% red + 30% blue; and the Control, with a white fluorescent lamp (WL). After the subcultures, the number of sprouts/shoots, height of seedlings, fresh mass and chlorophyll *a*, *b* and carotenoids were evaluated. Structural and ultrastructural changes were observed in the grouping of chloroplast thylakoid membranes, as well as an elongation of the hypodermal cells, and a reduction of leaf thickness in the treatment with a higher incidence of red light. The results demonstrated a reduction in the size of the cuttings produced *in vitro*, and an increase in productivity at the highest concentrations of red light incidence.

Keywords: Ornamental, micropropagation, chloroplast.

RESUMO

O uso de diferentes fontes de luz no cultivo in vitro, abre novas perspectivas para a micropropagação e o estudo do efeito nos explantes. O objetivo do presente trabalho foi avaliar diferentes combinações de fontes de luz de LED no desenvolvimento *in vitro* de mudas de *Heliconia orthotricha*. Explantes da cultivar Eclipse Total foram submetidos a quatro subcultivos em meio de cultivo de propagação (MS) e incubados sob os tratamentos de fontes de luz sendo B100: 100% azul, R100: 100% vermelho, B70R30: 70% azul + 30% vermelho, R70B30: 70% vermelho + 30% azul e o controle com lâmpada fluorescente branca (WL). Após os subcultivos foram avaliados o número de brotações, altura das plântulas, massa fresca e clorofila a, b e carotenoides. Observaram-se alterações no agrupamento dos tilacoides dos cloroplastos, bem como o alongamento das células da hipoderme, redução da espessura da lamina foliar em tratamento com maior incidência de luz vermelha. Os resultados demonstram redução de porte das mudas produzidas *in vitro* e aumento no número de brotações, nas maiores concentrações de incidência de luz vermelho.

Palavras-chave: Ornamental, micropropagação, cloroplasto.

INTRODUCTION

The genus *Heliconia* comprises a large variety of plants of interest to gardens and the cut flower market. They are tropically distributed and are present in the Central and South American regions. Heliconias have been in increasing demand on the Brazilian and International markets, with this being the crop with the highest growth rate in the ornamental plant sector "Castro et al. (2006)". The propagation of cuttings using tissue culture technique may be an efficient technique for large scale production, and for advanced studies of a crop "Modal et al. (2010)". In heliconia, Rodrigues (2005), Rodrigues et al. (2006) and Ulisses et al. (2010) have studied the *in vitro* system of propagation and acclimatization of some species of commercial interest. In *in vitro* propagation, the use of Light Emitting Diodes (LEDs) in the multiplication stage has replaced tubular fluorescent light. LEDs are efficient in the light generation process, have a long period of useful life with low heat production, and a specific wavelength "Yeh and Chung (2009) and Fang et al. (2011)". The use of LEDs in *in vitro* propagation has been evaluated in the cultivation of *Cymbidium orchids* "Huan and Tanaka, 2004"; strawberries "Rocha et al. 2010" and bananas "Duong et al. 2003" with significant results in the quality of the cuttings produced, and improved efficiency in the production process. In *Chrysanthemums*, morphological changes in leaf stomata have been observed *in vitro* under a blue LED light source (70%) associated with red (30%) that produced leaves *in vitro* with lower stomatal density when compared with fluorescent light treatment "Kim et al. (2003)". In

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the *in vitro* cultivation of sugarcane, Maluta et al. 2013 observed a reduction in the size of cuttings produced with greater intensity of red in the band from 620 to 630 nm. Furthermore, in evaluations by transmission microscopy in the leaves of seedlings, the study demonstrated changes in the grouping of chloroplast thylakoids, proportional to the concentration of the red color light source. The specific wavelength of the light source may act on some plants in the *in vitro* cultivation stage, changing the size and propagation rate. In view of the foregoing, the aim of this study was to evaluate the influence of different wavelengths provided by LEDs on the *in vitro* development of *Heliconia* cuttings.

MATERIAL AND METHODS

In the trials, *Heliconia ortotricha* cv. Total Eclipse cuttings were used at the stage of *in vitro* propagation in semisolid MS "Murashige and Skoog (1962)" culture medium, using 1.8 g L^{-1} Phytagel SIGMA®, in addition to 30 g L^{-1} of sucrose, MS vitamins and 4.0 mg L^{-1} benzylaminopurine (BAP).

The cuttings were submitted to a LED lighting system in the colors red LPEL-06R3-B (620-630 nm) and blue LPEL-06B3-B (455-475 nm) from Cromatek®. Four combinations of LED treatments were evaluated: 100% blue, (B100); 100% red, (R100); 70% blue + 30% red, (B70R30); 70% red + 30% blue, (R70B30). In the control treatment white fluorescent lamps (WL) were used, with the light intensity adjusted to 25 µmols m^2 s^{-1} in all the treatments. Fifty (50) explants were selected, per treatment, in ten flasks of 200 ml, with five explants per flask, containing 40 ml of culture medium for propagation, as previously described. The explants were propagated for four subcultures and incubated at a temperature of 25 °C ± 2 °C with a light period of 16/8 hours day/night.

The explants were evaluated on a weekly basis, with changes of culture medium of the treatments every three weeks, until the fourth subculture was completed, when the sprouts were measured, quantified and individualized, with all the data expressed by the mean number of sprouts per flask, length (cm) of explants and fresh mass (g). On completion of the measurements, samples of the leaves of sprouts obtained were collected, irrespective of their position, in different light sources (100 mg of leaf tissue), for the purpose of quantifying the contents of carotenoids and chlorophylls *a* and *b*, in acetone extract (80%). The pigments were quantified by spectrophotometry (chlorophyll a = 663 nm, chlorophyll b = 645 nm and carotenoids = 470 nm), according to Lichtenthaler (1987). Leaf tissue samples from five seedlings *in vitro* were evaluated by light and transmission electron microscopy (TEM) according to the methodology of Gratão et al., (2009). Semi-thin sections were observed and documented under a transmitted light microscope (Leica LMD 7000-Switzerland) and the ultra-thin sections were observed by transmission electron microscopy (JEOL JEM 1400 Tokyo-Japan) at 80 KV, with the images being digitized. The effect of light on the size and number of epidermal and hypodermal cells of the adaxial surface of the leaves (density per linear millimeter-cells/mm) and the thickness of the leaf blade (µm) were evaluated by using the Image J program.

The data obtained were submitted to the analysis of variance, comparing the measurements of the factor light source, for the final number of shoots per flask, fresh mass, and explant length - from the tip of the largest leaf to the end of the pseudostem - by the Duncan test at probability of 5%, and the results with reference to the chlorophylls *a* and *b* and carotenoids, number of cells, and thickness of leaves.

RESULTS AND DISCUSSION

The mean number of shoots was highest in treatment R100 that differed significantly from the control, followed by treatments B100 and R70B30 (Table 1).

In strawberry plants, Rocha et al. (2010), observed a larger number of shoots with treatments of blue, red and green lights, when compared with fluorescent light and Growlux, even at different concentrations of BAP. In the combination of 75% blue light associated with 25% red light, Huan and Tanaka (2004) reported a larger number of shoots of *Cymbidium* with this treatment compared with that of fluorescent light. In sugarcane, cultivar CTC-07, the number of shoots was higher in treatment with fluorescent light but have shown to be sensitive to the red light, with reduced production of shoots "Maluta et al. 2013". Both in sugarcane and in the present study with heliconias, the TEM studies revealed the thylakoids were shown to be sensitive to treatments with red light. The thylakoids were found to be aggregated in 3 and up to 5 bands, separated by an interband space, and distributed parallel to the larger axis of the chloroplast.

Therefore, in the treatments with red light, it was possible to find significantly alterations in the internal structures. In chloroplasts, with the breakdown of the thylakoids bands, disorganization of the thylakoid membrane, disappearance the grana stacking, malformed grain and chloroplasts and an increase in the number of starch granules, peroxisomes and mitochondria (Figure 1 B and D). Note that the control (WL) showed no internal structural differences compared to the other treatments (Figure 1 E).

Dekker and Boekema (2005) and Kiss et al. (2009) reported that this breakdown occurred due to the mechanism of compensation by the quality of incident light, and occurred when the light intensity exceeded the photosynthetic capacity of the plant. It was probably what happened to the heliconia plantlets in the *in vitro* system with only light source for red light. Contrary to the breakdown and entanglement of the thylakoids that occurred with red light, the treatment with blue light presented more grouped thylakoids (for the species studied), with less space between the bands (Figures 1 A and C). This thylakoid grouping would be a mechanism of compensation for the lack of other light sources and thus increase the photosynthetic efficiency of the plant.

The pigments evaluated showed no significant difference among the treatments. Except in B100 for chlorophyll *a*; the quantity recorded was higher than it was in the other treatments (Table 1). As previously mentioned, in this treatment the thylakoids of the chloroplasts were shown to be more grouped than they were in the other treatments. The better grouping of thylakoids is probably for the purpose of increasing photosynthetic efficiency due to the restriction of light sources and suggests that's the red light provoked important disturbances in metabolic functions and lipid composition. The blue light band is more absorbed by chlorophyll *a*, thus the increase in this pigment demonstrates a better adaptation of the photosynthetic apparatus of this species to absorb blue light more efficiently, which would explain the significant difference for this treatment "Shirley 1929; Taiz and Zieger 2006". In sugarcane, treatment exclusively with the blue light presented a similar result, with a larger quantity of chlorophyll *a* of the treatments studied "Maluta et al. 2013". The fresh mass and length of shoots were also influenced by the different light sources (Table 1). Treatment R100 presented the lowest mean fresh mass value and the treatment B70R30, showed the highest mean value for this parameter. In sugarcane, the fresh mass value was lower in the treatment with 70% blue and 30% red, and the treatment that showed the highest fresh mass value was composed of white fluorescent light "Maluta et al. 2013". The highest mean length value was observed with WL that is frequently undesirable in *in vitro* cultivation. The competition for light during the *in vitro* propagation process may result in etiolated seedlings and future losses in acclimatization "Barros et al. 2011". Size was affected by treatment with red light for the cultivar of *Heliconia ortotricha*, with the lowest result among the measurements evaluated (Table 1). Maluta et al. (2013) observed a similar result in sugarcane, and also reported accentuated reduction in size with red light. For sugarcane, they concluded that the reduction in size may favor the increase in propagation rate in the temporal immersion bioreactor (TIB) propagation system**.** In flower cultivation, the reduction in size *in vitro* induced by red light may also favor a higher propagation rate.

Table 1 – *In vitro* development of sprouts numbers, length, fresh weight, carotenoids content, chlorophyll *a* and chlorophyll *b* content from *in vitro* heliconia plants after four subcultures in different light treatments.

Light treatments	*Seedlings*	*length (cm)*	*Fresh weight (g)*	*Carotenoids, Chlorophyll (a), Chlorophyll (b) ..... (mg g^{-1}) .....*		
B100	8.80 ab	2.53 bc	2.11 b	2.13 a	1.19 a	0.21 a
R100	10.11 a	1.99 c	1.83 b	2.27 a	0.70 bc	0.22 a
B70R30	7.59 b	2.60 b	2.66 a	2.08 a	0.61 c	0.20 a
R70B30	8.88 ab	2.64 b	2.46 a	2.27 a	0.61 c	0.22 a
WL	7.76 b	4.02 a	2.64 a	2.69 a	0.80 b	0.26 a
CV (%)	*29.45*	*67.23*	*36.44*	*14.81*	*11.09*	*14.8*

Means followed by the same letter do not differ by Duncan test at 5% probability.

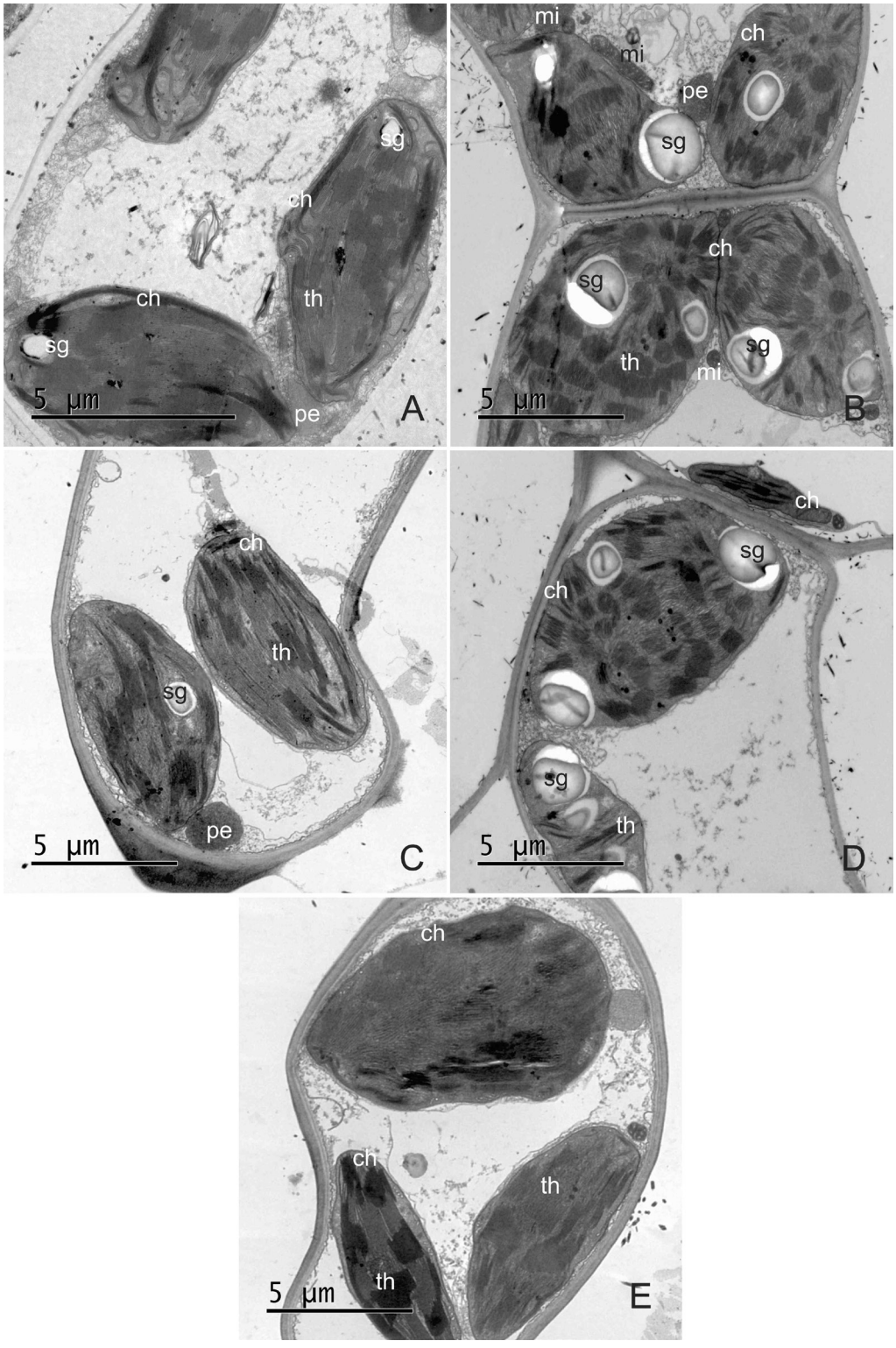

Figure 1 – Ultrastructure of mesophyll chloroplasts of leaves of *Heliconia orchotricha* in presence of different light treatments. A) B100, B) R100, C) B70R30, D) R70B30, E) WL. Note in R100 and R70B30 an increase of starch grains, and mitochondria and the distorted tilakoids. ch: chloroplast; sg=starch grain; mi=mitochondria; th: thylakoids, pe: peroxisome.

Histological analysis revealed structural changes such as the reduction in intercellular spaces and thickness of the leaf area in treatment R100 when compared with the other treatments, pointing out treatments B70R30 and R70B30 for showing the largest intercellular space in the leaf blade (Figures 2 B, C and D).

The small intercellular space in the mesophyll of leaves being thinner, and possibly more sensitive to temperature and humidity variations that may interfere in a process of acclimatization. The leaf thickness measurements proved the effect of R100 treatment as the one that showed a thinner leaf area differing significantly from that of the other treatments (Table 2).

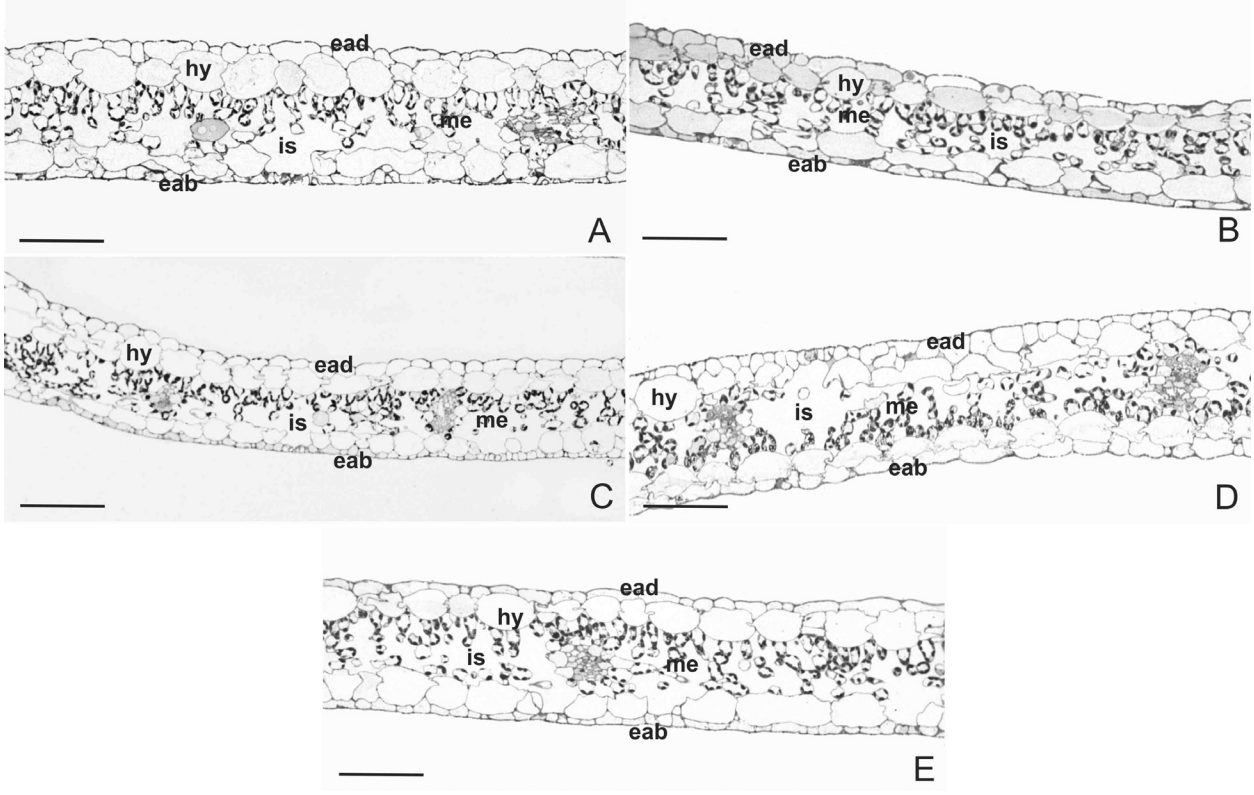

Figure 2 – Anatomical behavior of leaf cross-sections of *Heliconia orchotricha* in presence of different lights. A) B100, B) R100, C) B70R30, D) R70B30, E) WL. ead: epidermis adaxial; eab: epidermis abaxial; me: mesophyll; is: intercellular space; hy: hypodermal cell. Bars= 100µm.

Table 2 – Leaf thickness (µm), number of epidermal and hypodermal cells of the adaxial surface of the leaves (density per linear millimeter-cells/mm), from *in vitro* heliconia plants after four subcultures in different light treatments.

LEDs Treatments	Leaf Thickness (µm)	Adaxial epidermal	Adaxial hypodermal
B100	146.02b	37c	28a
R100	116.78c	48d	21b
B70R30	158.12a	59b	29a
R70B30	146.42b	63b	27a
WL	142.59b	77a	31a
CV (%)	4.12	15.69	13.5

Means followed by the same letter do not differ by Duncan test at 5% probability.

In the present trial, the combination with R70B30 presented the second highest thickness value of the leaf area *in vitro*. In Figure 2 B the hypodermal cells were shown to be more elongated when compared with the control and other treatments, in which a more rounded form of these cells was presented. In *in vitro* pineapple cultivation with ventilation and under natural light, Silva et al. (2013) found changes in leaf anatomy that provided greater thickness of the chlorophyll containing parenchyma when compared with the control treatment without ventilation and with artificial light. The elongated shape of this cells that occurred in R100 contributed to the reduction in leaf thickness in this treatment. Probably, this anatomic change would be an adaptation of the leaf to the quality of this light source, similar to the breakdown that occurred in the thylakoids of the chloroplasts as mentioned previously. The adaxial leaf epidermal and hypodermal cells showed a lower density per linear millimeter for treatment R100, with significant difference when compared with the other treatments (Table 2). When evaluating the light in the *in vitro* cultivation of birch, Saebo et al. 1995, observed a smaller leaf area in this culture under exposure to red light (640-680 nm) demonstrating the importance of the light source in the expansion of the leaf area. With blue light (410-510 nm), the authors reported a significant increase in leaf area, close to that of treatment *in vivo* that showed the greatest growth in this parameter.

CONCLUSIONS

The results presented in this trial indicate that in an *in vitro* system of seedling production, different sources of LED light may be used in the development of a crop. For the culture evaluated, the red light (620-630 nm) source was shown to be more adequate during the multiplication stage.

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