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IN VITRO PHOTOAUTOTROPHIC CULTIVATION OF *Melocactus zehntneri*: INSIGHTS FROM ALLOMETRIC CHARACTERISTICS FROM GERMINATION TO ACCLIMATIZATION

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Abstract

Melocactus zehntneri is a species of ornamental relevance suffering anthropogenic pressures, putting its preservation at risk. Thus, alternatives for propagating and conserving this species are needed. This study evaluated the effect of photoautotrophic cultivation of *M. zehntneri* to produce plants well acclimated to natural conditions. The seeds underwent different imbibition times before in vitro germination. Besides the growth room, a greenhouse hosted the photoautotrophic cultivation of seedlings. Vermiculite replaced agar in the culture medium. The imbibition pre-treatment in distilled water and the growth environments affected some parameters linked to germination. The greenhouse provided better characteristics for plants grown in vitro and for acclimatization than those kept in the growth room. Plants cultivated and acclimated ex vitro in a greenhouse photoautotrophic environment showed improved characteristics regarding ornamental significance.

Keywords: Cactus. Light quality. Tissue culture.

1. Introduction

Genus *Melocactus* species (*Cactaceae* family) exhibit a round-shaped cladode and a terminal cephalium with pink or red flowers. Such characteristics add high ornamental relevance to these plants (Silva et al. 2011; Pérez-Molphe-Balch et al. 2015; Lima-Nascimento et al. 2019). However, removing individuals from nature in an extractive manner for commercial exploitation has been drastically reducing natural populations (Goettsch et al. 2015). Also, this genus features exclusively sexual reproduction (Coelho et al. 2015) and slow growth, with individuals taking approximately ten years to reach the reproductive phase (Machado 2009; Pérez-Molphe-Balch et al. 2015). These factors put species of the *Melocactus* genus at risk of disappearance; therefore, *in vitro* cultivation is a relevant strategy for producing seedlings on a large scale and conserving these cacti (Dias et al. 2008).

Plant tissue culture is a set of techniques to cultivate vegetables or their parts under aseptic and controlled conditions. The choice and establishment of the *in vitro* explant are essential for the cultivation

process, as they provide material for the remaining steps (Nascimento et al. 2020). The most used initial explants in the *Melocactus* genus are seeds, and micropropagation uses the obtained *in vitro* plantlets (Torres-Silva et al. 2018). Successful *in vitro* cultivation depends on optimizing different growth steps, from the *in vitro* germination of seeds to *ex vitro* acclimatization (Resende et al. 2010). Abiotic conditions, such as irradiance, light spectral quality, culture medium, and temperature, may drastically interfere with explant growth and development (Dignart et al. 2009; Santos et al. 2020; Resende et al. 2021). These factors require adjustments depending on the species, and environmental conditions similar to natural ones may improve the rusticity of plants of *in vitro* origin.

Melocactus emerges in tropical environments with high irradiance and temperature. Therefore, using sunlight instead of artificial lamps may increase irradiance in *in vitro* cultivation. Thus, *in vitro* cultivation in a greenhouse environment is a suitable alternative (Batista et al. 2018). Moreover, replacing conventional *in vitro* photomixotrophic cultivation, which requires an exogenous sucrose supplement, with photoautotrophic cultivation (without adding sucrose or organic compounds) increases the photosynthetic rates of *in vitro* plants, improving their growth (Kozai and Kubota 2001; Martins et al. 2015; Ševčíková et al. 2019) and reducing costs.

Enhancing *in vitro* propagation conditions may help conserve *Melocactus zehntneri* (Britton & Rose) Luetzelb. This species is distributed in semiarid areas, such as the Caatinga biome in Brazil (Bravo Filho et al. 2018a; Zappi and Taylor 2020). Although *M. zehntneri* presents a Least Concern (LC) classification, its population has declined primarily due to the destruction of the Caatinga vegetation (Silva et al. 2011; IUCN 2019). The anthropogenic occupation through agricultural and livestock activities harms natural occurrence areas (Ribeiro et al. 2015; Antongiovanni et al. 2020). *Melocactus* rapidly multiplies *in vitro*, mainly due to advances in determining appropriate cultivation media for germination and the multiplication and acclimatization protocols. However, using natural light for germination and *in vitro* cultivation remains little explored. (Torres-Silva et al. 2018; Santos et al. 2020). In addition to conserving this species, it is important to invest in strategies for the large-scale production of rustic seedlings that are well acclimatized to *ex vitro* conditions. Thus, this study evaluated the effect of *in vitro* photoautotrophic cultivation of *M. zehntneri*.

2. Material and Methods

Seed disinfestation and culture media

Mature fruits of *M. zehntneri* were harvested from mother plants maintained in a greenhouse in São Cristóvão (10°55'34" S; 37°06'08" W), Sergipe, Brazil. The seeds were removed from the fruits, dried on paper towels at room temperature for 24 hours before disinfestation in 70% alcohol for 30 seconds in a laminar flow cabinet, and immersed in autoclaved distilled water containing two drops of neutral detergent for five minutes. Then, the seeds were immersed in a sodium hypochlorite solution (1.25% active chlorine) for five minutes and washed three times in autoclaved distilled water. The experiments used the following culture media (sterilized by autoclaving at 121°C for 20 minutes): Medium 1 (M1) - half-strength MS culture medium (Murashige and Skoog 1962) including vitamins, at a pH of 5.8, supplemented with 15 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol, and gelled with 7 g L^{-1} agar (Santos et al. 2020); Medium 2 (M2) - half-strength MS culture medium lacking vitamins, sucrose, and myo-inositol, at a pH of 5.8, with added vermiculite as a support agent instead of agar.

In vitro *germination: imbibition effect*

The study tested different imbibition times in distilled water: no imbibition (control) and 30, 60, 120, 180, and 240 minutes; then, the seeds were inoculated in M1. It was a completely randomized experimental design with ten replications per treatment, each comprising a test tube with five seeds. The tubes remained in a growth room (GR) at 25 ±2°C, under an artificial light source (white fluorescent lamps), a 12-hour photoperiod, and 40 µmol $m^{-2}s^{-1}$ of photosynthetically active radiation (PAR). Total seed germination percentage (G - %) and the germination speed index (GSI) were evaluated daily for up to 30

days of cultivation (Maguire 1962). Germination was considered from the beginning of radicle protrusion. The following formula determined the GSI: GSI = G1/N1 + G2/N2 + ...Gn/Nn (where: G1, G2, Gn = the number of germinated seeds in each count; Nn = the number of days after inoculation).

In vitro *germination: imbibition and growth environments*

Seeds were inoculated in M1, and two different imbibition pre-treatments - (i) no imbibition (control) or (ii) 30-minute imbibition - were combined with two growth environments: (i) growth room (GR), as described, and (ii) greenhouse (GH) at a temperature range of 20-40°C, with a shade net intercepting 80% of natural light, allowing the passage of 160 µmol m^{-2} s^{-1} PAR.

A portable USB-650 RED TIDE spectroradiometer (Ocean Optics) measured the spectral quality of radiation. A LI-COR Quantum Sensor Q41031 coupled to an IRGA LI-6400XT photosynthesis system measured radiation intensity and PAR. Despite the peaks in the blue band, the light quality of each wavelength decreased in the GR environment, including a reduction in the red region starting at 600 nm (Figure 1).

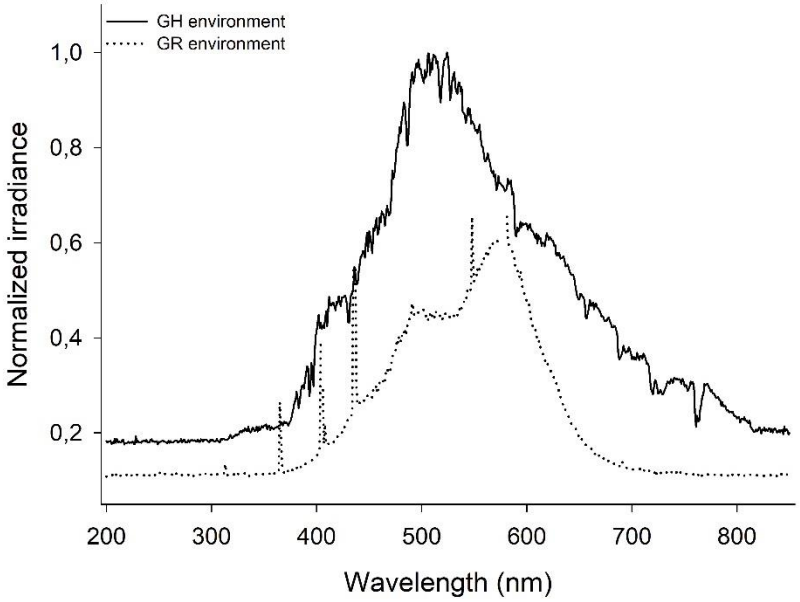

Figure 1. Normalized irradiance by wavelength in the two environments: growth room - GR (dashed line) and greenhouse - GH (solid line).

The study applied a completely randomized experimental design, and the tubes were placed in a 2x2 factorial scheme: two imbibition pre-treatments (control and 30 minutes) and two environments (GR and GH). The treatments comprised 20 replications, each composed of a test tube with ten seeds. Total seed germination percentage (G - %), GSI, mean germination time (MGT = ∑ni·ti/∑ni, where ni is the number of germinated seeds within a given time range (ti−1 and ti); the data are expressed in days), and mean germination rate (MGR= 1/MGT) were evaluated for up to 30 days of cultivation.

Initial photoautotrophic cultivation

The photoautotrophic cultivation experiment used one-year-old *in vitro* plants cultivated in M1. Healthy and well-developed *M. zehntneri* plants were sub-cultivated in M1 or M2 combined with two environments (GR and GH) in a completely randomized experimental design (2x2 factorial scheme). The treatments included 18 replications, each comprising a testing tube containing a single plant with an average height of 1.3 cm. After 90 days of cultivation, growth was analyzed through the following parameters: shoot height (cm) from the basis of the last aureole insertion to the tip and the length of the longest root (cm), both measured with a graduated ruler; cladode diameter (mm), measured with a digital caliper in the most dilated stem portion; and plant fresh weight (g), obtained with a digital analytical scale.

Also, the height-to-diameter relationship was estimated by dividing the shoot height by the cladode diameter (Gulmon et al. 1979), named the slenderness index (SI) throughout the following sections.

Ex vitro *acclimatization*

Plants obtained in the photoautotrophic cultivation experiment were transferred to plastic recipients (145 mL) holding 50 mL of washed sand and a humus substrate (1:1). The experiment was completely randomized in 2x2 factorial design depending on the origin: two media (M1 and M2) and two growth environments (GR and GH). The plants remained in GH, each receiving 10 mL of water every ten days.

The treatments applied nine replications for plant survival analysis and seven for plant growth analysis. The experimental unit included one recipient with one plant. After an experimental period of 60 days, the following characteristics were evaluated: plant survival (%), shoot height (cm), cladode diameter (mm), length of the longest root (cm), shoot and root fresh mass (g), shoot and root dry mass (g), and the slenderness index (SI). Dry mass was estimated after maintaining the plants in a forced-air circulation oven at 60°C until achieving constant weight.

Statistical analysis

SISVAR software, version 5.8, performed the statistical analyses (Ferreira 2014). Analysis of variance (ANOVA) compared the data, and the Scott-Knott or Tukey's test analyzed the means. All statistical analyses applied a 5% significance level (p<0.05).

3. Results

In vitro *germination: imbibition and growth environments*

The germination percentage (46%) was higher with 30 minutes of imbibition, although it did not differ from the control treatment (32%) and 60 (22%) and 120 minutes (22%) of imbibition (Figure 2A). Germination significantly decreased with imbibition times >120 minutes compared to 30 minutes (Figure 2A). The same trend occurred for GSI, showing no differences between the control and 30-minute imbibition treatments (0.21 and 0.25, respectively). Conversely, GSI remarkably decreased only in 180 and 240 minutes of imbibition compared to 30 minutes (Figure 2B).

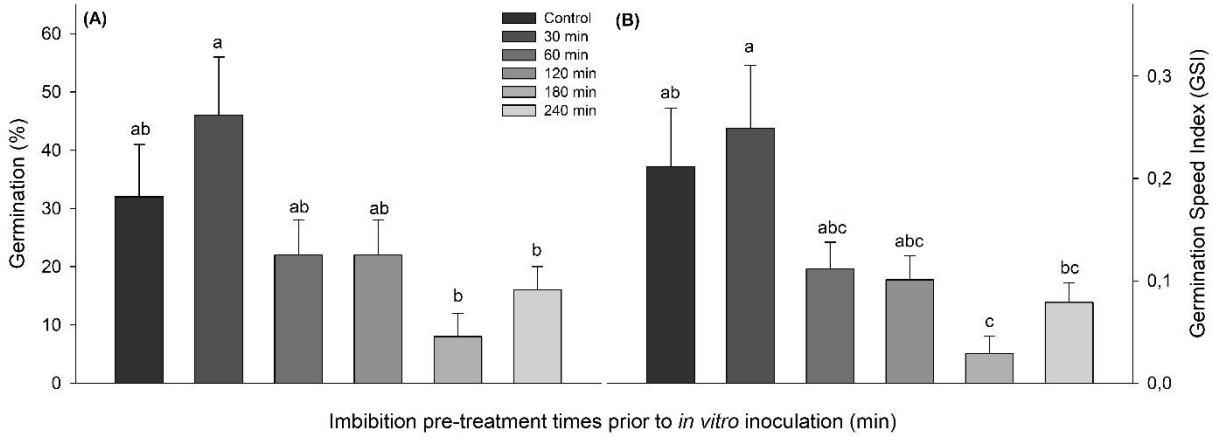

Figure 2. Germination percentage (A) and germination speed index (GSI) (B) of *Melocactus zehntneri* seeds subjected to different imbibition pre-treatment times before *in vitro* inoculation. Different letters indicate statistically significant differences (p<0.05) according to the Scott-Knott test. Columns show the means (n=10), and bars show the standard errors of the mean.

The interaction between the growth environment and imbibition time was not significant, nor were the germination percentage and mean germination time. However, the separate analysis of these two factors showed a statistical difference. Germination percentage in GH was >95% (Figure 3A). The 30-minute imbibition pre-treatment promoted a shorter mean germination time (5.37 days) than the control (6.87 days) (Figure 3B).

GSI values were also statistically different when analyzing growth environments and imbibition pre-treatments separately. GSI values were higher (1.71 and 1.09) for GH and 30 minutes of imbibition than GR and the control (0.31 and 0.92) (Figures 3C and 3D). The mean germination rate followed a similar pattern, and the highest results (0.17 and 0.16) occurred in GH and 30 minutes of imbibition compared to GR and the control (0.12 and 0.13) (Figures 3E and 3F).

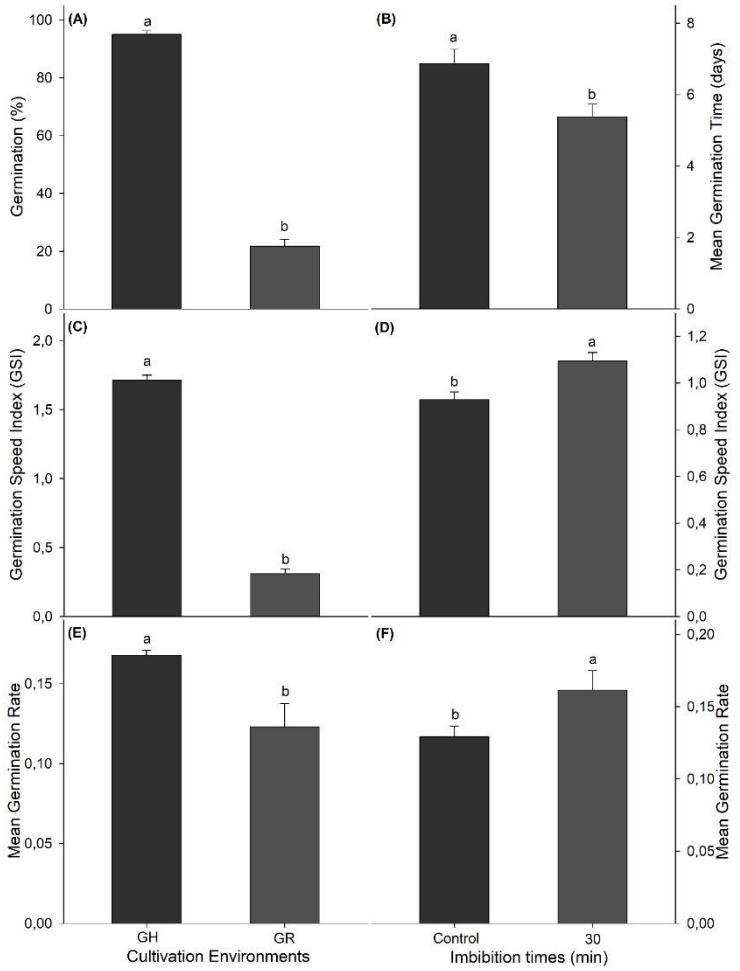

Figure 3. Germination percentage (A), mean germination time (B), germination speed index (GSI) (C and D), and mean germination rate (E and F) of *Melocactus zehntneri* seeds subjected to a growth room (GR) or greenhouse (GR) environment and imbibition pre-treatment times before *in vitro* inoculation. Different letters indicate statistically significant differences (p<0.05) according to Tukey's test. Columns show the means (n=20), and bars show the standard errors of the mean.

Early photoautotrophic cultivation

The interaction between the growth environment and culture medium was significant for shoot height, cladode diameter, and SI. Plants grown in M1 and maintained in GR were taller than those grown in GH. Conversely, the growth environment did not affect plants grown in M2 (Table 1). Contrary to shoot height, plants grown in M1 and GH showed the largest cladode diameter (8.84 mm) and the lowest SI (2.27), indicating the formation of globular-shaped plants among the treatments (Table 1, Figure 4).

The lengths of the longest root differed between growth environments and culture media separately. The values were higher in M1 (3.3 cm) or GH (3.1 cm) (Table 1). Root fresh mass differed only between the environments, with a higher mean of 0.99 g in GH (Table 1).

Table 1. Shoot height, cladode diameter, slenderness index, length of the longest root, and plant fresh mass of *Melocactus zehntneri* after 90 days of *in vitro* cultivation in different environments (GH - greenhouse; GR - growth room) and culture media (M1- agar as a support agent; M2- vermiculite as a support agent).

I.	Shoot height (cm)	
	M1	M2
GH	1.99±0.08^{bB}	2.59±0.05^{aA}
GR	2.42±0.07^{aA}	2.57±0.07^{aA}
	Cladode diameter (mm)	
	M1	M2
GH	8.84±0.15^{aA}	8.13±0.17^{aB}
GR	7.15±0.21^{bB}	7.65±0.13^{bA}
	Slenderness index	
	M1	M2
GH	2.27±0.11^{bB}	3.19±0.04^{aA}
GR	3.40±0.08^{aA}	3.36±0.08^{aA}
II.	M1	M2
Length of the longest root (cm)	3.32±0.19^{a}	1.94±0.13^{b}
III.	GH	GR
Length of the longest root (cm)	3.11±0.21^{a}	2.16±0.14^{b}
Plant fresh mass (g)	0.99±0.04^{a}	0.88±0.03^{b}

I. Lowercase letters in each column represent statistical comparisons between environments in each culture medium. Upper case letters in each row represent comparisons between the culture media in the same environment. Different letters indicate statistical significance by Tukey's test (p<0.05). Values are shown in mean (n=18) ± standard error of the mean. **II.** Different letters indicate statistically significant differences (p<0.05) between M1 and M2 by Tukey's test. Values are shown in mean (n=18) ± standard error of the mean. **III.** Different letters indicate statistically significant differences (p<0.05) between GH and GR by Tukey's test. Values are shown in mean (n=18) ± standard error of the mean.

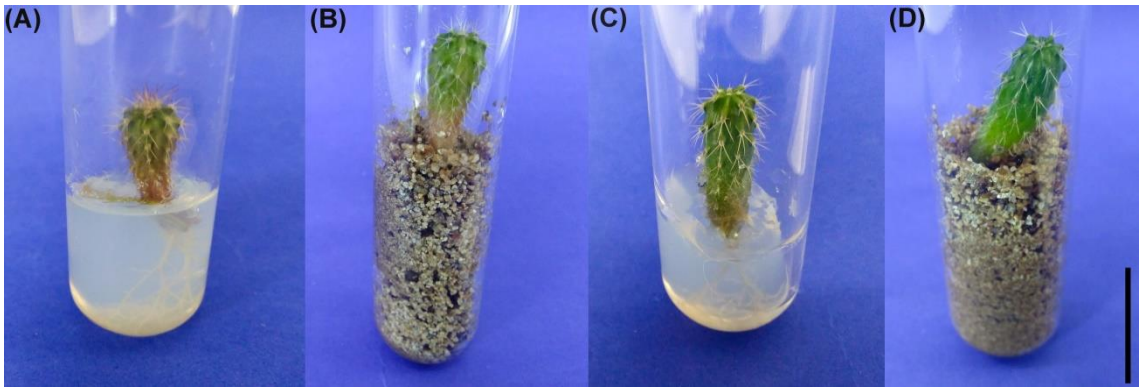

Figure 4. *Melocactus zehntneri* plants maintained in different cultivation environments and culture media after 90 days of *in vitro* cultivation: M1 (agar as a support agent) and GR (growth room) (A); M1 and GH (greenhouse) (B); M2 (vermiculite as a support agent) and GR (C); M2 and GH (D). Bars = 1.5 cm.

Ex vitro *acclimatization*

Plant survival percentage during *ex vitro* acclimatization did not differ between treatments, with an overall mean of 93%. However, combining growth environments and culture media in the photoautotrophic cultivation experiment influenced plant height and SI. Plants maintained in GR and cultivated in M1 and those kept in GH and cultivated in M2 were the tallest, with 2.3 and 2.2 cm,

respectively (Table 2). As for SI and root fresh mass, plants in GH and M1 showed the highest values of 1.31 and 0.40 g, respectively (Table 2). This SI pattern had appeared during initial growth. Regarding root dry mass, there were no differences between treatments, with an overall mean of 0.028 g.

The environment and culture medium of the previous step influenced the other analyzed variables separately. Plants grown in M1 (13.5 mm) or GH (14.3 mm) showed the highest cladode diameter means (Table 2). M1 influenced the increment of tissue mass, with higher values of shoot fresh (2.13 g) and dry (0.09) mass (Table 2). GH presented similar results, with higher values of shoot fresh (2.24 g) and dry (0.09) mass (Table 2). The length of the longest root exhibited the same pattern, so plants in M1 and GH had the highest mean of 4.61 cm (Table 2).

Table 2. Shoot height, slenderness index, and root fresh mass of *Melocactus zehntneri* after 60 days of *ex vitro* acclimatization in different growth environments (GH – greenhouse; GR – growth room) and culture media (M1 – agar as a support agent; M2 – vermiculite as a support agent).

I.	Shoot height (cm)	
	M1	M2
GH	1.98±0.07^{bB}	2.21±0.03^{aA}
GR	2.28±0.04^{aA}	1.97±0.07^{bB}
	Slenderness index	
	M1	M2
GH	1.31±0.04^{bB}	1.67±0.06^{bA}
GR	1.93±0.05^{aA}	2.03±0.06^{aA}
	Root fresh mass (g)	
	M1	M2
GH	0.40±0.01^{aA}	0.25±0.01^{aB}
GR	0.23±0.03^{bA}	0.17±0.02^{bB}
II.	M1	M2
Cladode diameter (mm)	13.52±0.51^{a}	11.50±0.55^{b}
Length of the longest root (cm)	4.61±0.29^{a}	3.46±0.20^{b}
Shoot fresh mass (g)	2.13±0.13^{a}	1.59±0.13^{b}
Shoot dry mass (g)	0.09±0.01^{a}	0.06±0.01^{b}
III.	GH	GR
Cladode diameter (mm)	14.27±0.36^{a}	10.80±0.35^{b}
Length of the longest root (cm)	4.61±0.32^{a}	3.46±0.15^{b}
Shoot fresh mass (g)	2.24±0.12^{a}	1.48±0.09^{b}
Shoot dry mass (g)	0.09±0.01^{a}	0.06±0.01^{b}

I. Lowercase letters in each column represent statistical comparisons between environments in each culture medium. Upper case letters in each row represent comparisons between culture media in the same environment. Different letters indicate statistical significance by Tukey's test (p<0.05). Values are shown in mean (n=7) ± standard error of the mean. **II.** Different letters indicate statistically significant differences (p<0.05) between M1 and M2 by Tukey's test. Values are shown in mean (n=7) ± standard error of the mean. **III.** Different letters indicate statistically significant differences (p<0.05) between GH and GR by Tukey's test. Values are shown in mean (n=7) ± standard error of the mean.

4. Discussion

Greenhouse (GH) cultivation highly enhanced the germination and initial development of *M. zehntneri*, a relevant response to the production of *in vitro* seedlings that may be used for micropropagation and *in vitro* conservation programs.

The absence of differences between the control and 30-minute imbibition treatments regarding germination percentage and the germination speed index (GSI) showed that the imbibition pre-treatment did not improve these traits under growth room (GR) conditions. Germination percentage remained low after 30 minutes of imbibition and became lower as the time increased (Figure 2). However, 30 minutes of imbibition reduced the mean germination time (Figure 3B) and increased the GSI (Figure 3D) and the mean germination rate (Figure 3F) compared to the control. Columnar cacti *Pilosocereus aurisetus* had presented

similar responses, as the imbibition pre-treatment did not enhance its germination rates (Reis et al. 2012). Likewise, *in* and *ex vitro* germination of *M. sergipensis* did not improve by immersing seeds in distilled water for six hours (Bravo Filho et al. 2019).

The germination of cactus species from xeric regions, such as *Eriosyce subgibbosa* (Guerrero et al. 2016), *Hylocereus* spp. (Zerpa-Catanho et al. 2019), *Acanthocereus tetragonus, Melocactus curvispinus,* and *Stenocereus griseus* (Lina and Eloisa 2018), have remained even when reducing water potential. Considering that *M. zehntneri* has a distribution predominantly restricted to dry areas, such as the Caatinga biome (Bravo Filho et al. 2018a; Zappi and Taylor 2020), its seeds might tolerate drought, and the imbibition treatments might hinder germination. However, further investigations on this topic are required.

In this context, the primary constraint for *M. zehntneri* germination was possibly related to light conditions. Thus, when testing different growth environments, the germination traits of this species improved. Regardless of the imbibition pre-treatment, the seeds maintained in GH showed a germination rate 73% higher than those in GR. The main difference between these environments is the higher amount and quality of light observed in GH (Figure 1). Three species of the genus *Melocactus* (*M. zehntneri*, *M. sergipensis*, and *M. violaceus*) demonstrated similar outcomes (Santos et al. 2020).

Previous experiments showed that *M. zehntneri* germination only occurs with light (200 µmol m^{-2} s^{-1}), which classifies this species as positive photoblastic (Meiado et al. 2016). Increased red wavelengths benefited *M. conoideus* germination (Rebouças and dos Santos 2007). Also, small seeds of the *Cactaceae* family, such as those observed in *Melocactus* species, require more irradiance for appropriate germination (Flores et al. 2011).

Thus, a higher germination percentage in GH might be associated with the role played by phytochromes. Higher amount and quality of light, especially in the red wavelength, may enhance phytochrome molecule conversion into their activated form, considering it is a light-induced pigment that promotes seed germination (Lymperopoulos et al. 2018; Yang et al. 2020). However, the usual light availability in conventional GRs (40-60 µmol m^{-2} s^{-1}) presents a limitation for the *in vitro* germination of this species because of the low photon flux.

Plants grown in GH and M1 conditions showed a more globular pattern during *in vitro* cultivation, as they had a lower SI. Moreover, plants in GH showed longer roots and more fresh mass. Although *M. zehntneri* acclimation was efficient regardless of the *in vitro* condition, confirmed by the high survival, plant origin influenced its shape.

M. sergipensis (Bravo Filho et al. 2018b; Bravo Filho et al. 2019) and *M. glaucescens* (Resende et al. 2010) also demonstrated this rusticity during acclimatization. However, GH quality and amount of light favored the observed growth and shape, whereas GR caused cladode elongation due to low irradiance. The globose cactus *Mammillaria compressa* exhibited a cylindrical shape during its seedling stage under artificial lighting (Flores et al. 2016).

The increased plant fresh mass and root length in GH may relate to the capacity to maintain photosynthetic activity, whereas a low photosynthetic activity usually relates to *in vitro* conventional conditions (Sáez et al. 2016; Batista et al. 2018). Increasing light availability may increase net photosynthesis, which is associated with a higher carbon gain and biomass accumulation (Oguchi et al. 2008). In this context, natural light may be an alternative to *in vitro* cultivation and *ex vitro* acclimatization of plants (Arencibia et al. 2017).

The globular shape of the genus *Melocactus* is relevant because it promotes a smaller exposure area with higher volume, thus reducing the transpiration area and increasing water and nutrient reserves (Lina and Eloisa 2018). As for *M. curvispinus*, volume relation is associated with higher survival during extended drought periods (Lina and Eloisa 2018). Thus, the GH environment is adequate for the *in vitro* cultivation of *M. zehntneri* during the initial development steps, benefiting the plant and reducing costs related to temperature control and artificial light. The viability of this environment for the *in vitro* cultivation of cactus species relates to their adaptation to high temperatures and irradiance levels (Dettke and Milaneze-Gutierre 2008).

It is worth noting that the photoautotrophic conditions tested in this study produced well-developed plants with low mortality rates even when subjected to higher temperatures in GH. The

vermiculite medium was viable for the *in vitro* cultivation of *M. zehntneri*, replacing agar as a support agent, although the latter increased growth.

5. Conclusions

Imbibition did not increase the *in vitro* germination of *M. zehntneri*. Cultivating seedlings in a greenhouse with vermiculite as the *in vitro* culture medium support promoted better characteristics for ornamental purposes.

Authors' Contributions: CAMPOS, J.A.: acquisition of data, analysis and interpretation, drafting the article, final approval; MENDONÇA, A.M.C.: analysis and interpretation, critical review of important intellectual content, final approval; NASCIMENTO, A.V.S.: acquisition of data, final approval; DIAS, G.S.: acquisition of data, final approval; SILVA, L.C.: conception and design, critical review of important intellectual content, final approval; DE SANTANA, M.C.: conception and design, critical review of important intellectual content, final approval; DA SILVA JÚNIOR, C.D.: critical review of important intellectual content, final approval; SANTOS, P.A.A.: conception and design, analysis and interpretation, critical review of important intellectual content, final approval.

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