

Silver nitrate, light spectrum and gas exchange on *in vitro* flowering of *Capsicum frutescens*

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ABSTRACT

The study aimed to evaluate the influence of silver nitrate on the *in vitro* flowering of *Capsicum frutescens* cv. Cayenne associated with different light spectra and gas exchange. After 36 days of germination in MS medium, two seedlings previously germinated, were subjected to experiments in glass flasks containing two types of culture medium: I) MS culture medium; or II) Culture medium with the addition of 40 μM of AgNO_3 . The explants were maintained in a growth room with a 16-hour photoperiod, temperature of $25 \pm 2^\circ\text{C}$, light intensity ($72 \mu\text{mol m}^{-2} \text{s}^{-1}$) from different light spectra. The experiment was conducted in a completely randomized design, 4 x 2 factorial scheme, with eight replicates, and the experimental unit composed of two seedlings. After 74 days of *in vitro* cultivation, the following parameters were evaluated: number of bifurcations, number of oxidized buds, number of open buds, number of green buds, number of aborted buds, number of leaves, root length, shoot length, total fresh and dry mass of shoots, and total fresh and dry mass of root. Thus, the combination of light spectrum, addition of gas exchange and AgNO_3 favors the growth and development of *Capsicum frutescens* cv. Cayenne.

Keywords: light-emitting diodes; gas-permeable membranes; spectral quality; pepper production; tissue culture.

INTRODUCTION

The genus *Capsicum* belongs to the Solanaceae family, with about 38 described species, six of which are considered domesticated: *C. annuum*, *C. assamicum*, *C. baccatum*, *C. chinense*, *C. frutescens* and *C. pubescens*.⁽¹⁾ The crop has great economic importance for Brazil, with a planted area of about 19,070 hectares, with a production of 462,955 tons of the fruit.⁽²⁾ Because it is a species whose main form of reproduction is sexual, it is susceptible to pathogens that cause a significant reduction in the yield and quality of the final product.⁽³⁾ Considering the susceptibility of peppers, *in vitro* cultivation has been gaining space for the supervision of plant species, helping in improvement methods, among which *in vitro* flowering stands out.

In vitro flowering allows perfecting crosses for genetic improvement and development of new cultivars; however, this event is considered a complex process regulated by environmental and physiological factors, and its occurrence under these conditions is rare.⁽⁴⁾ In the conventional *in vitro* culture environment, sealed flasks with reduced gas exchange are adopted, which can lead to morphophysiological disturbances in the plants, accumulation of ethylene and other volatile gases, and reduction of CO₂, which can limit the photosynthetic process, generating physiological anomalies in the plants.^(5,6) To avoid these disturbances, natural ventilation of the internal atmosphere of the flasks can be adopted, using gas-permeable membranes capable of allowing gas exchange between the *in vitro* and *ex vitro* culture environment, promoting improvements in photosynthetic conditions, increased growth rates, reduction of problems with leaf senescence and benefits during *ex vitro* plant acclimatization.⁽⁷⁾ Indeed, gas exchange is important in the morphogenic responses of plants of the genus *Capsicum*; in *Capsicum frutescens* it reduced leaf senescence,⁽⁸⁾ and in *Capsicum annuum* it promoted the increase of biomass and *ex vitro* acclimatization.⁽⁹⁾

The flowering process can induce seedling senescence due to the accumulation of ethylene in the *in vitro* culture environment.⁽¹⁰⁾ One of the alternatives that have been used to overcome the negative effects of this hormone is the addition of anti-ethylene compounds, such as AgNO₃, to the culture medium, gas-permeable membranes, and the spectral quality with different wavelengths.⁽¹¹⁾

With advances in lighting technology, researchers began to use light-emitting diodes (LEDs) in tissue culture, due to their specificity in emitting spectral qualities, Pawlowska,⁽¹²⁾ being used to regulate the levels of pho-

tosynthetically active radiation and photomorphogenesis radiation necessary for plant growth and development.⁽¹³⁾

The light absorbed through the plant photoreceptors is the form of energy that the plant uses to carry out photosynthesis, being influenced by the red LED, blue LED and red + blue LED.⁽¹⁴⁾ As they are the main energy sources for photosynthetic CO₂ assimilation in plants.⁽¹⁵⁾ According to the intensity, wavelength and direction in which they are being emitted, they can generate a series of morphophysiological responses.⁽¹⁶⁾

The wavelengths in the red light range (660 nm) and blue light range (400-500 nm) are the main focus of current research. Due to their regulatory effects on plant development, red light is efficiently absorbed by photosynthetic pigments and stimulates phytochrome. Blue light plays important morphogenic roles, including stomatal opening, stem elongation, and phototropism.⁽¹⁷⁾

The light spectrum and the effect of gas exchange are some of the necessary factors for the success of *in vitro* cultivation. As for the effects of *in vitro* flowering associating the techniques of light qualities, use of gas-permeable membranes and different AgNO₃ concentrations, few reports are found in the literature. Therefore, the study aimed to evaluate the influence of silver nitrate on the *in vitro* flowering of *Capsicum frutescens* cv. *Cayenne* associated with different light spectra and gas exchange.

MATERIAL AND METHODS

Study location and experimental design

The study was carried out in a growth room belonging to the Aromatic Extractives laboratory at the Federal University of Santa Maria, Frederico Westphalen, Rio Grande do Sul, Brazil.

The experiment was conducted in a completely randomized design (CRD), in a 4 x 2 factorial scheme, with four spectral qualities, from light-emitting diode (LED); white LED, red LED (660 nm), blue LED (450 nm) and red+blue LED in the proportion of 60 and 40%, respectively, and two concentrations of AgNO₃ (without AgNO₃ and with 40 µM of AgNO₃), with eight repetitions, and the experimental unit consisting of two seedlings per flask.

For the variables of photosynthetic pigments, chlorophyll a, chlorophyll b, total chlorophyll and total chlorophyll/carotenoids (Chlt/Crt), CRD was performed in a 4 x 2 factorial scheme, four replications, with the experimental unit consisting of a test tube containing five leaf discs.

Plant material and growing conditions

Under laminar flow hood conditions, seeds of *Capsicum frutescens* cv. Cayenne (ISLA®) were disinfected in 70% alcohol for one minute, followed by sodium hypochlorite (NaClO) at a concentration of 0.8% active chlorine, for 15 minutes, followed by four washes in autoclaved distilled water. After disinfection, the seeds were inoculated into glass flasks (350 mL) containing 50 mL of MS culture medium⁽¹⁸⁾ plus 100 mg L⁻¹ of myo-inositol, 30 g L⁻¹ of sucrose, gelled with 6.0 g L⁻¹ of agar and pH adjusted to 5.7 ± 0.1 before autoclaving.

They were kept in a growth room with a photoperiod of 16 hours, temperature of 25 ± 2 °C, with light intensity of (72 µmol m⁻² s⁻¹) from white LED. After 30 days of germination, two seedlings with about 10 leaves each were transferred to 350 mL glass flasks containing 50 mL of two types of culture medium: I) MS medium or II) MS medium + 40 µM of AgNO₃, following the methodology of Sharma.⁽¹⁹⁾ These were added to the same reagents mentioned above and also autoclaved at 120 °C, 108 kPa, for 15 minutes and sealed with rigid metal lids with two holes of 8 mm each covered by gas-permeable membranes, following the methodology of Saldanha *et al.*⁽²⁰⁾ for making the membranes.

Variables analyzed

The evaluation was performed 74 days after *in vitro* cultivation, and the following characteristics were analyzed: number of bifurcations, number of oxidized buds, number of open buds, number of greenish buds, number of aborted buds, number of leaves, root length (cm), shoot length (cm), total fresh and dry mass of the shoot (g), total fresh and dry mass of the root (g) and photosynthetic pigments (chlorophyll a and b, total chlorophylls and carotenoids given in µg cm⁻²). For the dry mass, the material was placed in an oven with forced air ventilation and a temperature of 60 °C until it reached a constant weight, which was later measured on a precision scale.

Quantification of photosynthetic pigments

For the determination of photosynthetic pigments, chlorophyll a and b, total chlorophyll and carotenoids, five leaf discs from the second to third leaves from the apex to the base of the plants, with 5 mm in diameter each, were incubated in 5 mL of dimethyl sulfoxide (DMSO) saturated with calcium carbonate (CaCO₃), remaining in glass tubes covered with aluminum and kept in the dark for 48 hours, following the methodology of Santos.⁽²¹⁾ After this period,

the absorbance of the samples was determined in a Bel Photonics® SP 1105 spectrophotometer using a 10 mm quartz cuvette with an optical path. The wavelengths and equations for calculating the concentrations of chlorophylls a, b, total chlorophylls and carotenoids were also based on the methodology described by Wellburn.⁽²²⁾

Statistical analysis

The variables were subjected to the Shapiro-Wilk test to check the normality of the data, and because they did not follow the assumptions of normality, the variables number of bifurcations, number of oxidized buds, number of open buds, root length, shoot length, number of leaves, shoot fresh mass, root fresh mass and shoot dry mass were transformed to $\sqrt{(x + 0.5)}$.

Variables were subjected to analysis of variance and, when significant, means were compared using Tukey's test at 5% probability level. Figures are presented with untransformed means. Statistical analyses were performed using the SISVAR software.⁽²³⁾

RESULTS

In the first 21 days of establishment, green buds and fully open flowers were observed, and up to 74 days at the end of the experiment, it was possible to observe green buds, open flowers and oxidized buds, confirming that the flowering pattern does not follow a constant chronological rhythm (Figure 1).

According to the analysis of variance, the number of bifurcations showed a significant difference only for AgNO₃ concentrations, at 5% probability level by the F test, and for the number of oxidized buds there was a significant difference caused by the interaction between light spectra and AgNO₃ concentration. For number of leaves, shoot and root length, total fresh and dry mass of shoots and roots, there was a significant difference only for the AgNO₃ concentration factor.

For photosynthetic pigments, carotenoids, there was a significant difference for the interaction between light spectra and AgNO₃ concentrations. There was a significant difference for the Chl/Crt ratio caused by light spectra and AgNO₃ concentrations.

For *in vitro* flowering variables, it was observed that the addition of AgNO₃ induced a greater number of bifurcations in the plants (6.74) (Figure 2a). For the number of oxidized buds, the use of blue LED and red LED stood out (1.63 and 2.50, respectively), leading to a lower number of

oxidized buds (Figure 2b). Regarding the concentrations of AgNO_3 , the treatments without the presence of the reagent showed a smaller number of oxidized buds compared to the treatments that contained AgNO_3 (2.61 and 6.71) (Figure 2b). For the number of flowers in relation to the different light spectra, it was possible to observe that there was a greater production of flowers under blue LED and white LED (0.57 and 0.51), respectively (Figure 2c), and in the presence of AgNO_3 there was greater formation of flowers (Figure 2d).

In the presence of AgNO_3 , greater root length and shoot length (75.42 and 68.44 mm, respectively) were observed (Figure 3a). The same result was observed for fresh mass of shoots and roots (Figure 3b), number of leaves (Figure 3c), and dry mass of shoots and roots (Figure 3d).

Carotenoids, when evaluating the AgNO_3 factor decomposed within the light spectrum factor, in the absence of AgNO_3 , there was no significant difference between the spectral qualities. However, when AgNO_3 was added, it was possible to observe that plants kept under blue LED had a greater increase in carotenoids when compared to those under red + blue LED. When comparing the decomposition of spectral qualities within AgNO_3 concentrations,

there was a significant difference only for the blue LED, in which there was superiority with the addition of AgNO_3 (Figure 4a).

For the Chlt/Crt variable, there was superiority for the blue LED and red LED + blue LED, when compared to the white LED. For the AgNO_3 concentration factor, without its addition, there was a greater increase in this ratio, being significantly higher (Figure 4b).

DISCUSSION

This work describes for the first time the *in vitro* flowering development of *Capsicum frutescens* cv. Cayenne under light spectra and in the absence and presence of 40 μM of AgNO_3 when added to the culture medium, resulting in seedlings with significant differences.

Vegetative development is the stage in which the plant can develop lateral structures that allow its establishment in the middle. Reproductive development occurs in the transition from vegetative development when floral meristems are formed to flower formation.⁽²⁴⁾ Valera⁽²⁵⁾, when setting up the phenological scale of the ‘biquinho’ pepper (*Capsicum chinense*), defined that the ideal temperature for the beginning of the bifurcation and subsequent flowering



Figure 1: In vitro flowering of *Capsicum frutescens* cv. Cayenne. Normal flower at 35 days (a); internal top view of the culture flasks showing two flowers exposed and fully opened at 35 days (b); visualization of green buds at 35 days (c); flowers fully opened in vitro at 21 days (d); top view of the flower and green buds still closed at 35 days (e).

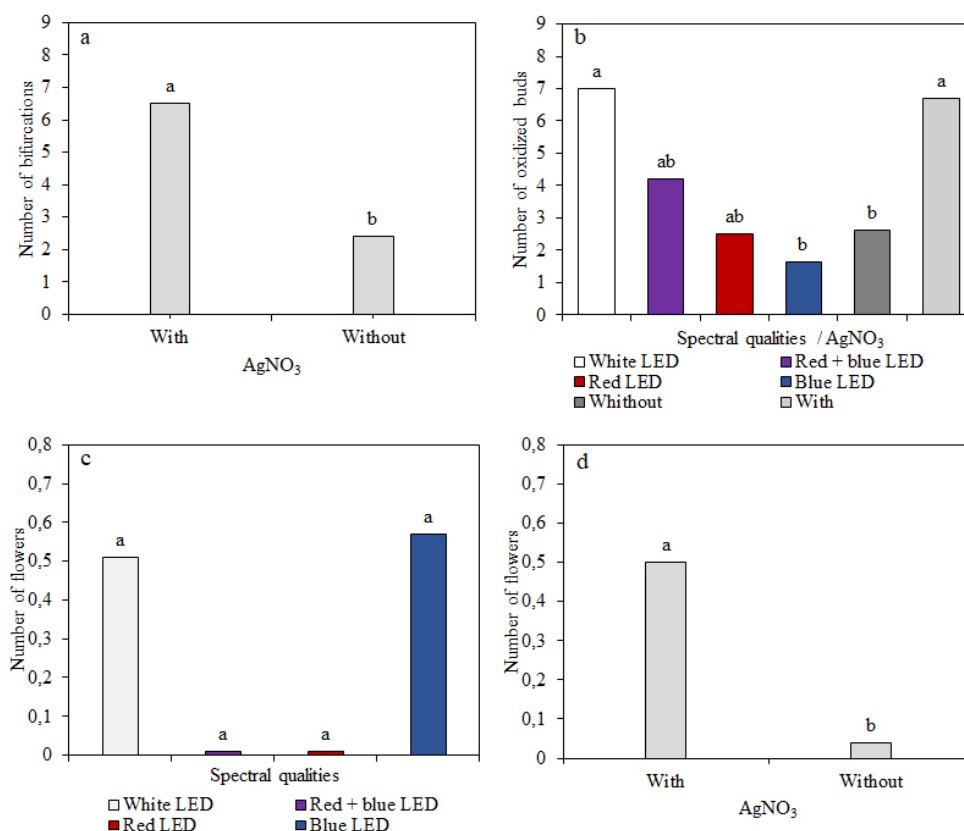


Figure 2: Flowering of *Capsicum frutescens* cv. Cayenne submitted to different spectral qualities in the presence or absence of AgNO₃. Number of bifurcations for the factor presence or absence of AgNO₃ (a); number of oxidized buds for the spectral quality factor and for the presence or absence of AgNO₃ factor (b); number of flowers for the spectral qualities factor (c); and number of flowers for the factor presence or absence of AgNO₃ (d). *Equal and lowercase letters do not differ by Tukey's test at 5% probability level.

is 24.5 °C, and this bifurcation is defined after the plant changes from a structure containing only a main stem to a second-order and third-order stem, commonly called bifurcations under field conditions.

From the moment the plant begins its bifurcation process, it will soon be ready to produce its first flowers. In the present work, when in the presence of AgNO₃, there was a greater number of bifurcations, in opposition to the treatments without AgNO₃, which favored the flowering of the plant, since the presence of new stems will lead to new floral buds (Figure 2a).

According to Silva⁽²⁶⁾, the phenomenon of *in vitro* flowering is divided into three stages: induction to flowering, floral determination and extension of floral structure. At the stage of floral determination for the flowering variable, number of oxidized buds, and light spectra, a higher number of oxidized buds occurred for seedlings under white LED and lower number for those under blue LED (Figure 1b).

For the presence or absence of AgNO₃ in MS medium, the number of oxidized buds was higher for the 40 µM

AgNO₃ concentration when compared to MS medium without AgNO₃ (Figure 1b), which contrasts with the work of Gammoudi⁽²⁷⁾, who observed the influence of ethylene on bud induction and development of eight pepper cultivars, where MS medium was added with 58.86 µM of AgNO₃, and observed that oxidation was higher in the media without AgNO₃.

De Oliveira⁽²⁸⁾ found that adding AgNO₃ at a concentration of 2.0 µmol L⁻¹ to the culture medium favored the emergence and permanence of green leaves in explants of *Annona glabra* L.⁽²⁹⁾, were able to improve the *in vitro* flowering protocol to achieve a maximum of seven flowers per plant, using 40 µM of AgNO₃ in *C. frutescens*. In another study with ten different cultivars of the *Capsicum* genus, Haque⁽³⁰⁾ observed on average 15 flowers per plant, also in the presence of AgNO₃, which is higher than the values reported in previous studies. In the present study, it was possible to reach a maximum of four flowers per plant at 72 days of cultivation in the presence of the 40 µM concentration of AgNO₃ under the white LED

spectral quality. The result achieved can be attributed to the constant presence of ethylene in the flasks of the light spectra, which influenced the *in vitro* flowering of the species under study. Similar data were observed during *in vitro* flowering studies in different genotypes of pea (*Pisum sativum*), where accumulated ethylene prevented the growth and development of flower buds ready for flowering.⁽³¹⁾

In the present study, it was observed that in culture medium with 40 μM AgNO_3 *Capsicum frutescens* seedlings responded better to flowering (Figure 1d) and, regarding the number of flowers in the different light spectra, there was a better response under the blue and white LED in MS medium plus 40 μM AgNO_3 (Figure 1c).

About the use of auxins and cytokinins combined with AgNO_3 for plant regeneration, Venkatachalam *et al.*⁽³²⁾, when working with *Prosopis cineraria* plants, verified the increase in the number of shoots at the concentration of 0.59 $\mu\text{mol L}^{-1}$ of AgNO_3 , which consequently influences the emergence of new leaves, which agrees with

the results found in the present study, in which, in the presence of 40 μM of AgNO_3 , the number of leaves tends to increase when compared to plants not subjected to the medium without AgNO_3 (19.536 and 5.628, respectively) (Figure 3b). Moreover, it was favorable for root and shoot length (75.416 and 68.437 cm, respectively) (Figure 3a).

The growth variables are influenced by the presence of AgNO_3 . The fresh mass of the shoot and root, in the presence of AgNO_3 , obtained superior results compared to the absence of AgNO_3 (Figure 3c). High values of shoot and root dry mass (0.292 g and 0.151 g, respectively) were observed (Figure 3d). Kumar⁽³³⁾, found contrasting results in a study with cotton (*Gossypium hirsutum*) culture, in which from cotyledonary nodes inoculated in culture medium with the addition of 11.77 μM of AgNO_3 and under gas exchange conditions, there was a reduction in seedling shoot dry mass, which was possibly influenced by the levels of Ag^+ ions, which can elevate ethylene production⁽³⁴⁾, and consequently have detrimental effects on seedling development.

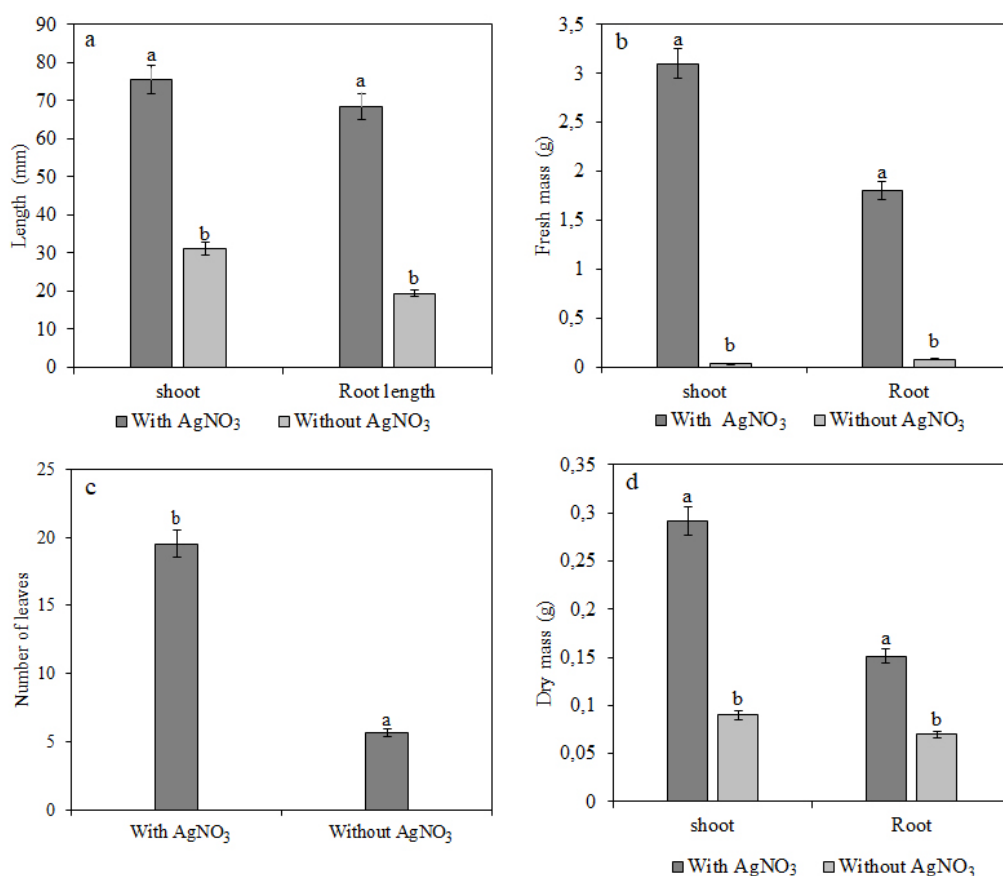


Figure 3: Growth of peppers *Capsicum frutescens* cv. Cayenne subjected to different spectral qualities and in the presence (40 μM) or absence of AgNO_3 . Shoot length and root length for spectral qualities factor (a); shoot fresh mass and root fresh mass (b); number of leaves (c); and shoot dry mass and root dry mass (d) all for factor and in the presence or absence of AgNO_3 . Lowercase letters for do not differ from each other by Tukey's test, at 5% probability level.

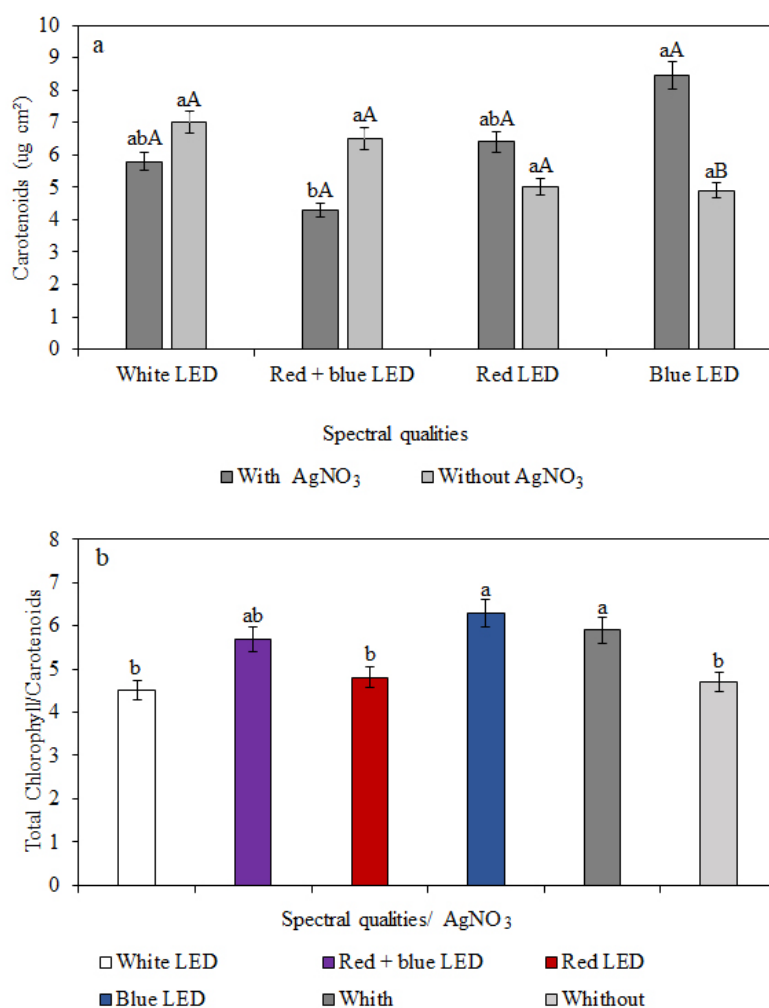


Figure 4: Photosynthetic pigments from peppers *Capsicum frutescens* cv. Cayenne in vitro subjected to different spectral qualities, in the presence or absence of AgNO₃. Carotenoids under different spectral qualities and in the presence (40 µM) or absence of AgNO₃ (a); Total chlorophyll/carotenoids under different spectral qualities and in the presence or absence of AgNO₃ (b). Equal small letters indicate no difference between the qualities of light at each concentration of AgNO₃ (a and b); Equal capital letters in the same quality of light indicate no difference between the concentrations of AgNO₃ by the Tukey test, at 5% probability level.

The increase in carotenoids in seedlings grown under AgNO₃ concentration was higher than in those grown without the addition and in the presence of blue LED (8.44 µg cm⁻²) (Figure 4a). Carotenoids can limit the damage caused to membranes due to excess light, as they are free radical scavengers and can efficiently dissipate energy causing chlorophyll excitation, i.e., they are able to increase the photosynthetic efficiency of the plant.⁽³⁵⁾

The Blue light is also necessary to promote the opening of stomata, improve access to CO₂, transpiration and nutrient uptake.⁽³⁶⁾ For Chl/Crt, under the presence of AgNO₃, higher values were observed, indicating that seedlings under these conditions become more tolerant to possible environmental stresses, and without silver nitrate this ratio is higher (Figure 4b).

The light source provided by blue LED and red LED and their associating with the culture medium in the addition of 40 µM of AgNO₃ decreased the number of oxidized buds, promoting a greater number of green buds. Plant responses are triggered by changes in light intensity, direction, and quality, and are driven by specific photoreceptors.⁽³⁷⁾ Thus, the use of spectral qualities and AgNO₃ can be an alternative in the *in vitro* production of *Capsicum frutescens*, seeking healthy seedlings, but more studies with different concentrations of AgNO₃ associated with spectral qualities are needed, in order to find a protocol that is efficient for the production of the crop.

CONCLUSION

The use of AgNO₃ resulted in greater flower production in *Capsicum frutescens* cv. Cayenne. The growth and de-

velopment of the seedlings cultivated under white LED and blue LED, both with the presence of 40µM silver nitrate in the culture medium, were superior to the others, so their use is suggested for *in vitro* cultivation.

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CONFLICT OF INTERESTS

There is no conflict of interests in carrying this research and publishing this manuscript.

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