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Original Paper

Influence of macronutrients, sucrose and LED on *in vitro* culture of *Lomatozona artemisiifolia* (Asteraceae - Eupatorieae)

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Abstract

Balanced levels of macronutrients and sucrose may ensure the success of micropropagation of the endangered ones. This study aimed to evaluate the effect of levels of salts in the culture medium on *in vitro* culture of *Lomatozona artemisiifolia*, as well as to determine the influence of light emitting diode (LED) on the shoot proliferation and rooting of the species. Nodal segments were used to evaluate the different macronutrient concentrations of MS medium (25, 50 and 100%), as well as sucrose concentrations (0.0 mM; 0.34 mM; 0.68 mM and 1.03 mM). Five light conditions were evaluated at shoots proliferation and rooting [100% blue (455 nm); 100% red (630 nm); 30% blue + 70% red; 30% red + 70% blue or fluorescent white]. Low levels of macronutrients in MS (50% and 25%) and sucrose (0.00 mM and 0.34 mM) resulted in plants with higher height, number of shoots and higher production of photosynthetic pigments. The 100% red light promoted rooting of 100% of the plants, and in 100% red or 70% red + 30% blue, higher plants were observed. These results demonstrate that under *in vitro* conditions, *L. artemisiifolia* has low nutritional needs, typical of plants that live in rupestrian fields.

Key words: culture medium, micropropagation, rock plants, spectral light, sucrose concentration.

Resumo

Níveis balanceados de macronutrientes e sacarose podem garantir o sucesso na micropropagação de espécies em perigo de extinção. Este estudo teve como objetivo avaliar o efeito de níveis de sais no meio de cultura no cultivo *in vitro* de *Lomatozona artemisiifolia*, além de determinar a influência de diodos emissores de luz (LED) na proliferação de brotos e enraizamento da espécie. Segmentos nodais foram utilizados para avaliar as diferentes concentrações de macronutrientes do meio MS (25, 50 e 100%), bem como de concentrações de sacarose (0,0 mM; 0,34 mM; 0,68 mM e 1,03 mM). Cinco condições luminosas foram avaliadas na proliferação de brotos e enraizamento [100% azul (455 nm), 100% vermelho (630 nm), 30% azul + 70% vermelho, 30% vermelho + 70% azul ou branco fluorescente]. Baixos níveis de macronutrientes no meio MS (50% e 25%) e sacarose (0,00 mM e 0,34 mM) resultaram plantas com maior altura, número de brotos e número de folhas, além de maior produção de pigmentos fotossintéticos. Observou-se que 100% de luz vermelha promoveu enraizamento de 100% das plantas, e em 100% vermelho ou 70% vermelho + 30% azul, observou-se médias mais elevadas em altura de plantas. Esses resultados demonstram que, em condições *in vitro*, *L. artemisiifolia* apresenta baixas necessidades nutricionais, típicas de plantas que vivem em campos rupestres.

Palavras-chave: meio de cultura, micropropagação, plantas rupestres, espectros de luz, concentração de sacarose.

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Introduction

The Cerrado domain has faced the degradation of its plant resources in several ways (Brandão *et al.* 2017). Currently, the list of endangered species in this domain is significant due to the constant deforestation (Nakajima *et al.* 2012), to the competition among invasive species with native species and incorrect management of fire clearing intensifies burning. In addition, it may often decrease the productive capacity of an ecosystem (Martinelli *et al.* 2014), therefore, giving rise to a threat to the biodiversity of species, especially endangered species, which may disappear.

Lomatozona artemisiifolia Baker is described as an endangered species (Martinelli & Moraes 2013). This plant has probable medicinal potential with insecticidal action (Moreira 2018), due to the large amount of trichomes, which may indicates the secretion of metabolites (Trindade *et al.* 2014). The species is characterized as a subshrub (30 to 60 cm), which is traditionally propagated by seeds, which have a low germination rate (20–50%) (Moreira 2018). The occurrence is restricted to the state of Goiás (Brazil), recorded only in the regions of Serranópolis, Mossâmedes and Jataí (Nakajima *et al.* 2012). This Asteraceae usually grows in regions of rupestrian Cerrado (CNCFlora 2012), which does not have many information records (Nakajima *et al.* 2012).

The Cerrado is the Brazilian phytogeographic domain that concentrates the largest number of Asteraceae endangered species (Nakajima *et al.* 2012), in addition to this domain having a large number of rock species from this family (Almeida *et al.* 2014). Because the Cerrado has been subjected to intense pressures in recent years regarding its deforestation (Brandão *et al.* 2017), large-scale propagation studies of the species are necessary. Tissue culture has useful methods for large-scale plant propagation. With this set of techniques, it is possible to obtain plantlets much faster than in conventional propagation (Butt *et al.* 2015). Studies on micropropagation of several species of the Asteraceae family have been developed (Abraham & Thomas 2018), such as *Artemisia lacinata* Willd. (an endangered species) (Kodym *et al.* 2018); *Calendula marítima* Guss. (a rock plant) (Angela *et al.* 2016) and *Saussurea lappa* (Decne.) Sch.Bip. (an endangered and medicinal plant) (Nisa 2018). However, few studies cover nutritional characteristics of the culture medium for rock species (Oliveira *et al.* 2015).

Several factors may influence *in vitro* shoot proliferation, such as culture medium components (Faria *et al.* 2004), light wavelength (Massa *et al.* 2008), temperature (Pereira-Netto 2001), among others (Espinosa-Leal *et al.* 2018). In addition, balanced levels of sucrose may interfere with the morphogenesis process. This carbohydrate is largely used in tissue culture as a carbon source to provide energy for explants (Paiva Neto & Otoni 2003), however, their optimal levels may differ from one species to another (Faria *et al.* 2004; Sorace *et al.* 2008). Therefore, balanced levels of sucrose in *in vitro* cultivation may promote an increase in shoots as well as in number of leaves and pigment content (Faria *et al.* 2004), which enhances the quality of the produced plantlets. Balanced levels of MS culture medium macronutrient concentrations (Murashige & Skoog 1962) may also determine the success in micropropagation (Terrer & Tomás 2001), given that different species have their own nutritional requirements.

Light Emitting Diode (LED) have also been used in tissue culture to improve plantlet quality, as well as accelerate plant growth and improve rooting, which may be reflected in the high survival of plantlets in the acclimatization (Massaro *et al.* 2018). LED light can act similarly to plant growth regulators (PGR) (Folta & Childers 2008). Thus, it is may be used both to shoot proliferation (Massa *et al.* 2008) and to rooting *in vitro* (Folta & Childers 2008) through the modulation between light and phytochromes and cryptochromes (Briggs & Olney 2001).

These advantages are due to the fact that LED lights are considered PAR (Photosynthetically Active Radiation) lights (Gupta & Jatothu 2013), which guarantee photosynthetic efficiency. This process reflects in photo-morphogenesis which promotes desirable characteristics in the produced plantlets (Stutte 2009; Yeh & Chung 2009; Silva *et al.* 2016).

Thus, by considering the increasing deforestation in the Cerrado domain and the fact that *L. artemisiifolia* is an endangered species, *in vitro* multiplication protocols become necessary for this species. Therefore, the objective of this work was to develop a *L. artemisiifolia* micropropagation protocol by evaluating the influence of different sucrose and macronutrient concentrations in the medium, as well as the influence of different spectral light (LED) on the shoots proliferation and rooting, aiming further studies of acclimatization and population reinforcement with the plantlets produced.

Material and Methods

Collection of plant material

The experiments were performed at the Plant Tissue Culture Laboratory (LabCulTive) of the Universidade Federal de Goiás (UFG), in Goiânia, state of Goias, Brazil. The determination of the botanical material was performed and the registration exsiccate with registration no. 4253 is deposited in the Herbarium of UFG. Seeds were collected in the "Professor José Ângelo Rizzo" Biological reserve, Parque Estadual da Serra Dourada, Mossâmedes, state of Goiás in July, 2017. The seeds were placed in paper bags in glass jars at room temperature (25ºC ± 2).

In vitro establishment

Two hundred *Lomatozona artemisiifolia* seeds (from 3 plant matrices) were placed in a nylon sachet-like package (5 × 7 cm). These seeds were placed in an autoclaved bottle together with 20 drops of neutral detergent, and washed in running water for 20 min. After that, asepsis was done with immersion in 70% alcohol solution for one minute, followed by immersion in sodium hypochlorite (2.5% active chlorine) for 10 min. Under aseptic conditions, the seeds were triple washed in autoclaved and distilled water and inoculated into 200 mL glass flasks containing 30 mL MS medium (Murashige & Skoog 1962) with half the macronutrient concentration supplemented with 0.7% agar (w/v), and 1.03 mM sucrose.

A natural ventilation system was used, with 0.8 mm opening polypropylene cap, covered with two layers of 0.45 µm micro-porous tape (Micropore Cremer AVS-0.45 air vent) and one layer of polytetrafluoroethylene (PTFE) tape between them (Saldanha *et al.* 2012). After closure of the flasks, they were sealed with three layers of PVC film.

The flasks containing the explants were kept in a growth room at 25 ± 2 ºC, and photoperiod of 16 hours of light and 8 hours of dark with fluorescent white light under 25 µmol.m^{-2}.s^{-1} irradiance. After 15 days, plantlets with two complete leaf pairs were transferred to flasks with MS medium. After 60 days, the 200 plantlets were assigned to the shoot proliferation experiments.

Influence of different macronutrient and sucrose concentrations

Under aseptic conditions, leaves and roots of *L. artemisiifolia* plantlets were removed. The 144 stem segments were excised at approximately 10 mm in length, containing two axillary buds. They were inoculated in 200 ml flasks containing MS medium supplemented with 7% agar (w/v) and their respective treatments, which consisted of different concentrations of MS medium macronutrients (Murashige & Skoog 1962) [25% (1/4 MS - MS basal medium with macronutrients at quarter strength); 50% (1/2 MS, MS basal medium with macronutrients at half strength); and 100% (MS basal medium with macronutrients at full strength) (w/v)], combined at different sucrose concentrations (0.00 mM; 0.34 mM; 0.68 mM and 1.03 mM). The *in vitro* cultures were kept in a growth room under the same conditions as the previous item.

The evaluation was carried out after 60 days of cultivation. Under aseptic conditions, the shoots were removed from the flasks and washed in distilled and autoclaved water. The following were evaluated: shoot height; number of shoots and number of leaves. The experimental design was completely randomized, in 3×4 factorial design (three levels of macronutrient concentration × four sucrose levels), resulting in twelve treatments and thirty replications (per treatment). One experimental unit consisted of one bottle containing one nodal segment.

Light-Emitting Diodes on shoot proliferation and rooting

Under aseptic conditions, the leaves, shoot apex and roots of the explants were removed. Nodal segments measuring 10 mm were inoculated in MS culture medium (25% macronutrients) and their respective treatments, characterized by different light conditions: 100% blue; (455 nm); 100% red (660 nm); 70% red + 30% blue; 70% blue + 30% red; and control with white fluorescent light bulb (Osram® brand, 25 µmol.m^{-2}.s^{-1}). The Tecnal® LED system was used, which consists of four LED row shelves (196 red and 196 blue), containing a panel with photoperiod control. The explants were grown in growth room under the same conditions as the previous item. After 45 days, the shoots formed were evaluated, observing shoot height, number of shoots and presence or absence of roots. The experimental design was completely randomized with five treatments and twenty replicates.

Pigment contents

Quantification of chlorophyll and carotenoid content in relation to different sucrose and

macronutrient levels of the MS medium was done using the Lichtenthaler protocol (Lichtenthaler 1987). The experiment was performed in triplicate. Thus, 150 mg of leaves from each treatment of the previous item were fragmented. Then, they were transferred into 2-mL plastic micro-tubes and addition of 1.5 mL of acetone. The micro-tubes were kept on a rotary shaker for 24 h at low temperature (8 ºC). After that, the cetonic solutions were removed and their absorbance was determined through a spectrophotometer. Calculations of the concentrations of chlorophyll a (Ca), chlorophyll b (Cb) and carotenoids (C) were performed and expressed in µg per ml extract.

Statistical analysis

Data were submitted to analysis of variance (Anova), and the means submitted to Tukey test with 95% probability. Data that did not meet Anova's assumptions were submitted to Box-Cox transformation. The program used for data analysis was Minitab (Minitab 2010) and GraphPad Prism (Radushev 2007) was used for the graphs.

Results and Discussion

Influence of different macronutrient and sucrose concentrations

Treatment with 50% of macronutrient concentration associated with 0.34 mM sucrose obtained the highest mean in shoot height (20.53 mm) (Fig. 1a) compared to 100% MS macronutrients combined with 1.03 mM sucrose, corresponding to an increase of 351.21% (Tab. 1). In relation to number of shoots and number of leaves, the treatment with 25% macronutrients combined with 0.00 mM sucrose promoted an increase of 220,61% and 152,09% (respectively) in relation to 100% MS macronutrients combined with 1.03 mM sucrose (Fig. 1b). We observed that the treatment with 100% macronutrients combined with 1.03 mM sucrose obtained the lowest averages, therefore, suggesting a likely toxicity due to excess of salts in the culture medium. Further studies should be performed to confirm such a toxicity relationship.

The development of cells and tissues in *in vitro* plant culture requires balancing between PGRs, organic substances and mineral nutrients. In a study involving *Dendranthema grandiflora* Tzevele cv. Rage (Asteraceae) the concentration of 75% macronutrients was ideal for the species, characterizing an increase in the number of shoots and roots in this concentration. However, the authors describe a decrease in the number of roots at 100% macronutrient concentration, which may have caused toxicity due to the high salt concentration (Borges *et al.* 2011).

Lower macronutrient concentrations (from 50% to 25%) associated with low sucrose

Figure 1 – a-b. Effect of different concentrations of sucrose combined with different concentrations of macronutrients on the micropropagation of *Lomatozona artemisiifolia* – a. 50% macronutrients + 0.34 mM sucrose; b. 25% macronutrients and no sucrose. Scale bar = 60 mm.

Table 1 – Means of the variables analyzed for *Lomatozona artemisiifolia* micropropagation in relation to the percentage of MS medium macronutrients combined with different sucrose concentrations in the MS medium.

Macro MS (%)	Sucrose (mM)	Height (mm)	Shoots	Leaves
25	0	11.47abc	4.2a	11.47a
25	1	11.8abc	3.77ab	11.8ab
25	2	14.55abc	2.93abc	14.55bcd
25	3	14.38ab	2.14abc	14.38bcd
50	0	10.13abcd	2.23abc	10.13bcd
50	1	20.53a	3.13ab	20.53abc
50	2	12.26abc	3.13ab	12.26bc
50	3	6.65cd	1.72cd	6.65de
100	0	7.07bcd	2.97abc	7.07bcd
100	1	8.27bcd	3.23abc	8.27bc
100	2	9.48bcd	2.35bcd	9.48cd
100	3	4.55d	1.31d	4.55e

* Means that do not share a letter are significantly different by the test of Tukey with 95% probability.

concentrations (from 0.00 mM to 0.68 mM) produced better results for the variables analyzed for *L. artemisiifolia* propagation (Fig. 2). Probably, this relationship is explained by the fact that plant communities that grow in rupestrian field environments have lower nutritional requirements than plants that live in fertile soil environments (Oliveira *et al.* 2015).

Rupestrian fields are known for their nutrient-poor soils, and despite their recognition as a global biodiversity hotspot, little is known about the diversity of phosphorus acquisition strategies and other aspects of plant mineral nutrition in this region (Oliveira *et al.* 2015). These plants have specialized roots that act on nutrient absorption. The roots secrete acidic substances (for example, carboxylates), which degrade the rock to which they are attached, and thus obtain necessary macronutrients for their nutrition, such as phosphorus (Teodoro *et al.* 2019). Therefore, further studies involving this macronutrient may be carried out to clarify the relationship between *L. artemisiifolia* and phosphorus.

A significant increase was found in shoot proliferation when sucrose was not used (Fig. 2). Carbon is known to act in the culture medium as a way of supplying the plant for energy needs (Durand *et al.* 2016). However, as stated earlier, *L. artemisiifolia* is a species that grows in rupestrian field environments, which have nutrient scarcity (Oliveira *et al.* 2015).

Although this experiment had been developed in natural ventilation system (Saldanha *et al.* 2012), combined with fluorescent white light, probably, plants have approached a condition similar to photoautotrophic, in which the plant can produce energy using the available light source without the addition of an external carbon source (Nguyen *et al.* 2016).

White fluorescent lights have continuous spectrum (Lister *et al.* 2004), over which shiny lines overlap. These are caused by mercury and phosphorus vapor, which generates emission peaks at 450 nm and 550 nm. (Onorato *et al.* 2016). For many species, this luminous condition is sufficient for shoot proliferation, as in sugarcane (Silva *et al.* 2016). Thus, further studies should be performed using bioreactors to confirm the photoautotrophic condition in fluorescent light.

The treatment corresponding to 25% macronutrients combined with 0.00 mM sucrose achieved higher means for number of leaves (Tab. 1). This variation occurred probably since *L. artemisiifolia* is considered an undomesticated allogamous species, which explains the wide variation. Thus, the relationship between macronutrients, sucrose and number of leaves of *L. artemisiifolia* should be further investigated. In a study involving *Gerbera hybrida* (Asteraceae), the number of leaves was also affected by the excess of macronutrients, especially the NH_4^+ K^+ ions (Niedz *et al.* 2014).

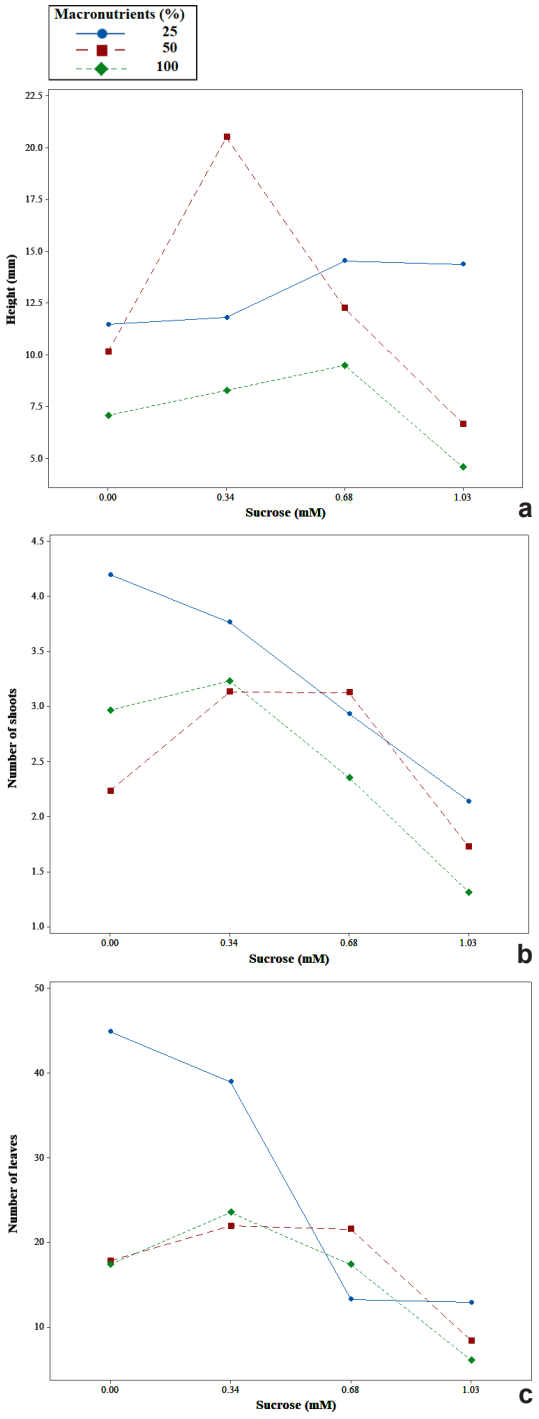

Figure 2 – a-c. *Lomatozona artemisiifolia* propagation in relation to different levels of the MS medium macronutrients combined with different sucrose levels – a. height of the shoots; b. number of shoots; c. number of leaves.

Light-Emitting Diodes on shoot proliferation and rooting

Higher percentages of red LEDs (100% red and 70% red + 30% blue) resulted in higher shoot height, with averages of 37.5 mm and 37.43 mm respectively. 100% red light also provided more plants with roots, with 100% rooted plantlets; however, there was no significant difference between the number of shoots and the evaluated light condition (Fig. 3). 100% of the rooted plantlets of *L. artemisiifolia* survived after acclimatization, which was carried out using vermiculite substrate, in a greenhouse with controlled temperature, irrigation and humidity [27 ºC ± 2, sprinkling for 5 min (3 times a day) and 70% humidity].

Red light plays a fundamental role in modulating plant morphogenetic responses. This relationship occurs in various ways, among which, stretching of the hypocotyl and facilitation of rooting (Demotes-Mainard *et al.* 2016). The formation of roots under red light irradiation occurs through the action of phytochromes, which are found in the leaf and modulate the morphogenetic responses through the capture of red or far-red light (Smith 2000). In *Calendula officinalis* L. (Asteraceae), the red-light led to an early flowering and improved growth compared with white light-grown plants. In addition, the pigment content has increased considerably (Aliniaeifard *et al.* 2018). Thus, further studies may be carried out to confirm the potential for increased chlorophyll under LED irradiance.

Lower means of plant height and number of rooted plants were observed when 100% blue light were used. The wavelength of blue light is responsible for the activation of cryptochromes (Pedmale *et al.* 2016). In *Arabidopsis*, blue light plays a key role in inhibiting etiolation of hypocotyl by influencing the expression of genes linked to the production of auxins and gibberellins. Thus, plants gain short height and consequently rooting is impaired by inhibition of auxins (Folta *et al.* 2003). However, further studies may be conducted to prove the relationship between blue light and chlorophyll production in *L. artemisiifolia*.

No difference was observed between light conditions and number of shoots. However, this relationship was determined in this work when we found a high shoot proliferation with low percentages of macronutrients and sucrose levels. Also, in the shoot proliferation of *Campomanesia rufa* (Berg) Niedenzu, the red and blue LED

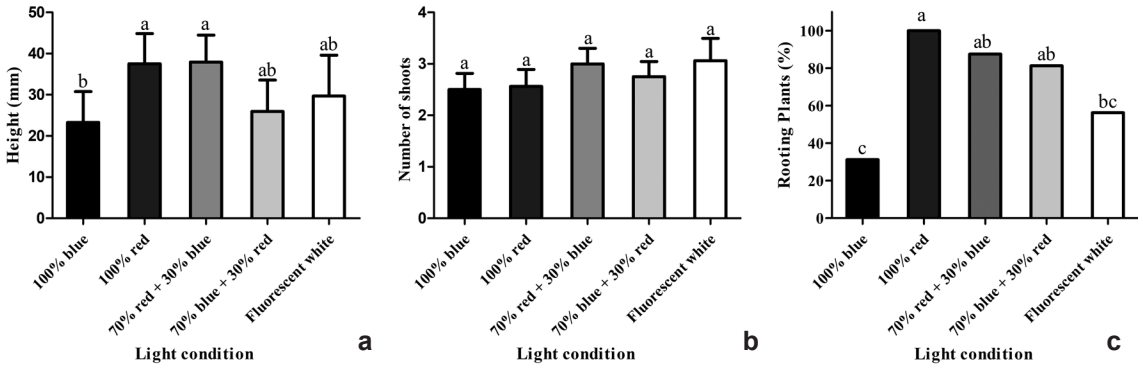

Figure 3 – a-c. Response of *Lomatozona artemiziifolia* to the different light conditions – a. height of plants; b. number of shoots; c. percentage of rooted plants. Bars represent the respective standard errors.

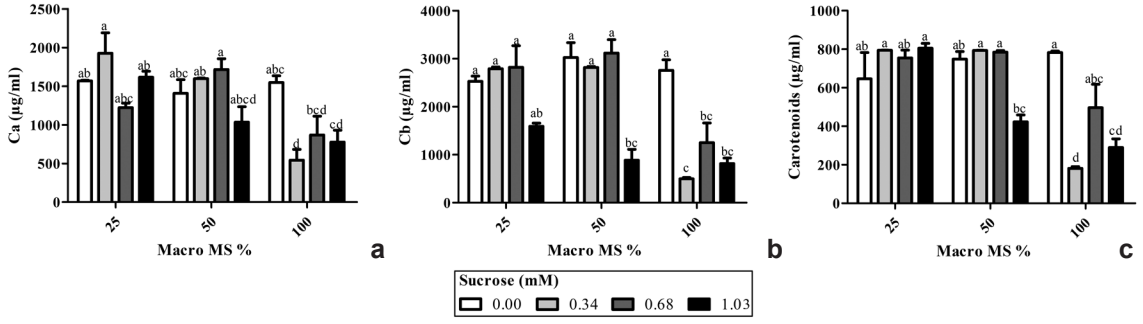

Figure 4 – a-c. Production of chlorophyll *a* (a), *b* (b) and carotenoids (c) in *Lomatozona artemisiifolia* micropropagation using different percentages of macronutrients in MS medium combined with different sucrose levels in culture medium. Bars represent the respective standard errors.

light combinations did not differ from the control (conventional white light) (Sant'Ana *et al.* 2018). On the other hand, other studies demonstrate that blue light may act as a cytokine-like growth regulator, inhibiting hypocotyl growth and increasing shoot proliferation (Vandenbussche *et al.* 2007; Pedmale *et al.* 2016). In addition, blue light may also produce distinct results in combination with plant growth regulators found in the culture medium (Vandenbussche *et al.* 2007). For this reason, further studies should be conducted using new blue light combinations associated with plant growth regulators to evidence this relationship.

Pigments contents

The best treatments for chlorophyll *a* production were 25% macronutrients combined with 0.34 mM sucrose and 50% macronutrients combined with 0.68 mM sucrose (Fig. 4). In general, higher sucrose levels associated with high macronutrient levels were detrimental to pigment production, including carotenoids, in which a decrease in production was observed in treatments with 100% macronutrients (Fig. 4). In a study carried out with *Calendula officinalis* L., it was observed that *in vitro* carotenoid production was optimized by modifying the concentration of macronutrients in the culture medium and enhancing to sucrose concentration (Legha *et al.* 2012).

In relation to plant tissue culture, the most commonly used carbon source in MS medium is sucrose at a concentration of 3% (1.02 mM) (Shahzad *et al.* 2017). In a study with *Chrysanthemum leiophyllum* (Asteraceae), the authors found that the excess of salts promoted a decrease in the number of shoots, as well as in the other growth characteristics (Kuritskaya *et al.* 2016).

Proliferation of *Lomatozona artemisiifolia* shoots was successfully performed using MS

culture medium with 25% macronutrients combined with 0.00 mM sucrose, with an average of 4 shoots per explant.

The decrease in Macronutrient and sucrose concentrations influenced positively the pigment contents and concentrations of 100% macronutrients and the increase of sucrose level impaired their production.

The best rooting condition for *L. artemisiifolia* shoots was under 100% red LED, which promoted 100% rooted shoots. High percentages of red lights promoted higher plantlets. However, the different light conditions did not influence the shoot proliferation.

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