RONILSON CARLOS DE ARAÚJO

MICROPROPAGAÇÃO DE *ADENIUM OBESUM*

LAVRAS – MG
2023

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Curso de Pós-Graduação em Agronomia/Fitotecnia, área de concentração em Produção Vegetal, para aobtenção do título de Doutor.

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LAVRAS – MG
2023

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LAVRAS – MG
2023

Dedico este trabalho a minha
famíliae amigos.

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Muito obrigado!

“Aprenda a encerrar ciclos. Quanto mais você alimenta o que não é para você, maisvocê adia o que realmente é.”

(@Motiva_Meta

RESUMO

Adenium obesum é uma planta nativa de áreas áridas e semiáridas da África e da Península Arábica, cultivada em todo o mundo, apresenta hábito de crescimento compacto, raízes suculentas, caudex vistoso e floração exuberante. Objetivou-se: **a)** avaliar o efeito de LEDs coloridos e meios de cultura na germinação e crescimento inicial; **b)** avaliar diferentes fotoperíodos e concentrações de meio MRA no crescimento inicial e; **c)** determinar o período de tempo mais eficaz na assepsia de sementes. Do 3º ao 32º foram avaliadas diariamente o número de sementes germinadas e contaminações e aos 60 dias avaliou-se: **a)** germinação; **b)** índice de velocidade de germinação; **c)** tempo médio de germinação; **d)** velocidade média de germinação; **e)** número de folhas; **f)** comprimento das folhas; **g)** comprimento total da planta; **h)** massa fresca e, **i)** massa seca da planta inteira; **j)** clorofila-a; **k)** clorofila-b; **l)** clorofila total (a+b) e, **m)** carotenoide. O delineamento experimental foi inteiramente casualizado. As avaliações iniciaram no primeiro dia após o semeio. Houve diferença significativa entre os tratamentos utilizados para todas as variáveis analisadas. As respostas da germinação de sementes de *A. obesum* variou em função da qualidade espectral do LED e do meio de cultura. O melhor LED para suplementação luminosa é o vermelho para o meio MRA e o LED roxo para o meio MS, para o aumento da germinação com menor índice de mortalidade. Para o desenvolvimento inicial das plântulas de *A. obesum*, o melhor foi o LED vermelho para ambos os meios de cultura com alterações positivas em todas as variáveis morfológicas. O melhor LED foi o branco para o meio MS e o LED verde para o meio MRA, proporcionando melhor quantidade nos teores de pigmentos. Cada fase da cultura exige uma qualidade espectral diferente, com finalidade de promover a germinação e crescimento inicial. A germinação *in vitro* de *A. obesum* ocorreu nos diferentes tempos de imersão na solução de NaClO com destaque para 30 min que apresentou menor porcentagem de contaminação e mudas imperfeitas, com maior percentual de mudas perfeitas quando comparado aos demais tempos de assepsia. A solução de hipoclorito de sódio (30%) a 30 min de imersão influencia positivamente a germinação de sementes de *A. obesum*, possibilitando produção de mudas perfeitas livres de contaminantes.

Palavras-chave: Rosa do deserto. Cultura de tecidos. Cultivo *in vitro*. Planta ornamental. Sementes. LED.

ABSTRACT

Adenium obesum is a plant native to arid and semi-arid areas of Africa and the Arabian Peninsula, cultivated all over the world, presents compact growth habit, succulent roots, showy caudex and lush flowering. The objective was: **a)** to evaluate the effect of colored LEDs and culture media on germination and initial growth; **b)** evaluate different photoperiods and concentrations of MRA medium in the initial growth and; **c)** determine the most effective period of time in seed asepsis. From the 3rd to the 32nd the number of germinated seeds and contaminations were evaluated daily and at 60 days the following were evaluated: **a)** germination; **b)** germination speed index; **c)** average germination time; **d)** average germination speed; **e)** number of leaves; **f)** leaf length; **g)** total length of the plant; **h)** fresh mass and, **i)** dry mass of the whole plant; **j)** chlorophyll-a; **k)** chlorophyll-b; **l)** total chlorophyll (a+b) and, **m)** carotenoid. The experimental design was completely randomized. The evaluations began on the first day after sowing. There was a significant difference between the treatments used for all variables analyzed. The germination responses of *A. obesum* seeds varied according to the spectral quality of the LED and the culture medium. The best LED for light supplementation is red for MRA medium and purple LED for MS medium, for increased germination with lower mortality rate. For the initial development of *A. obesum* seedlings, the best was the red LED for both culture media with positive changes in all morphological variables. The best LED was white for the MS medium and the green LED for the MRA medium, providing a better amount of pigment contents. Each phase of the crop requires a different spectral quality in order to promote germination and initial growth. The *in vitro* germination of *A. obesum* occurred in the different times of immersion in the NaClO solution, with emphasis on 30 min, which presented a lower percentage of contamination and imperfect seedlings, with a higher percentage of perfect seedlings when compared to the other times of asepsis. The sodium hypochlorite solution (30%) at 30 min of immersion positively influences the germination of *A. obesum* seeds, allowing the production of perfect seedlings free of contaminants

Keywords: Desert rose. Tissue culture. *In vitro* cultivation. Ornamental plant. Seeds, LED.

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FIRST PART

INTRODUCTION

Adenium obesum, known as desert rose, is an angiosperm of the *Apocynaceae* family native to arid and semi-arid areas of Africa and the Arabian Peninsula. This species is widely cultivated all over the world due to its exotic and attractive appearance. In addition, the plant has a habit of compact growth, juicy *roots*, thick structure of the trunk with flashy caudex, which acts as primary organs for water and nutrient reserves ensuring its survival in arid places, good branching with cultural aspect and exuberant flowering with high level of divergence in flower colors.

Seeds mainly perform the propagation of *A. obesum*, as they are considered more efficient and less costly to producers for providing plants with more swollen caudex. Propagation from cuttings is easier, but they are not well accepted in the ornamental market because they produce underground stems and do not present the same exuberance of plants propagated via seeds.

With the growing demand for flower growers and landscapers, due to its high ornamental value, its commercial production is still recent, requiring scientific studies on the most appropriate methods of propagation, crop management and commercial production of potted plants.

Numerous factors can influence the germination process, such as luminosity and seed asepsis. However, the great challenge of tissue culture is to improve the supply of light in sufficient quantity and quality, which will allow the germination and growth of plants more efficiently. In general, research has shown that sodium hypochlorite is an efficient disinfestatoin the control of various pathogens, without harming the physiological quality of seeds, and may replace other chemicals, such as fungicides, which are more expensive and were previouslyused for the same purpose.

In view of this report and the importance of this ornamental plant, the objective was to: **a)** evaluate the effects of LEDs of different colors and different culture media on seed germination and *in vitro* initial growth of *A. obesum* seedlings; **b)** evaluate the effect of MRA medium concentrations and its interaction with different photoperiods on the initial growth of *A. obesum* plants grown *in vitro*, and; **c)** determine the most effective period of time in the asepsis of *A. obesum* seeds for *in vitro* cultivation.

SECOND PART

1º ARTICLE

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IN VITRO GERMINATION OF *ADENIUM OBESUM* UNDER THE EFFECTS OF CULTURE MEDIUM AND LIGHT EMITTING DIODES OF DIFFERENT COLORS

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Abstract

Adenium obesum seeds germinated and *in vitro* cultivated under lighting with different wavelengths of light from light emitting diodes (LEDs) (white LED - 6400 K - λ = 525 nm; blue LED - λ = 430 nm; green LED - λ = 520 nm; orange LED - λ = 595 nm; purple LED - λ = 440 nm "2 blue LEDs + 1 red LED"; and red LED - λ = 670 nm) and control - absence of LED, combined with different culture media (MS and MRA). This work aimed to evaluate the influence of LED lighting conditions and culture media on seed germination, initial *in vitro* growth of *A. obesum* seedlings and production of photosynthetic pigments and soluble sugars. The study demonstrated a significant effect of red LED light combined with MRA medium and purple LED combined with MS medium, promoting the highest germination rates and the lowest mortality rates. The best initial in vitro development of *A. obesum* seedlings occurred under red LED in both culture media, which generated positive changes in the morphological variables analyzed. The highest pigment contents were obtained by combining white light with MS medium and green light with MRA medium. This is the first report to provide evidence of the stimulating effect of light quality on germination, early growth, production of photosynthetic pigments by *A. obesum* seedlings *in vitro*.

Keywords: LED, Seeds, luminosity, culture media, flowers.

Introduction

Adenium obesum Roem. & Schult, popularly known as desert rose, is an angiosperm of the Apocynaceae family native to tropical Africa and Arabia, but it is currently found in most tropical and subtropical countries (Talukdar, 2012; Akhtar et al., 2016). Threatened with extinction in some African countries due to the destruction of its natural habitat and great demand for the plant (Talukdar, 2012).

The plant has a compact growth habit, succulent roots, and a thick trunk structure with a showy caudex, which acts as the primary organ for water, nutrient reserves and ensures its survival in arid locations. The plant also has good branching with a sculptural aspect and exuberant flowering with a high level of flower color divergence (Colombo et al., 2016).

According to Colombo et al. (2015), the propagation of *A. obesum* is performed mainly by seeds because this gives the plants a more swollen caudex than propagation from cuttings. Propagation by cuttings is easier, but these plants are not well accepted on the ornamental market because they produce underground stems and do not have the same exuberance as plants propagated by seeds. Commercial production is still new, but with the increasing demand for florists and landscapers due to the high ornamental value of *A. obesum* (Santos et al., 2015a), scientific studies on the most appropriate methods for propagation, crop management, and commercial production of its seedlings are needed (Colombo et al., 2018).

According to Marcos Filho (2005), numerous factors can influence the germination process, among them is the luminosity. The seeds are classified according to the responses to light: a) positive photoblastics (benefited by light); b) negative photoblastics (damaged by light); c) neutral photoblastics, when indifferent or insensitive to light.

The use of light emitting diodes (LEDs) in plant seed germination can provide significant electricity savings (Santos et al., 2015b) and improve germination rates, early seedling development and photosynthetic pigment concentrations due to expectral properties of LEDs, which can regulate physiological, morphological and anatomical attributes (Rocha et al., 2015). Although LEDs have been used for various plants, such as chrysanthemum (Wozny & Miler, 2016), strawberry (Rocha et al., 2010), blackberry (Rocha et al., 2013a), sugarcane (Rocha et al., 2013b; Ferreira et al., 2016), and potato (Rocha et al, 2015), there are no evaluations of this light source for *A. obesum* seed germination and no *in vitro* germination protocol specific for this species (Portes et al., 2018). According to Samuoliené et al. (2013) & Dong et al. (2014), the great challenge of tissue culture is to provide controlled light intensity of sufficient quantity and quality for seed germination and plant development.

However, scientific studies related to the *in vitro* cultivation of plants of the genus *Adenium* are scarce in the literature (Kanchanapoom et al., 2010).

The objective of this research was to evaluate the influence of the quality of different LED lamps and culture media on seed germination, in vitro initial growth, photosynthetic pigment production by *A. obesum* seedlings *in vitro*.

Materials and Methods

Location of the experiment

The experiment was conducted in a growth room of the Laboratory of Plant Tissue Culture of the Federal University of Lavras, located in the municipality of Lavras, Minas Gerais, Brazil (44°57′50″ W, 21°13′40″ S, 919 m altitude), from January to April 2020.

Plant material

A. obesum seeds were donated by Rosa do Deserto Garden Center and obtained from the mother plant (Fig. 1A) at approximately 15 years of age, in the city of Divinópolis, Minas Gerais, Brazil.

Seed preparation

The seeds were placed in a container with 100 mL of 70% alcohol for 1 min and then transferred to a container containing 100 mL of 30% sodium hypochlorite solution (2.5% w/v active chlorine) and 1 drop of neutral detergent under agitation for 20 min. The seeds were washed three times with autoclaved deionized water to remove the disinfectant in a laminar flow chamber sanitized with 70% alcohol. Soon after, the seeds were sown in 100-mL test tubes containing 20 mL (Fig. 1B) of MS (Murashige & Skoog, 1962) medium or MRA medium (Table 1).

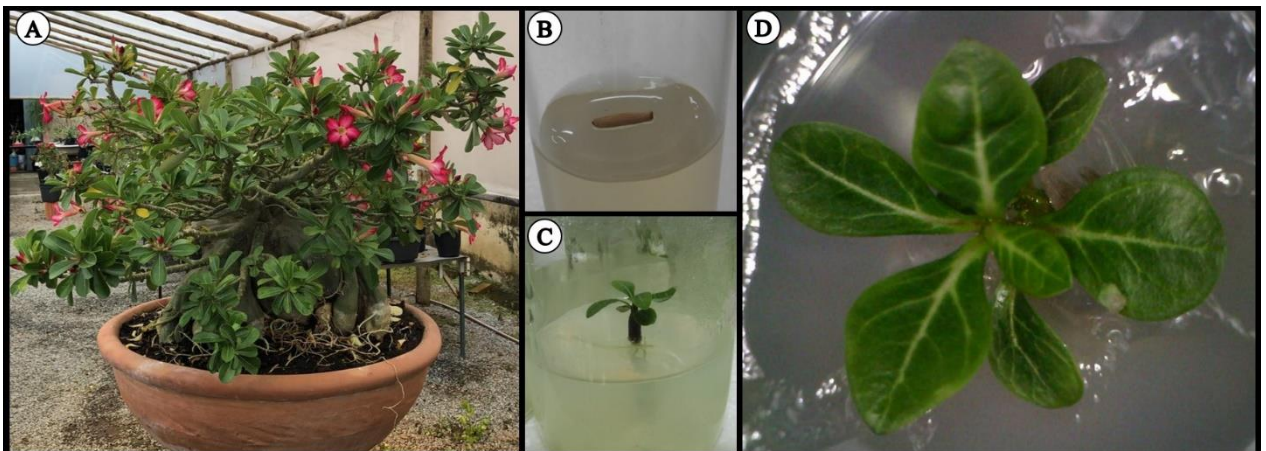

Fig. 1 *A. obseum* propagation. A, mother plant; B, *in vitro* seed establishment; C, plant with 30 days; D, plant with 60 days

In addition, 5.5 g L^{-1} agar and 30 g L^{-1} sucrose were added to the media (Table 1).

Table 1 Concentration of nutrients in different culture media used for *in vitro* germination of *A. obesum*.

Compounds	MS (mg L^{-1})	*MRA (mg L^{-1})
NH_4NO_3	1650	226.02
KNO_3	1900	793.64
KH_2PO_4	170	233.82
KI	0.83	-
$CaCl_2 \cdot 2H_2O$	440	779.15
$Mg(NO_3)_2$	-	1525.64
$MgSO_4 \cdot 7H_2O$	370	534.86
H_3BO_3	6.2	6.98
$Na_2MoO_4 \cdot 2H_2O$	0.25	-
$CoCl_2 \cdot 6H_2O$	0.025	-
$MnSO_4 \cdot 4H_2O$	22.3	31.6
$ZnSO_4 \cdot 7H_2O$	8.6	20.04
$CuSO_4 \cdot 5H_2O$	0.025	7.065
$Na_2EDTA \cdot 2H_2O$	37.25	97.21
$FeSO_4 \cdot 7H_2O$	27.85	72.65
Myo-inositol	100	-
Glycine	2	-
Thiamine (HCl)	0.5	-
Nicotinic acid	0.5	-
Pyridoxine HCl	0.5	-

* Culture medium developed by the authors

The pH of the medium was adjusted to 5.7 ± 0.3 before autoclaving at 121 ± 1 °C and 1.05 atm pressure for 20 min. After sowing, the tubes were sealed with parafilm and kept in a growth room at 25 ± 2 °C (day/night) with a 16/8 h photoperiod under different LEDs, and at 70% relative humidity in a phytotron.

Light sources

Photon flux measurements of light emitting diodes (Led Tec-LUX, Tecnal®, Piracicaba, Brazil): white LED 6400 K (λ = 525 nm); purple LED (2 blue LEDs, 1 red LED); blue LED (λ = 430 nm); green LED (λ = 520 nm); orange LED (λ = 595 nm); red LED (λ = 670 nm) were carried out 18 cm from the lamps, approximately at the height of the top of the

plant using a spectroradiometer (Li-1400, LI-COR Biosciences, USA), 60 days after germination. The control light condition was no light (dark; Fig. 2).

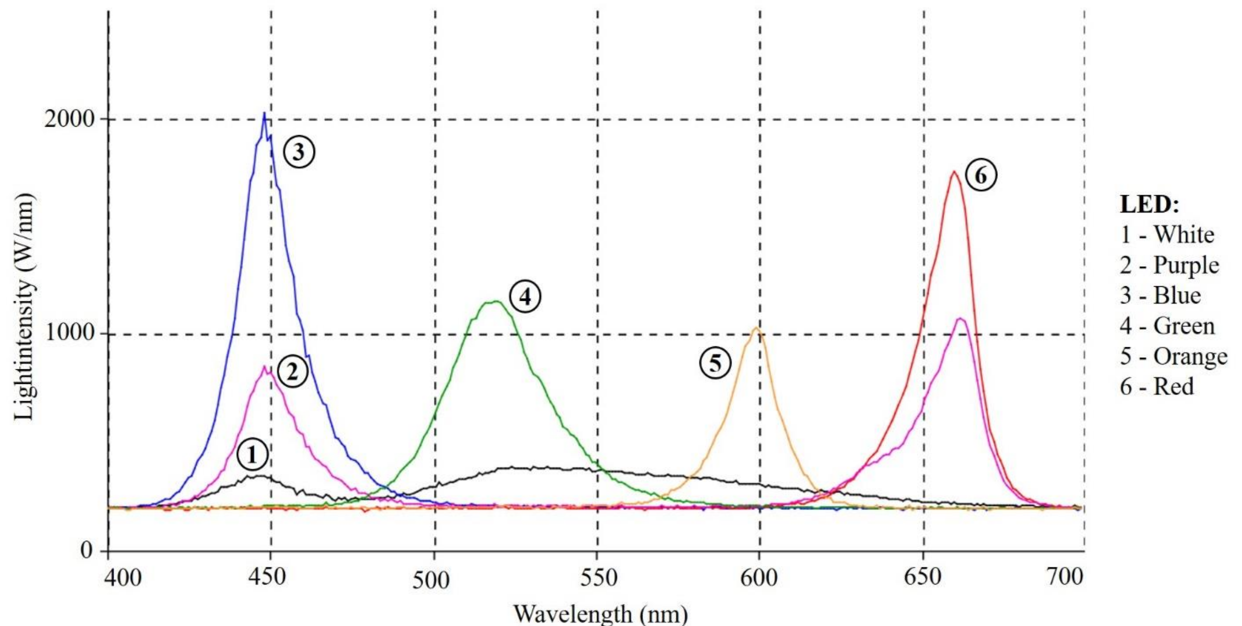

Fig. 2 Light spectra evaluated during *in vitro* cultivation of *Adenium obesum*. 1) white LED 6400 K (λ = 525 nm); 2) purple LED (2 blue LEDs, 1 red LED); 3) blue LED (λ = 430 nm); 4) green LED (λ = 520 nm); 5) orange LED (λ = 595 nm); 6) red LED (λ = 670 nm)

Analysis

Germination

The evaluations began on the third day after sowing and continued daily for 30 days. Evaluations consisted of observation and individual counting of germinated seeds. The seeds with root emission (approximately 2 mm) were considered germinated seeds. The calculated variables were as follows:

a) Germination: calculated from the formula germination (%) = N/A × 100, where N = number of seeds germinated at the end of the test, and A = total number of seeds placed to germinate (Laboriau, 1983).

b) Germination speed index (GSI): calculated by the formula GSI = $G_1/N_1 + G_2/N_2 + ... G_n/N_n$, where G_1, G_2, ..., G_n = number of normal seedlings in the first, second, …, nth observations; N_1, N_2, ..., N_n = number of days (or hours) after sowing. Unit: dimensionless (Maguire, 1962).

c) Mean germination time (MGT): calculated by the formula MGT = $(\sum n_i\ t_i)/\sum n_i$, where n_i = number of seeds germinated by day i, t_i = incubation time, and i = 3 to 30 days. Unit: days (Laboriau, 1983).

d) Mean germination speed (MGS): calculated by the formula MGS = 1/t, where t = mean germination time. Unit: days (Laboriau, 1983).

Morphological characteristics

At 60 days after sowing, we evaluated the number of leaves (NL); length of leaves (LL), which was measured in the vertical position from one end to the other; total plant length (TPL), which was measured from the root end to the stem apex; whole-plant fresh weight (WPFW); and whole plant dry weight (WPDW), for which plant samples were stored in paper bags and transferred to a forced-air oven at 60 °C for 96 h and then weighed on a CELTAC FA2104N digital scale.

Content of photosynthetic pigments

The pigment analysis was performed at the Laboratory of Plant Tissue Culture of the Department of Agriculture of the Federal University of Lavras. Fresh leaves (± 0.015 g) of desert rose (*A. obesum*) were transferred to test tubes containing 3 mL of 80% acetone. The test tubes were wrapped in aluminum foil to protect the sample from light and prevent pigment degradation. After 24 h in a refrigerator at ~4 °C, the absorptivity of the samples was measured in an ELISA Multiskan GO (Thermo Fisher Scientific) spectrophotometer at wavelengths of 470, 645, 652, and 663 nm, as described by Scopel et al. (2011).

The equations proposed by Lichtenthaler and Wellburn (1983) and Zhang et al. (2009) were used to determine the levels of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids. The wavelength readings were performed in triplicate, and three replicates per treatment were used. The analysis was performed using Skanit Software 5.0 for Microplate Readers RE version 5.0.0.42.

Experimental design and statistical analysis

The experimental design was completely randomized with three replicates of four seeds each in a 2×7 factorial scheme (two culture media with six cold-colored LEDs (white, blue, green, orange, red, purple) and the dark control), totaling 14 treatments. The data were subjected to analysis of variance, and the means were compared using the Scott-Knott test at 5% probability within SISVAR statistical software (Ferreira, 2011).

Results

Effect of different LEDs on the germination of *A. obesum*

The treatments with the different LEDs promoted significant responses in the germination, GSI of seeds and the percentage of postemergence deaths of *A. obesum* seedlings (Table 2).

Germination: A significant effect was observed between the different LEDs (Table 2). Higher germination rates were observed under the red LED (100 ± 4.23%) in MRA medium, and under the purple LED (83.33 ± 4.23 in MS medium.

GSI: Significant differences between the different LEDs occurred in the MS and MRA media (Table 2). The best result was observed with the red LED in MRA medium (1.6 ± 0.10) and purple LED in MS medium (1.49 ± 0.10).

Postemergence death: A significant effect of LED color was seen on postemergence death percentage (Table 2). The highest percentage of seedling deaths was observed with the red LED (58.33 ± 3.53%), which did not differ significantly from the purple LED in MS medium (50 ± 3.53%), and the dark condition (33 ± 3.53%) did not differ significantly from the blue and green LEDs in MRA medium (30 ± 3.53%). While the lowest death percentage was observed with red LEDs in MRA medium (7 ± 3.53%).

Table 2 Germination (%), germination speed index (GSI) and postgermination deaths (%) in *A. obesum* seeds germinated in different culture media under different LEDs in a controlled-environment growth room.

Color LEDs*	Germination (%)	GSI	Deaths (%)
Dark, MS	$50.0^{d} \pm 4.23$	$0.51^{c} \pm 0.10$	$16.66^{c} \pm 3.53$
Dark, MRA	$75.0^{c} \pm 4.23$	$0.88^{b} \pm 0.10$	$33.33^{b} \pm 3.53$
White LED, MS	$50.0^{d} \pm 4.23$	$0.53^{c} \pm 0.10$	$16.66^{c} \pm 3.53$
White LED, MRA	$75.0^{c} \pm 4.23$	$1.22^{b} \pm 0.10$	$22.22^{c} \pm 3.53$
Blue LED, MS	$66.64^{c} \pm 4.23$	$0.99^{b} \pm 0.10$	$25.0^{b} \pm 3.53$
Blue LED, MRA	$83.33^{b} \pm 4.23$	$1.03^{b} \pm 0.10$	$30.0^{b} \pm 3.53$
Green LED, MS	$58.33^{d} \pm 4.23$	$0.48^{c} \pm 0.10$	$14.33^{c} \pm 3.53$
Green LED, MRA	$83.33^{b} \pm 4.23$	$0.85^{b} \pm 0.10$	$30.0^{b} \pm 3.53$
Orange LED, MS	$41.67^{e} \pm 4.23$	$0.40^{c} \pm 0.10$	$33.33^{b} \pm 3.53$
Orange LED, MRA	$83.33^{b} \pm 4.23$	$0.86^{b} \pm 0.10$	$20.0^{c} \pm 3.53$
Purple LED, MS	$83.33^{b} \pm 4.23$	$1.49^{a} \pm 0.10$	$50.0^{a} \pm 3.53$
Purple LED, MRA	$41.67^{e} \pm 4.23$	$0.33^{c} \pm 0.10$	$20.0^{c} \pm 3.53$
Red LED, MS	$75.0^{c} \pm 4.23$	$1.05^{b} \pm 0.10$	$58.33^{a} \pm 3.53$

Data expressed as the mean value ± SD (n=3). Diferent superscript letters (a-e) within column for each parameter indicate signifcant diferences between means (Scott-Knott test; $p<0.05$)

*white LED 6400 K (λ = 525 nm); 2) purple LED (2 blue LEDs, 1 red LED); 3) blue LED (λ = 430 nm); 4) green LED (λ = 520 nm); 5) orange LED (λ = 595 nm); 6) red LED (λ = 670 nm)

Effect of LEDs on the initial growth of *Adenium obesum*

Regardless of the culture medium, a significant effect of LEDs was observed on the whole-plant fresh and dry weights (Table 3), on the number and length of leaves, and on the whole-plant length (Table 4).

Whole-plant fresh weight: A significant effect of LED color was seen (Table 3). Greater fresh weight occurred with the use of the red LED in MS medium (2.01 ± 0.15 g) and in MRA medium (1.83 ± 0.15 g).

Whole-plant dry weight: Both the medium and color treatments showed significant effects (Table 3). Higher dry weight occurred with the use of the red LED in MS medium (0.08 ± 0.006 g) and in MRA medium (0.07 ± 0.006 g).

Table 3 Whole-plant fresh weight (WPFW), and whole-plant dry weight (WPDW) (B) in *A. obesum* seedlings grown in different culture media under different LEDs in a controlled-environment growth room.

Color LEDs*	WPFW (g)	WPDW (g)
Dark, MS	$0.17^{b} \pm 0.15$	$0.009^{c} \pm 0.006$
Dark, MRA	$0.42^{b} \pm 0.15$	$0.007^{c} \pm 0.006$
White LED, MS	$0.32b^{b} \pm 0.15$	$0.016^{c} \pm 0.006$
White LED, MRA	$0.38^{b} \pm 0.15$	$0.021^{c} \pm 0.006$
Blue LED, MS	$0.31^{b} \pm 0.15$	$0.016^{c} \pm 0.006$
Blue LED, MRA	$0.67^{b} \pm 0.15$	$0.035^{b} \pm 0.006$
Green LED, MS	$0.34^{b} \pm 0.15$	$0.002^{c} \pm 0.006$
Green LED, MRA	$0.26^{b} \pm 0.15$	$0.011^{c} \pm 0.006$
Orange LED, MS	$0.22^{b} \pm 0.15$	$0.008^{c} \pm 0.006$
Orange LED, MRA	$0.39^{b} \pm 0.15$	$0.017^{c} \pm 0.006$
Purple LED, MS	$0.54^{b} \pm 0.15$	$0.034^{b} \pm 0.006$
Purple LED, MRA	$0.75^{b} \pm 0.15$	$0.040^{b} \pm 0.006$
Red LED, MS	$2.01^{a} \pm 0.15$	$0.080^{a} \pm 0.006$
Red LED, MRA	$1.83^{a} \pm 0.15$	$0.070^{a} \pm 0.006$

Data expressed as the mean value ± SD (n=3). Diferent superscript letters (a-c) within column for each parameter indicate signifcant diferences between means (Scott-Knott test; $p<0.05$)

*white LED 6400 K (λ = 525 nm); 2) purple LED (2 blue LEDs, 1 red LED); 3) blue LED (λ = 430 nm); 4) green LED (λ = 520 nm); 5) orange LED (λ = 595 nm); 6) red LED (λ = 670 nm)

Number of leaves: The LED color had a significantly different effect in different culture media (Table 4). A greater number of leaves was observed with the use of the red LED) in MS medium (12.33 ± 0.8 and in MRA medium (9 ± 0.8).

Length of leaves: Both treatments showed a significant effect (Table 4). Plants with longer leaves were observed under the red LED in MRA medium (17.35 ± 1.22 mm) and in MS medium (16.84 ± 1.22 mm).

Whole plant length: A significant effect was observed by both treatments (Table 4). A greater whole-plant length was observed with the use of the red LED in MS medium (128.52 ± 6 mm) and in MRA medium (111.21 ± 6 mm).

Table 4 Number of leaves, length of leaves (mm), and whole-plant length (mm) of *A. obesum* seedlings grown in different culture media under different LEDs in a controlled-environment growth room.

Color LEDs*	Number of leaves	Length of leaves (mm)	Plant length (mm)
Dark, MS	0.0^{e} ± 0.8	0.0^{d} ± 1.22	69.55^{c} ± 6
Dark, MRA	0.0^{e} ± 0.8	0.0^{d} ± 1.22	72.56^{c} ± 6
White LED, MS	7.0^{d} ± 0.8	12.42^{c} ± 1.22	84.50^{c} ± 6
White LED, MRA	7.6^{c} ± 0.8	13.86^{b} ± 1.22	72.61^{c} ± 6
Blue LED, MS	6.6^{d} ± 0.8	10.68^{c} ± 1.22	80.56^{c} ± 6
Blue LED, MRA	6.6^{d} ± 0.8	11.11^{c} ± 1.22	42.38^{d} ± 6
Green LED, MS	5.6^{d} ± 0.8	11.75^{c} ± 1.22	71.45^{c} ± 6
Green LED, MRA	6.6^{d} ± 0.8	9.37^{c} ± 1.22	73.13^{c} ± 6
Orange LED, MS	6.0^{d} ± 0.8	10.52^{c} ± 1.22	76.57^{c} ± 6
Orange LED, MRA	7.0^{d} ± 0.8	13.90^{b} ± 1.22	83.85^{c} ± 6
Purple LED, MS	4.3^{d} ± 0.8	13.25^{b} ± 1.22	53.9^{d} ± 6
Purple LED, MRA	7.3^{d} ± 0.8	14.58^{b} ± 1.22	54.5^{d} ± 6
Red LED, MS	12.3^{a} ± 0.8	16.84^{b} ± 1.22	128.52^{a} ± 6
Red LED, MRA	9.0^{b} ± 0.8	17.35^{b} ± 1.22	111.21^{b} ± 6

Data expressed as the mean value ± SD (n=3). Diferent superscript letters (a-d) within column for each parameter indicate signifcant diferences between means (Scott-Knott test; $p<0.05$)

*white LED 6400 K (λ = 525 nm); 2) purple LED (2 blue LEDs, 1 red LED); 3) blue LED (λ = 430 nm); 4) green LED (λ = 520 nm); 5) orange LED (λ = 595 nm); 6) red LED (λ = 670 nm)

Effects of LED color on the levels of photosynthetic pigments

The concentrations of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids in the leaves of the *A. obesum* seedlings grown in the MS and MRA media varied in response to the different LEDs (Table 5).

Chlorophyll *a*: Both treatments showed a significant effect (Table 5). A high level of chlorophyll *a* was observed with the white LED (0.31 ± 0.03 µg cm^{-2} of leaf), but this did not differ significantly from the green (0.26 ± 0.03 µg cm^{-2} of leaf) and orange LED values in MS medium (0.27 ± 0.03 µg cm^{-2} of leaf). The green LED chlorophyll *a* concentration (0.26 ± 0.03 µg cm^{-2} of leaf) did not differ significantly from the purple (0.25 ± 0.03 µg cm^{-2} of leaf) and red LED values in MRA medium (0.24 ± 0.03 µg cm^{-2} of leaf).

Chlorophyll *b*: Significant differences were observed between LED colors (Table 5). The highest chlorophyll *b* was obtained with the white LED in MS medium (0.38 ± 0.04 µg cm^{-2} of leaf), but this value did not differ significantly from that under the orange (0.36 ± 0.04 µg cm^{-2} of leaf), green (0.34 ± 0.04 µg cm^{-2} of leaf), blue (0.27 ± 0.04 µg cm^{-2} of leaf), or red LED in MS medium (0.24 ± 0.04 µg.cm $^{-2}$ of leaf). The chlorophyll *b* concentration in MRA medium did not differ between the green (0.31 ± 0.04 µg cm^{-2} of leaf), red (0.29 ± 0.04 µg cm^{-2} of leaf), purple (0.29 ± 0.04 µg cm^{-2} of leaf), white (0.28 ± 0.04 µg cm^{-2} of leaf), and blue LED groups (0.23 ± 0.04 µg cm^{-2} of leaf).

Total chlorophyll: LED color had a significant effect on total chlorophyll (Table 5). The highest level of total chlorophyll was observed with the white LED in MS medium (1.33 ± 0.14 µg cm^{-2} of leaf), but this did not differ significantly from the orange (1.26 ± 0.14 µg cm^{-2} of leaf), green (1.22 ± 0.14; 0.88 ± 0.11 µg cm^{-2} of leaf), blue (0.94 ± 0.14; 0.67 ± 0.11 µg cm^{-2} of leaf), and red LED values in MS medium (0.88 ± 0.14; 0.64 ± 0.11 µg cm^{-2} of leaf). The total chlorophyll in MRA medium was similar between the green (1.11 ± 0.14 µg cm^{-2} of leaf), red (1.07 ± 0.14 µg cm^{-2} of leaf), white (1.02 ± 0.14 µg.cm^{-2} of leaf), purple (1.00 ± 0.14 µg cm^{-2} of leaf), and blue LEDs (0.74 ± 0.14 µg cm^{-2} of leaf).

Carotenoid: LED color showed a significant effect on carotenoid (Table 5). The highest level of carotenoids was observed under the white LED in MS medium (0.96 ± 0.11 µg cm^{-2} of leaf), and under the orange (0.96 ± 0.11 µg cm^{-2} of leaf) but this values did not differ significantly from under green (0.88 ± 0.11 µg cm^{-2} of leaf), red (0.64 ± 0.11 µg cm^{-2} of leaf), and blue LEDs in MS medium (0.67 ± 0.11 µg cm^{-2} of leaf). The carotenoid concentration in MRA medium was similar between the green (0.81 ± 0.11 µg cm^{-2} of leaf), red (0.79 ± 0.11

μg cm^{-2} of leaf), white (0.74 ± 0.11 μg cm^{-2} of leaf), and purple LEDs (0.72 ± 0.11 μg cm^{-2} of leaf).

Table 5 Chlorophyll-a (μg cm^{-2}), chlorophyll-b (μg cm^{-2}), total chlorophyll (μg cm^{-2}), and carotenoids (μg cm^{-2}) in *A. obesum* seedlings grown in different culture media under different LEDs in a controlled-environment growth room.

Color LEDs*	Chlorophyll-a	Chlorophyll-b	Total chlorophyll	Carotenoids
Dark, MS	0.005^{c} ± 0.03	0.009^{c} ± 0.04	0.026^{c} ± 0.14	0.020^{c} ± 0.11
Dark, MRA	0.001^{c} ± 0.03	0.020^{c} ± 0.04	0.110^{c} ± 0.14	0.080^{c} ± 0.11
White LED, MS	0.310^{a} ± 0.03	0.380^{a} ± 0.04	1.330^{a} ± 0.14	0.960^{a} ± 0.11
White LED, MRA	0.210^{b} ± 0.03	0.280^{a} ± 0.04	1.020^{a} ± 0.14	0.740^{a} ± 0.11
Blue LED, MS	0.220^{b} ± 0.03	0.270^{a} ± 0.04	0.940^{a} ± 0.14	0.670^{a} ± 0.11
Blue LED, MRA	0.170^{b} ± 0.03	0.230^{a} ± 0.04	0.740^{a} ± 0.14	0.510^{b} ± 0.11
Green LED, MS	0.260^{a} ± 0.03	0.340^{a} ± 0.04	1.220^{a} ± 0.14	0.880^{a} ± 0.11
Green LED, MRA	0.260^{a} ± 0.03	0.310^{a} ± 0.04	1.110^{a} ± 0.14	0.810^{a} ± 0.11
Orange LED, MS	0.270^{a} ± 0.03	0.360^{a} ± 0.04	1.260^{a} ± 0.14	0.910^{a} ± 0.11
Orange LED, MRA	0.150^{b} ± 0.03	0.160^{b} ± 0.04	0.550^{b} ± 0.14	0.390^{b} ± 0.11
Purple LED, MS	0.160^{b} ± 0.03	0.180^{b} ± 0.04	0.640^{b} ± 0.14	0.470^{b} ± 0.11
Purple LED, MRA	0.250^{a} ± 0.03	0.290^{a} ± 0.04	1.000^{a} ± 0.14	0.720^{a} ± 0.11
Red LED, MS	0.210^{b} ± 0.03	0.240^{a} ± 0.04	0.880^{a} ± 0.14	0.640^{a} ± 0.11
Red LED, MRA	0.240^{a} ± 0.03	0.290^{a} ± 0.04	1.070^{a} ± 0.14	0.790^{a} ± 0.11

Data expressed as the mean value ± SD (n=3). Diferent superscript letters (a-d) within column for each parameter indicate signifcant diferences between means (Scott-Knott test; $p<0.05$)

*white LED 6400 K (λ = 525 nm); 2) purple LED (2 blue LEDs, 1 red LED); 3) blue LED (λ = 430 nm); 4) green LED (λ = 520 nm); 5) orange LED (λ = 595 nm); 6) red LED (λ = 670 nm)

Discussion

Light has a great influence on seed germination, growth and morphological characteristics of plants (Yuanchun et al., 2021). Different lighting sources have already been used in *in vitro* growth rooms, such as high-pressure sodium lamps, fluorescent lamps, and LEDs (Goméz & Izzo, 2018). According to Ouzounis et al. (2014), LED technology has great potential for market expansion. However, the results available from previous studies are controversial, as they have studied different plant species and cultivars, as well as various experimental conditions, which make more investments in research necessary.

LED lamps can focus the radiation near the plant canopies, require less energy for the photon flux to reach the target, and can be placed near the plants without overheating them or causing phytotoxicity (Goméz and Izzo, 2018; Al Murad et al., 2021). According to Van Iersel & Gianino (2017), there is the possibility of changing the light spectrum using specific heat-resistant adhesive tapes, eliminating the need to change the LED lights, which varies according to the physiological change of the culture, with spectra appropriate for the phase germination, vegetative and reproductive growth.

Effect of different LEDs on the *in vitro* germination of *A. obesum*

The seeds of *A. obesum* have *in vitro* germination of approximately four days, considering the rupture of the integument and the protrusion of the primary root (Colombo et al., 2015; Varella et al., 2015; Portes et al., 2018). The results obtained in (Table 3) show that the lower the MGT is, the more vigorous the sample can be considered. A similar result was obtained by Cabral et al. (2017), where *Piper marginatum* Jacq. seeds germinated *in vitro* under different light filters (blue, green, red, extreme red, and transparent) resulted in higher GSI when the seeds were subjected to red light. This work demonstrates that the quality and quantity of light directly influenced the germination and initial growth of the plant. Furthermore, the physiological quality of seeds can also influence the response to these variations.

According to Nakagawa (1999), the higher the GSI is, the higher the germination speed is, suggesting that the seed lot is more vigorous. In a study by Júnior et al. (2018) on two lettuce cultivars subjected to four light intensities (transparent, red, distant red, and dark), the results showed that both showed excellent germination indices and GSI with red light.

The incidence of the red and blue LED light spectrum most likely alters the structure of inactive phytochrome by converting it into active phytochrome (Taiz et al., 2017), triggering a series of reactions and stimulating the germination process of *A. obesum* seeds. Another important aspect is related to the species' origin in arid regions, which have a high incidence of light and high temperatures (Colombo et al., 2018). Excess light and high concentrations of some nutrients in the culture medium may have been related to high postemergence seedling mortality rates in some treatments (Table 2). According to Silva et al. (2016), excess light can promote photovoltaic changes in plants, leading to the production of reactive oxygen species, which can be detrimental to root and vegetative development.

The process of seed germination in some species can be positively or negatively affected by specific light spectra, which can exert a strong effect on plant life affect their growth through photosynthetic rate and photomorphogenesis (Simlat et al., 2016). Light

quality and intensity can promote different physiological, morphological, and phytochemical responses in the processes of seed germination and plant development (Barrales-López et al., 2015). Studies involving the use of different light sources to induce seed germination *A. obesium* are limited.

Effect of LEDs on the initial growth of *A. obesum*

The success of an *in vitro* culture system is related to the quality of light, it is important to identify the best spectra and models that provide the production of plants with desired characteristics (Miler et al., 2019). The results shown in (Table 3 & Table 4) show the strong effects of the red LED on the seedlings grown in both culture media. This color favored the initial development faster than other LEDs.

According to Chen et al. (2014), the spectral quality of light, as it effects the plant through the wavelengths absorbed by phytochrome through specific photoreceptors, activates enzymes associated with the synthesis of auxins, which affects several morphological (Table 3 & Table 4) and physiological aspects (Table 5). This improves the plant's initial development, increases shoot height and internode lengths, and promotes root growth, allowing faster acclimatization and increasing field survival rates.

Other studies have focused on spectral quality using red light in other cultures: a) *Jatropha curcas* and *Protea cynaroides* (Daud et al., 2013; Wu and Lin., 2013), *Stevia rebaudiana* (Simlat et al., 2016) had increased root formation; b) chrysanthemums (Kim et al., 2004), *Cattleya loddigesii* (Araújo et al., 2009) had increased shoot length; c) *Dendrobium phalaenopsis* (Sorgato et al., 2016) had increased fresh weight; d) *Cattleya* (Cybularz - Urban et al., 2007) had increased shoot length; and in *in vitro* culture: a) *Vaccinium corymbosum* (Hung et al., 2016), b) *Scrophularia takesimensis* (Jeong; Sivanesan, 2015), and c) *Oncidium* spp. (Chung et al., 2010) all had significant increases in leaf expansion, number of leaves, number of roots, dry mass, and chlorophyll content, while, d) *Plectranthus amboinicus* (Silva et al., 2017) showed increased plant growth. These results corroborate those obtained in the present study, e) *Stevia rebaudiana* Bertoni (Shulgina et al., 2021) stimulates adventitious shoot growth, and increases their number of leaves. Therefore, the effect of light is species specific.

According to Taiz et al. (2017), many plant species can obtain dry mass gain, stem elongation, expansion of the leaf area and improvement in photosynthetic efficiency under red LED (600-700 nm), being able to grow and complete its life cycle. However, with the

addition of low proportions of blue light, plant growth and development improves significantly.

Silva et al. (2014) reported that red LEDs can increase the growth rate of plants compared to white LEDs because they output a combination of low intensities of red and blue lights and other low-efficiency light wavelengths. In a study conducted by Cioć et al. (2019), higher photosynthetic photon flux densities (20 µmol m^{-2} s^{-1}) in the micropropagation of *Gerbera* j*amesonii* Bolus shoots increased leaf blade area and width and the proportions of circularity and elongation.

The results of this study corroborate those of Rocha et al. (2014), in which they evaluated the use of LEDs in the multiplication and rooting *in vitro* of two raspberry cultivars, and observed the highest percentages of rooting in the cultivar Batum (95.9%) and Dorman Red (90.2%) were obtained under the red LEDs, which contributed to the greater length of the rooted shoots.

The differences in the rates of increase of the variables analyzed in each culture medium may be related to the nutritional content provided to the seedlings.

Effect of different LEDs on the levels of photosynthetic pigments

The chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids (Table 5) were affected by the treatments with different artificial lightings and culture media because chlorophyll is involved in light absorption. The highest levels of pigments were observed in the white LED in combination with MS medium and under the green LED with MRA medium. Similar results were obtained by Roni et al. (2017) in *Lisianthus* when testing red, blue, and white monochromatic LEDs. However, the chlorophyll content in the leaves treated with white LEDs was higher than that in the leaves treated with the other LEDs.

In the *in vitro* culture environment, plants suffer from the stress caused by light intensity, which is responsible for determining the direction of morphogenesis. Very high irradiation affects photosynthetic capacity during the gas exchange process, destroying photopigment synthesis and the photosynthetic apparatus, and very low irradiation makes photosynthesis ineffective (Cioć et al., 2018).

The leaves of the plants have the ability to reflect, transmit or absorb light, converting chemical energy through photosynthetic photons, which can be used during the morphogenetic process, identifying the quality and quantity of light by photoreceptors (Batista et al., 2018). In the presence of light, the synthesis of chlorophylls and photo-oxidation usually occur, a reduction in the levels of total chlorophyll is expected in the face of

excess light. However, the concentration of carotenoids directly interferes with the increase in photo-oxidation and may prevent chlorophyll from photo-destruction, which in turn can affect the plant's biomass yield (Alvarenga et al., 2015; Arriaga et al., 2020; Yuanchun et al., 2021).

White LEDs (460-560 nm) have a light spectrum with a higher proportion of blue and green wavelengths and a lower proportion of red wavelengths. Studies conducted with LEDs show that plants need the spectral quality of a broad light spectrum to optimize their photosynthetic processes and that their responses vary according to the species and/or cultivar used (Fraszczak et al., 2015). Cope and Bugbee (2013) evaluated the effects of three types of white LEDs, with 11, 19, and 28% blue light, on the growth and development of *Raphanus sativus*, *Glycine max*, and *Triticum* spp. and observed that white LED lamps with 11% blue light promoted the greatest plant height. Light bulbs with 28% blue light resulted in more compact plants. Ferreira et al. (2016) found that white LEDs provided higher chlorophyll and carotenoid contents in the *in vitro* multiplication phase of sugarcane (RB 867515).

The blue light (400-500 nm) is an energy source responsible for the photosynthetic assimilation of CO_2, chlorophyll synthesis, chloroplast development, stomatal opening, and photomorphogenesis in plants chloroplast development, stomatal opening in plants. The exchange of air and water with the external environment is carried out by the stomata, which are influenced by the quality of light. It is possible to suggest from the results that the largest leaves improved the light interception by *A. obesum* seedlings. (Taiz et al., 2017; Batista et al., 2018; Rahman et al., 2021; and Yan et al., 2021).

Plants under adequate light can fully self-regulate to allow us to obtain the best condition of light energy absorption and transformation (Fraszczak et al., 2015). Green light also affects the action of blue light, by inhibiting or reducing its effects. The effect of the reversal of blue light–stimulated stomatal opening by green light may be an ecological adaptation to avoid excessive loss of leaf water by the stomata in shaded environments, which are rich in this green region of the light spectrum, because the photosynthetic potential is small in these sites (Aasamaa & Aphalo, 2016).

Although the wavelengths in the blue and red regions are more effective at promoting photosynthesis, the green spectrum can penetrate leaves more efficiently and increase carbon fixation, especially in shaded environments (Wang & Folta, 2013). Improvements in photosynthesis measurement techniques for in vitro culture are needed. It is important to note that the shape of the culture vessel and the characteristics of the material (thickness and composition of the glass or plastic) affect the spectral irradiance (Batista et al., 2018).

Conclusions

Red LEDs combined with MRA medium and purple LEDs combined with MS medium promoted the greatest germination rates and lowest mortality rates. The best initial *in vitro* development of *A. obesum* seedlings occurred under the red LED in both culture media, and these conditions promoted positive changes in the morphological variables analyzed. White LEDs combined with MS medium and green LEDs combined with MRA medium provided the highest levels of photosynthetic pigments. However, further investigations are needed, associated with the conscious use of the quality of LED light in controlled in vitro environments with new experiments to be planned, emphasizing the production of biomass, increased germination and development rate, and the growth of seedlings, ensuring plant uniformity.

Author contribution statement

Elaboration of the research project, collection of data analysis and interpretation, preparation of the article, final approval of the version to be published.

Competing Declaration of Interest

No interest to declare.

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2º ARTICLE
Industrial Crops & Products
Under review

INFLUENCE OF PHOTOPERIOD AND MRA CULTURE MEDIUM ON THE INITIAL GROWTH *in vitro* OF *Adenium obesum*

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Abstract

The cultivation of desert rose (*Adenium obesum*) is a relatively recent activity in Brazil, and little information is available on photoperiods and nutritional requirements for *in vitro* culture. As a result, low-quality products are inefficiently produced at a high cost. Thus, the objective of this study was to evaluate the effect of photoperiods and their interaction with concentrations of MRA medium on the initial growth of *A. obesum* plants cultivated *in vitro*. The plants were grown *in vitro* under different photoperiods (08/16; 12/12; 16/08; 20/04) combined with different MRA medium concentrations (0, 50, 100, 150, 200%). The experimental design was completely randomized in a 4x5 factorial scheme. At 70 days, the number of leaves, leaf length, leaf diameter, root length, shoot length, caudex diameter, whole-plant fresh and dry weight, and chlorophyll *a*, *b*, total and carotenoid contents were evaluated. The 0% + 12/12, 50% + 08/16, 100% + 12/12, 150% + 12/12, and 200% + 08/16 combinations between MRA medium concentrations and photoperiods were more favorable for *in vitro* cultivation of *A. obesum* up to 70 days.

Keywords: Plant nutrition; micropropagation; substrate; succulent.

Abbreviations: MRA, Ronilson Araújo medium; WPFW, whole-plant fresh weight; WPDW, whole-plant dry weight; N, nitrogen; P, phosphorus; K, potassium; Ca, calcium; CO_2, carbon dioxide; Mg, magnesium; S, sulfur; B, boron; CAM, crassulacean acid metabolism; CV, coefficient of variation; Cl, chlorine; Cu, copper; Fe, iron; DF, degrees of freedom; Mn, manganese; Mo, molybdenum; Ni, nickel; Zn, zinc; F, photoperiod; g, grams;

1. Introduction

The genus *Adenium*, popularly known as desert rose, is a member of the family Apocynaceae, which is native to tropical Africa, south Sahara from Senegal to Sudan and Kenya, and through Saudi Arabia, Oman, and Yemen (Plaizier 1980; Oyen 2008; Talukdar 2012; Akhtar et al. 2016). These plants are valued for their floral diversity and architectural shapes and well-developed caudex (Dimmitt et al. 2009).

The micropropagation of ornamental species combined with nutritional balance enables better plant production in terms of quantity, quality, uniformity, and effective disease control (Wang et al. 2008; Martinez-Ballestra et al. 2010; Marschner 2012; Veatch-Blohm et al. 2012), and these characteristics determine the sales price. Thus, it is important to pay attention to the adequate amounts and balance of nutrients in the *in vitro* culture medium.

Chemical elements are essential for plants to complete their life cycle, where macronutrients (N, P, K, Ca, Mg and S) are absorbed in greater amounts by plants, while micronutrients (B, Cl, Cu, Fe, Mn, Mo, Ni and Zn) are absorbed in smaller amounts, and these nutrients are responsible for vegetative growth and development (Arnon & Stout 1939; Taiz et al. 2017). Furthermore, McBride et al. (2014) reported that N, P, K, Ca and Mg are the nutrients present at higher levels in *Adenium obesum* (desert rose).

The initial development of plants grown *in vitro* is expensive, as it requires using culture media with higher added value. The formulation of new media using cheaper raw materials, without affecting the quality of the plants, tends to increase profitability. In this vein, the tests (germination, number of leaves, length of leaves, whole-plant length and photosynthetic pigments) performed by Araújo et al. (2021) with MRA medium demonstrated satisfactory results in *A. obesum* production. Another factor that burdens production costs is the agar gelling agent, but medium sand can be used as an alternative.

Regarding the photoperiod, most studies on *A. obesum* report the use of 16 hours of light (Varella et al. 2015; Kanchanapoom et al. 2010; Araújo et al. 2021). According to Chang (1974), photoperiodism is a response to variation in day length. Therefore, photoperiod influences the formation of pigments, flowers, fruits, seeds, bulbs and tubers, as well as vegetative growth, dormancy and plant death, branching, leaf shape, abscission and leaf drop, pubescence and root development.

As the cultivation of *A. obesum* is a relatively recent activity in Brazil, there is little information regarding nutritional requirements and photoperiods for *in vitro* cultivation, resulting in low-quality products produced inefficiently and at a high cost, justifying the importance of experiments designed to improve the nutrition of ornamentals (Neto et al. 2015).

Therefore, the objective of this study was to evaluate the effect of photoperiods and their interaction with concentrations of MRA medium on the initial growth of *A. obesum* plants cultivated *in vitro*.

2. Materials and Methods

2.1. *Experiment location*

The experiment was carried out at the Plant Tissue Culture Laboratory of Federal University of Lavras, located in Lavras, Minas Gerais, Brazil.

2.2. *Seed preparation*

A. obesum seeds were first immersed in 70% alcohol for 1 min and then transferred to a 30% sodium hypochlorite solution (2.5% w/v active chlorine), and 1 drop of Tween® 20 was added under stirring for 2 min.

Next, in a laminar flow chamber sanitized with 70% alcohol, the seeds were triple-washed with deionized and autoclaved water to remove the disinfectant. Subsequently, the seeds were inoculated in 200-mL flasks containing 20 g of coarse sand and 10 mL of deionized water. The flasks were sealed with parafilm and kept in a growth room at 25 ± 2 °C with a photoperiod of 16 h provided by a white LED lamp (6400 K-λ = 525 nm) and mean irradiance of 49.4 µmol m^2 s^{-1} for 24 days.

After 24 days, 10 mL of MRA medium was added to each vial (Araújo et al. 2021). The pH of the medium was adjusted to 5.7 ± 0.3 before autoclaving at 121 ± 1 °C and 1.05 atm for 20 min. Then, the flasks were sealed with parafilm and kept in a growth room at 25 ± 2 °C with four photoperiods (8/16; 12/12; 16/8; 20/4) under the white LED lamp (6400 K - λ = 525 nm) and mean irradiance of 49.4 µmol m^2 s^{-1}.

At 24 days, the plants had the following characteristics: 2.5 cm ± 0.2 shoot height, 0.1 cm ± 0.1 caudex diameter, and two pairs of fully expanded leaves (Fig. 1A).

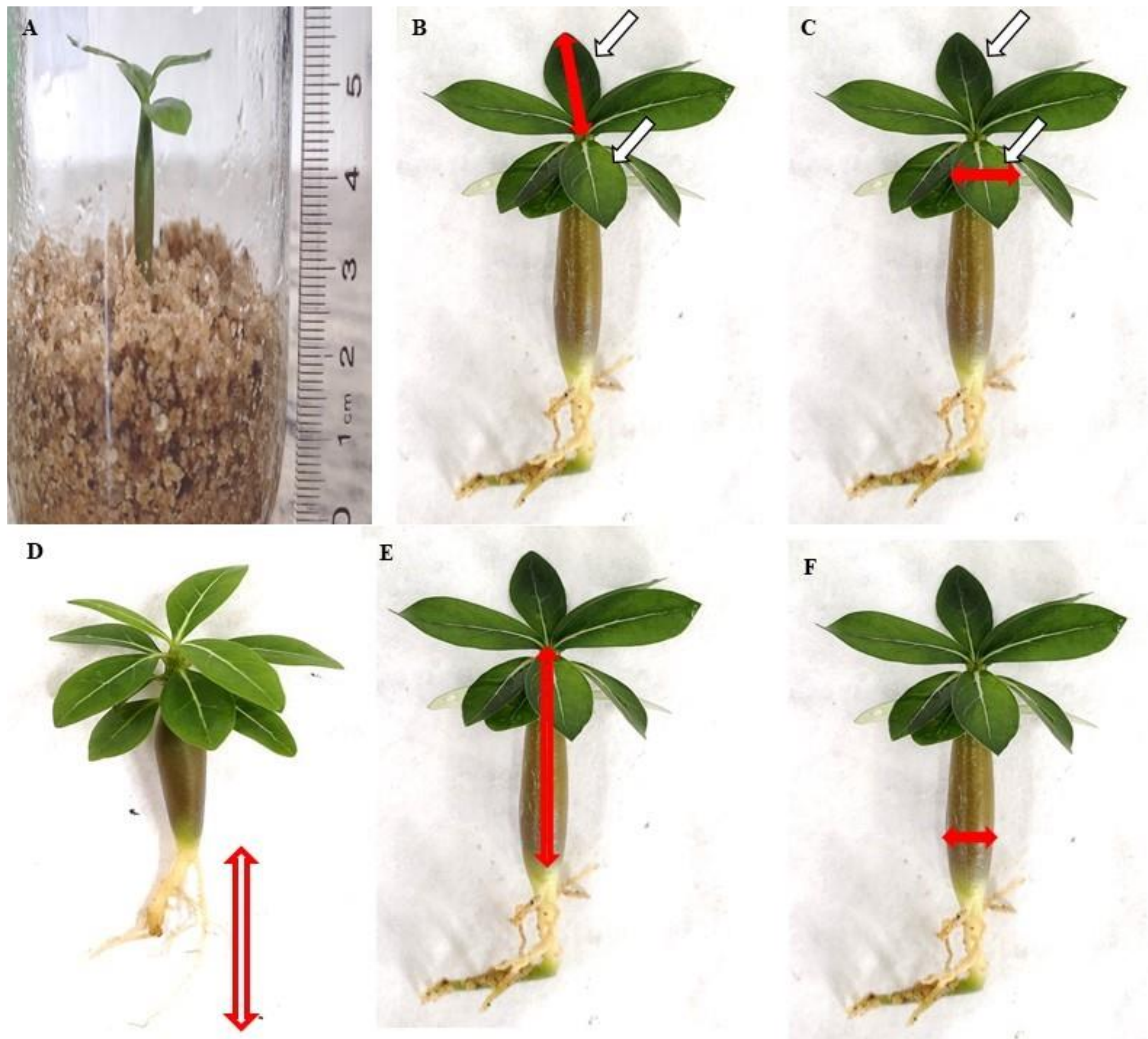

Fig. 1 *Adenium obesum* plant at 24 days of cultivation (A); indication of the 2nd pair of leaves with the correct position for measuring leaf length (B), leaf width (C), root length (D), shoot length (E) and caudex diameter (F).

2.3. *Analyses*

2.3.1. *Phytotechnics*

At 70 days after sowing, the following characteristics were evaluated: number of leaves; leaf length (Fig. 1B) and; width (Fig. 1C), measured from the 2nd pair of leaves, counted from the shoot apex; root length (Fig. 1D); shoot length (Fig. 1E), measured from the base to the apex of the stem; caudex diameter (Fig. 1F), measured at 0.5 cm above the stem base; whole-plant fresh weight; and whole-plant dry weight, for which plant samples were stored in paper bags and transferred to a forced ventilation oven and dried at 60 °C for 96 h and weighed on a CELTAC FA2104N digital scale.

2.3.2. *Photosynthetic pigments*

Fresh leaves (± 0.015 g) of *A. obesum* were transferred to test tubes containing 3 mL of 80% acetone. The test tubes were wrapped in aluminum foil to avoid contact of the sample with light to prevent pigment degradation. After 24 h in a refrigerator at ± 4 °C, the absorbance of the samples was measured in an ELISA Multiskan GO spectrophotometer (Thermo Fisher Scientific) at wavelengths of 470, 645, 652 and 663 nm, (Scopel et al. 2011).

To determine the chlorophyll *a*, *b*, total and carotenoid contents, the equations proposed by Lichtenthaler and Wellburn (1983) and Zhang et al. (2009) were used. The wavelength readings were performed in triplicate, with three repetitions per treatment. The analysis was performed using the Skanit Software for Microplate Readers RE version 5.0.0.42.

2.4. ***Experimental design and statistical analysis***

The experimental design was completely randomized in a 5 x 4 factorial scheme, with five concentrations of MRA culture medium (0, 50, 100, 150 and 200%) and four photoperiods (8/16; 12/12; 16/8; 20/04), with five replications of three plants each, totaling 20 treatments.

The data were subjected to analysis of variance, and the means were compared by Tukey's test at 5% probability using the SISVAR statistical software (Ferreira 2011).

3. Results

A significant interaction effect was observed between the factor's photoperiod and MRA medium concentration on the initial growth of *A. obesum* plants *in vitro*.

The breakdown of the effects of the photoperiod factor within the culture medium concentrations is shown in Tables 1 and 2. These factors (photoperiod and culture medium) affect the phytotechnical variables and photosynthetic content of *A. obesum* plants grown *in vitro*.

Table 1. Means of phytotechnical variables: number of leaves (NL), leaf length (LL; mm), leaf width (LW; mm), root length (RL; mm), shoot length (SL; mm), caudex diameter (CD; mm), whole-plant fresh weight (WPFW; g) and whole-plant dry weight (WPDW; g) of *A. obesum* plants after 70 days of *in vitro* cultivation.

MRA concentration	Photoperiod	Phytotechnical variables*							
		NL	LL	LW	RL	SL	CD	WPFW	WPDW
0	08/16	8,33^{B}	14,57^{B}	5,90^{A}	23^{B}	34,4^{B}	4,9^{A}	0,6^{A}	0,04^{A}
	12/12	9,60^{A}	16,00^{B}	6,20^{A}	28^{B}	33,44^{B}	4,3^{A}	0,6^{A}	0,04^{A}
	16/08	9,40^{A}	18,00^{A}	6,92^{A}	26^{B}	38,5^{A}	5^{A}	0,6^{A}	0,04^{A}
	20/04	8,10^{B}	13,57^{B}	5,67^{A}	36^{A}	34,20^{B}	4,8^{A}	0,6^{A}	0,03^{A}
50	08/16	7,46^{C}	12,50^{B}	4,64^{B}	14,60^{B}	37,40^{A}	5,1^{C}	0,6^{C}	0,03^{C}
	12/12	8,86^{B}	14,70^{B}	5,60^{B}	25,6^{A}	41^{A}	5,4^{C}	0,1^{B}	0,7^{B}
	16/08	8,93^{B}	18,54^{A}	6,79^{A}	28,74^{A}	40^{A}	6,7^{A}	1,33^{A}	0,14^{A}
	20/04	10,3^{A}	16,16^{B}	6,56^{A}	26,45^{A}	39,9^{A}	6,25^{B}	1,26^{A}	0,07^{B}
100	08/16	9,00^{A}	9,80^{C}	6,35^{B}	23,50^{A}	36,50^{B}	5^{C}	0,6^{B}	0,12^{A}
	12/12	7,00^{B}	23,70^{A}	8,14^{A}	25,7^{A}	43,46^{A}	5,8^{B}	1,14^{A}	0,06^{B}
	16/08	10,00^{A}	11,80^{C}	4,84^{C}	23^{A}	35,20^{B}	4,7^{C}	0,71^{B}	0,04^{C}

	20/04	$7,33^{B}$	$15,40^{B}$	$6,11^{B}$	$30,33^{A}$	$37,75^{B}$	$6,2^{A}$	$1,30^{A}$	$0,08^{B}$
150	16/8	$7,60^{A}$	$13,10^{B}$	$5,75^{A}$	$29,30^{A}$	$37,60^{B}$	$4,4^{A}$	$0,75^{A}$	$0,06^{A}$
	12/12	$8,00^{A}$	$17,17^{A}$	$6,12^{A}$	$24,00^{A}$	$41,10^{A}$	$4,3^{A}$	$0,77^{A}$	$0,06^{A}$
	08/16	$6,4^{B}$	$14,5^{B}$	$6,05^{A}$	$25,80^{A}$	$35,80^{B}$	$4,7^{A}$	$0,7^{A}$	$0,06^{A}$
	20/04	6^{C}	$14,30^{B}$	$6,89^{A}$	$24,42^{A}$	$23,42^{C}$	$3,3^{B}$	$0,29^{B}$	$0,06^{A}$
200	08/16	$8,00^{A}$	$12,90^{A}$	$4,32^{B}$	$20,90^{B}$	$32,69^{C}$	$3,25^{B}$	$0,3^{B}$	$0,02^{C}$
	12/12	$2,00^{D}$	$8,76^{B}$	$3,53^{B}$	$35,38^{A}$	$39,96^{B}$	$3,6^{B}$	$0,45^{B}$	$0,06^{B}$
	16/08	$3,60^{C}$	$9,35^{B}$	$3,08^{B}$	$19,10^{B}$	$43,50^{A}$	3^{B}	$0,35^{B}$	$0,03^{C}$
	20/04	$8,80^{A}$	$13,67^{A}$	$5,57^{A}$	$22,73^{B}$	$37,98^{B}$	$5,6^{A}$	$1,26^{A}$	$0,09^{A}$
Means	-	7.74	14.42	5.75	25.66	37.20	4.81	0.80	0.06
CV (%)	-	16.93	24.81	27.87	33.74	12.07	20.56	41.80	68.90

*Means followed by the same letter, on the column, do not differ by Tukey's test at 5% probability.

Table 2. Means of photosynthetic pigments: chlorophyll *a* (*Chl-a*; µg cm^{-2}), chlorophyll *b* (*Chl-b* ;µg cm^{-2}), total chlorophyll (*Chl-T*; µg cm^{-2}) and carotenoids (*Car*; µg cm^{-2}) of *A. obesum* plants after 70 days of *in vitro* cultivation.

MRA Concentration	Photoperiod	Content of photosynthetic pigments*			
		Chl a	*Chl b*	*Chl T*	*Car*
0	08/16	$1,52^{B}$	$0,89^{B}$	$2,42^{B}$	$0,41^{B}$
	12/12	$1,91^{A}$	$1,12^{A}$	$3,03^{A}$	$0,54^{A}$
	16/08	$1,48^{B}$	$0,80^{B}$	$2,29^{B}$	$0,40^{B}$
	20/04	$1,59^{B}$	$0,91^{B}$	$2,51^{B}$	$0,44^{B}$
50	08/16	$1,02^{C}$	$0,51^{C}$	$1,52^{C}$	$0,28^{C}$
	12/12	$1,96^{A}$	$0,90^{A}$	$2,86^{A}$	$0,53^{A}$
	16/08	$1,37^{B}$	$0,75^{B}$	$2,11^{B}$	$0,37^{B}$
	20/04	$1,44^{B}$	$0,61^{C}$	$2,06^{B}$	$0,38^{B}$
100	08/16	$1,28^{A}$	$0,60^{A}$	$1,88^{A}$	$0,94^{A}$
	12/12	$1,18^{A}$	$0,51^{A}$	$1,69^{A}$	$0,91^{B}$
	16/08	$1,15^{A}$	$0,52^{A}$	$1,67^{A}$	$0,91^{B}$
	20/04	$0,59^{B}$	$0,39^{B}$	$0,98^{B}$	$0,82^{C}$
150	08/16	$1,45^{A}$	$0,80^{B}$	$2,27^{A}$	$0,38^{A}$
	12/12	$1,43^{A}$	$0,86^{A}$	$2,30^{A}$	$0,32^{A}$
	16/08	$1,23^{A}$	$0,75^{B}$	$1,98^{B}$	$0,33^{A}$
	20/04	$1,20^{A}$	$0,56^{C}$	$1,77^{B}$	$0,17^{B}$
200	08/16	$0,77^{B}$	$0,37^{B}$	$1,15^{B}$	$0,40^{A}$
	12/12	$0,65^{B}$	$0,35^{B}$	1^{B}	$0,40^{A}$
	16/08	$1,39^{A}$	$0,66^{A}$	$2,06^{A}$	$0,34^{A}$
	20/04	$0,76^{B}$	$0,37^{B}$	$1,12^{B}$	$0,34^{A}$
Means	-	1.27	0.66	1.94	0.35
CV (%)	-	23.34	17.77	20.77	21.93

*Means followed by the same letter in the column do not differ by Tukey’s test at 5% probability.

The **0% concentration of MRA medium** provided better results when associated with the following photoperiods:

- **16/08:** number of leaves (9.4), leaf length (18 mm), leaf diameter (6.92 mm), shoot length (38.5 mm), caudex diameter (5 mm), whole-plant fresh weight (WPFW) (0,6 g) and whole-plant dry weight (WPDW) (0.04 g) **(Table 2)**;
- **12/12:** number of leaves (9.6), leaf diameter (6.2 mm), caudex diameter (4.33 mm), whole plant fresh weight (WPFW) (0.6 g) and whole-plant dry weight (WPDW) (0.04 g) **(Table 2)**, chlorophyll *a* (1.91 $\mu g.cm^{-2}$), chlorophyll *b* (1.12 $\mu g.cm^{-2}$), total chlorophyll (3.03 $\mu g.cm^{-2}$) and carotenoids (0.54 $\mu g.cm^{-2}$) **(Table 3)**;
- **08/16:** leaf diameter (5.9 mm), caudex diameter (4.9 mm), whole-plant fresh weight (WPFW) (0.6 g) and whole-plant dry weight (WPDW) (0.04 g) **(Table 2)**;
- **20/04:** leaf diameter (5.67 mm), root length (36 mm), caudex diameter (4.8 mm), whole-plant fresh weight (WPFW) (0.6 g) and whole-plant dry weight (WPDW) (0.03 g) **(Table 2)**. (Fig. 2A-D).

Figure 2 shows the appearance of the *A. obesum* plants at 70 days of in vitro culture in the treatments with different of MRA culture medium concentrations and photoperiods.

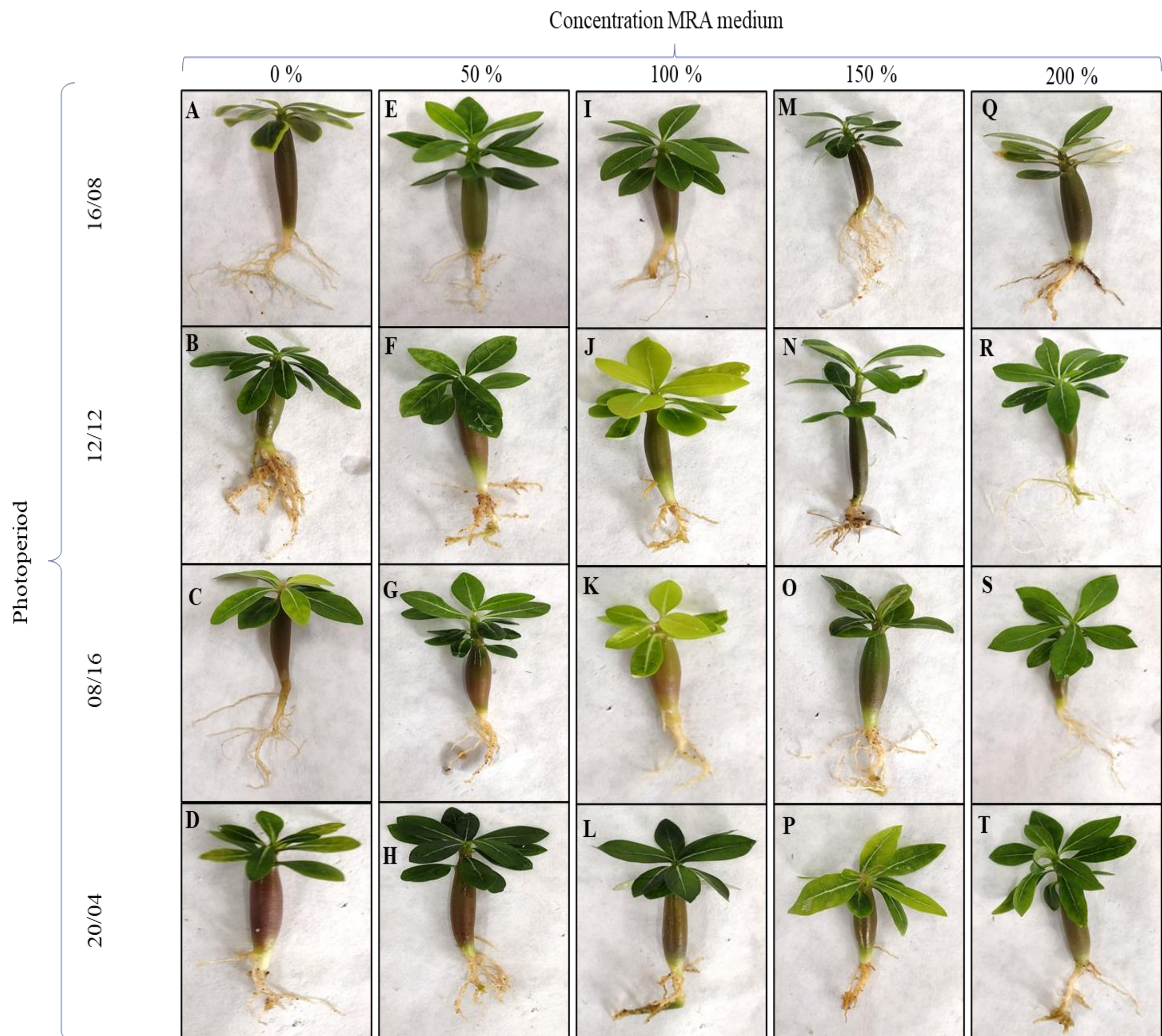

Fig. 2 *A. obesum* plants at 70 days of *in vitro* cultivation in different MRA culture medium concentrations and photoperiods: **a) 0% MRA:** 16/08 (A), 12/12 (B), 08/16 (C), 20/04 (D); **b) 50% MRA:** 16/08 (E), 12/12 (F), 08/16 (G), 20/04 (H); **c) 100% MRA:** 16/08 (I), 12/12 (J), 08/16 (K), 20/04 (L); **d) 150% MRA:** 16/08 (M), 12/12 (N), 08/16 (O), 20/04 (P); **e) 200% MRA:** 16/08 (Q), 12/12 (R), 08/16 (S), 20/04 (T).

The **50% concentration of MRA medium** provided better results when associated with the following photoperiods (Fig. 2E-H):

- **16/08:** leaf length (18.54 mm), leaf diameter (6.79 mm), root length (28.74 mm), caudex diameter (6.7 mm), shoot length (40 mm), whole-plant fresh weight (WPFW) (1.33 g) and whole-plant dry weight (WPDW) (0.14 g) **(Table 2)**;
- **12/12:** root length (25.6 mm), shoot length (41 mm) **(Table 2)**, chlorophyll *a* (1.96 $\mu g.cm^{-2}$), chlorophyll *b* (0.90 $\mu g.cm^{-2}$), total chlorophyll (2.86 $\mu g.cm^{-2}$) and carotenoids (0.53 $\mu g.cm^{-2}$) **(Table 3)**;
- **08/16:** shoot length (37.4 mm) **(Table 2)** and;

- **20/04:** number of leaves (10.3), leaf diameter (6.56 mm), root length (26.45 mm), shoot length (39.9 mm) and whole-plant fresh weight (WPFW) (1.26 g) **(Table 2)**.

The **100% concentration of MRA medium** provided better results when associated with the following photoperiods (Fig. 2I-L):

- **16/08:** number of leaves (10), root length (23 mm), whole-plant dry weight (WPDW) (0.14 g) **(Table 2)**, chlorophyll *a* (1.15 $\mu g.cm^{-2}$), chlorophyll *b* (0.52 $\mu g.cm^{-2}$) and total chlorophyll (1.67 $\mu g.cm^{-2}$) **(Table 3)**;
- **12/12:** leaf length (23.7 mm), leaf diameter (8.14 mm), root length (25.7 mm), shoot length (43.46 mm), whole-plant fresh weight (WPFW) (1.14 g) **(Table 2)**, chlorophyll *a* (1.18 $\mu g.cm^{-2}$), chlorophyll *b* (0.51 $\mu g.cm^{-2}$) and total chlorophyll (1.69 $\mu g.cm^{-2}$);
- **08/16:** number of leaves (9) and root length (23.5 mm) **(Table 2)**, chlorophyll *a* (1.28 $\mu g.cm^{-2}$), chlorophyll *b* (0.60 $\mu g.cm^{-2}$), total chlorophyll (1.88 $\mu g.cm^{-2}$) and carotenoids (0.94 $\mu g.cm^{-2}$) **(Table 3)**;
- **20/04:** root length (30.33 mm), caudex diameter (6.2 mm) and whole-plant fresh weight (WPFW) (1.30 g) **(Table 2)**.

The **150% concentration of MRA medium** provided better results when associated with the following photoperiods (Fig. 2M-P):

- **16/08:** number of leaves (7.6), leaf diameter (5.75 mm), root length (29.3 mm), caudex diameter (4.4 mm), whole-plant fresh weight (WPFW) (1.11 g) **(Table 2)**, chlorophyll *a* (1.23 $\mu g.cm^{-2}$) and carotenoids (0.33 $\mu g.cm^{-2}$) **(Table 3)**;
- **12/12:** number of leaves (8), leaf length (17.17 mm), leaf diameter (6.12 mm), root length (24 mm), shoot length (41.10 mm), caudex diameter (4.3 mm), whole-plant fresh weight (WPFW) (1.12 g) **(Table 2)**, chlorophyll *a* (1.43 $\mu g.cm^{-2}$), chlorophyll *b* (0.86 $\mu g.cm^{-2}$), total chlorophyll (2.30 $\mu g.cm^{-2}$) and carotenoids (0.32 $\mu g.cm^{-2}$) **(Table 3)**;
- **08/16:** leaf diameter (6.05 mm), root length (25.8 mm), caudex diameter (4.7 mm), whole-plant fresh weight (WPFW) (1.08 g) **(Table 2)**, chlorophyll *a* (1.45 $\mu g.cm^{-2}$), total chlorophyll (2.27 $\mu g.cm^{-2}$) and carotenoids (0.38 $\mu g.cm^{-2}$) **(Table 3)**;
- **20/04:** leaf diameter (6.89 mm), root length (24.42 mm) **(Table 2)**, chlorophyll *a* (1.20 $\mu g.cm^{-2}$) **(Table 3)**.

The **200% concentration of MRA medium** provided better results when associated with the following photoperiods (Fig. 2Q-T):

- **16/08:** shoot length (43.50 mm) **(Table 2)**, chlorophyll *a* (1.39 $\mu g.cm^{-2}$), chlorophyll *b* (0.66 $\mu g.cm^{-2}$), total chlorophyll (2.06 $\mu g.cm^{-2}$) and carotenoids (0.34 $\mu g.cm^{-2}$) **(Table 3)**;
- **12/12:** root length (35.38 mm) **(Table 2)** and carotenoids (0.40 $\mu g.cm^{-2}$) **(Table 3)**;
- **08/16:** number of leaves (8), leaf length (12.90 mm) **(Table 2)** and carotenoids (0.40 $\mu g.cm^{-2}$) **(Table 3);**

- **20/04:** number of leaves (8.80), leaf length (13.67 mm), leaf diameter (5.57 mm), caudex diameter (5.6 mm), whole-plant fresh weight (WPFW) (1.26 g) and whole-plant dry weight (WPDW) (0.09 g) **(Table 2)** and carotenoids (0.34 $\mu g.cm^{-2}$) **(Table 3)**.

4. Discussion

The *in vitro* cultivation experiments of *A. obesum* using different MRA culture medium concentrations and photoperiods resulted in significant responses in the initial growth in *A. obesum* for the number of leaves, leaf length, leaf diameter, root length, shoot length, caudex diameter, whole-plant fresh weight, whole-plant dry weight, chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids. This can be explained by the stressful environment in *in vitro* cultivation, which is associated with low gas exchange, high relative humidity, and high concentrations of nitrogen and sucrose, directly affecting physiological and biochemical processes, and decreased absorption of water and mineral salts, affecting plant growth (Murashige 1977; Nguyen et al. 2016; Araújo et al. 2021).

The MRA culture medium is rich in macro- and micronutrients, however, the nutritional requirements of *A. obesum* are quite specific, varying greatly between stages, environments and growing seasons (Araújo et al. 2021). The inadequate supply of an essential element (excess or deficiency) can result in damage to plant development, starting with changes at the molecular level and the formation of chemical compounds (Malavolta 2008), which can cause visible symptoms in different parts of the plant, depending on the severity of the nutritional imbalance, plant species and variety, and environmental factors (Rosa et al. 2012).

Araújo et al. (2021) reported that the MRA medium has the following composition: nitrogen (226.02 mg L^{-1}), calcium (779.15 mg L^{-1}), manganese (31.6 mg L^{-1}) and zinc (20.04 mg L^{-1}). However, in the present study, these amounts were changed at the different concentrations (0, 50, 100, 150 and 200%). The superior root growth performance of *A. obesum* plants grown *in vitro* at the 200% concentration and 12/12 photoperiod is attributed to the nutrients required in the root formation process. The synthesis of proteins, amino acids and nucleic acids is related to nitrogen, which also has an important role in the production of tryptophan. Endogenous auxin levels are influenced by zinc and manganese, and root growth and development are functions of calcium (Rosa et al. 2012).

A. obesum is a plant species with crassulacean acid metabolism (CAM), in which the stomata open predominantly at night to absorb atmospheric CO_2 and close during the day to prevent water loss. CAM plants have the following enzymes: a) PEPCase, which is responsible for fixing CO_2 at night; and b) Rubisco, which acts during the day in the Calvin cycle. This behavior saves water, resulting in the low accumulation of dry matter and providing good adaptation to drought in *A. obesum*. In addition, *A. obesum* plants can remain from 100 to 200 days without opening their stomata during the day (Marenco and Lopes 2009). The low dry matter accumulation obtained by Marenco and Lopes (2009) was also observed in the present study with the 0% concentration of MRA medium and the different photoperiods.

The photoperiod, that is, the time of exposure to light, regulates the number and anatomy of

leaves, leaf expansion and the biosynthesis of photosynthetic pigments. The activation of expression genes, transcription factors and different photoreceptors is responsible for this regulation (Bou-Torrent et al. 2015; Seiler et al. 2017).

Light is considered the most influential factor in the *in vitro* cultivation of plants and is responsible for several physiological processes and early development. It is visually possible to perceive the results of excess light intensity on plants due to the photooxidative effect, which increases the respiratory rate and reduces net photosynthesis. When combined with the different MRA medium concentrations, excess light led to a reduction in the absorption of water and nutrients and increased plant biomass, resulting in bleached or yellowed leaves. In the present study, plants grown under photoperiods with longer hours of light had long, pale leaves with irregular shape and size and reduced growth in the initial phase of development.

Singh et al. (2017) reported that plants reduce chlorophyll levels and accumulate anthocyanins in response to light stress, which may explain the low levels of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids in some treatments with longer hours of light exposure.

The greater shoot length in *A. obesum* plants can be explained by the exposure time to white LED (6400 K - λ = 525 nm), composed of a greater proportion of blue and green light, which favors photosynthesis (Table 4) and optimizes the light passing through the leaves and consequently favors plant growth (Lin et al. 2013; Fraszczak et al. 2015).

Although sand may be considered an unusual substrate type, it is a great substrate for succulent and cactus species, as they need a very permeable substrate that accumulates little water.

5. Conclusions

The 0% + 12/12, 50% + 08/16, 100% + 12/12, 150% + 12/12, and 200% + 08/16 combinations between MRA medium concentrations and photoperiods were more favorable for *in vitro* cultivation of *A. obesum* up to 70 days.

Author contribution statement

Study design, data collection, analysis and interpretation, drafting of the manuscript, final approval of the version to be published.

Declaration of competing interests

There are no conflicts of interest to declare.

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3º ARTICLE
Journal of Applied Research on Medicinal and Aromatic Plants
Under review

SODIUM HYPOCHLORITE IS EFFECTIVE IN ASEPSIS OF *Adenium obesum* SEEDS GERMINATED *IN VITRO*

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Abstract

Asepsis is an important step in *in vitro* micropropagation, as fungi and bacteria can interfere with seed germination and vigor. The objective of this study was to evaluate the effect of immersion time on sodium hypochlorite on sanitary attributes and germination on the asepsis of *A. obesum* seeds inoculated *in vitro*. The seeds were sanitized with solution containing 30% commercial sodium hypochlorite (NaClO) (2.5% active chlorine) for four periods of time (10, 20, 30 and 40 min) and then soed in MS medium. The experimental design was completely randomized. Evaluations started on the first day after sowed, followed daily for 32 days. There was a significant difference between the treatments used for all variables analyzed. The *in vitro* germination of *A. obesum* occurred in the different immersion times in the NaClO solution, with emphasis on 30 min, which presented lower percentage of contamination and imperfect seedlings, with a higher percentage of perfect seedlings when compared to the other asepsis times. Sodium hypochlorite solution (30%) at 30 min of immersion positively influences the germination of *A. obesum* seeds, enabling the production of perfect seedlings free of contaminants.

Keywords: Contamination, germination, exposure time, active chlorine.

Introduction

The ornamental plant *Adenium obesum*, known as Desert Rose, native to Africa and the Arabian Peninsula, grows in desert and semi-arid regions, adapted to hot and dry climates (Hossain et al., 2013; Akhtar et al., 2019).

The seeds have brown coloration, integument and brown gold pappus (bristling) at both ends, which helps in the dispersion by the wind. These organs are also the main ones for the propagation of the species, being considered more efficient and less costly for producers, since they allow the obtaining of plants with more swollen and showy caudex, important for the production of rootstocks (Colombo et al., 2015) and seedlings for propagation and *in vitro* micropropagation studies.

Seed asepsis is a promising procedure for reducing pathogens during the *in vitro* micropropagation stage. If performed inefficiently, because it is arranged in a medium rich in chemical and mineral compounds, contamination by fungi and bacteria can occur, when present, interfere in the germination and vigor of seeds.

To avoid this, research focusing on the asepsis of *in vitro* propagation structures, such as seed, for different species, has already been conducted, and it is determined that factors such as concentration and time of exposure to disinfectant, as well as the age and origin of the material, interfere in the success of this stage (Carmello & Cardoso, 2018).

In general, research results have shown that disinfectant, sodium hypochlorite, is efficient in controlling various pathogens without harming the physiological qualities of seeds, and may replace other chemicals (fungicides) (Coutinho et al., 2000; Tomazi et al., 2019), and that were previously used for the same purpose.

Sodium hypochlorite (NaClO) is a salt of hypochlorus acid that has 10 to 13% active chlorine. For the disinfection of *A. obesum* seeds propagated *in vitro*, active chlorine in the form of sodium hypochlorite is already used, however, in the literature, several exposure times and concentrations are found, respectively: **a)** 10 min, 25% commercial sodium hypochlorite (2.0% to 2.5% active chlorine) (Portes et al., 2018); **b)** 6 min, 2.5% commercial sodium hypochlorite (Varella et al., 2015); **c)** 3 min, 50, 20 e 10% commercial sodium hypochlorite (2,0% a 2,5% active chlorine) (Rasad et al., 2015); **d)** 30 min, 10% commercial sodium hypochlorite (0,5% active chlorine) (Kanchanapoom et al., 2010); **e)** 20 min, 30% commercial sodium hypochlorite (2,5% active chlorine) (Araújo et al., 2021).

The *in vitro* cultivation of *A. obesum* seeds assists in the development and/or improvement of micropropagation and regeneration protocols, in addition to providing important information on plant growth and initial development (Araújo et al., 2021). Reports

in the literature on asepsis and seed germination of *A. obesum in vitro* are scarce, which makes this type of scientific research important, since it expands the possibilities of study and discoveries about the species.

In this context, the objective was to determine the most effective period of time in the asepsis of *Adenium obesum* seeds for *in vitro* cultivation.

Materials and methods

The experiment was carried out at the Plant Tissue Culture Laboratory of the Federal University of Lavras, located in the municipality of Lavras, Minas Gerais, Brazil (44°57′50" W, 21°13′40" S, 919 m altitude).

A completely randomized experimental design was used, with four exposure times (10, 20, 30 and 40 min) and a concentration of 30% commercial sodium hypochlorite (2.5% active chlorine w/v), with 16 replicates with 10 seeds each, totaling 160 seeds per treatment. The weight of six hundred and twenty seeds was 10.25 g, with average length and diameter of 10.78 mm and 1.59 mm, respectively. The moisture content was 12%.

The seeds of *A. obesum* (Fig.1), were placed in a container with 100 mL of 70% alcohol for 1 minute and then transferred to a container containing 100 mL of sodium hypochlorite solution 30% (2.5% of active chlorine) and 1 drop of neutral detergent under agitation for 10 min (T0), 20 min (T1), 30 min (T3) and 40 min (T4). Then in the laminar flow chamber sanitized with álcool 70%, the triple washing was performed with deionized and autoclaved water to remove the disinfectant.

Fig. 1. Fruits of *A. obesum* in the development phase, 40 days after pollination (a) and seeds ready to

be soed (b).

Soon after, the seeds were soed individually in 100 mL test tubes containing 15 mL of MS culture medium (Murashige and Skoog 1962), supplemented with 30 g L^{-1} sucrose and 5.5 g L^{-1} agar, with pH adjusted to 5.7 ± 0.3 before autoclaving at 121 ± 1 °C and pressure of 1.05 atm for 20 min. After sowing, the tubes were sealed with parafilm and kept in a growth room at 25 ± 2 °C with a photoperiod of 16 h and white LED (6400 K - λ = 525 nm) for 30 days.

Evaluations

Evaluations started on the first day after sowed, followed daily for 32 days, observing and determining in each container: **a)** bacteria-contaminated seeds (BC) (%) e **b)** fungal -contaminated seeds (FC) (%), **c)** germination (G) (%), **d)** Germination speed index (GSI), **e)** Mean germination time (MGT), **f)** Mean germination speed (MGS), **g)** Percentage of perfect seedlings (P), that is, those with radicle emission, coethylene leaves, leaf beginnings and secondary roots and **h)** Percentage of imperfect seedlings (I), that is, those malformed, atrophied and or with reduction of the caulinar, basal, apical and shoot stem region.

Contamination by fungi and bacteria was estimated using all vials. The variables were calculated as follows:

a) Bacteria-contaminated seeds (%) (BC):

Obtained by expression:

$$\text{BC}\ (\%) = \frac{\text{N} \times 100}{\text{A}}$$

Where: **N**= number of seeds contaminated by bactéria.

A= number of seeds sanded.

b) Fungal -contaminated seeds (%) (FC):

Obtained by expression:

$$\text{FC}\ (\%) = \frac{\text{N} \times 100}{\text{A}}$$

Where: **N**= number of seeds contaminated by bacteria.

A= number of seeds sanded.

c) Germination (%) (G):

Calculated by the formula of Laboriau (1983):

$$\text{G}\ (\%) = \frac{\text{N}}{\text{A}} \times 100$$

Where: **N**= number of seeds contaminated by bacteria.

A= number of seeds sanded.

d) Germination speed index (GSI):

Calculated by the formula of Maguire (1962):

$$GSI = \frac{G1}{N1} + \frac{G2}{N2} + \cdots \frac{Gn}{Nn}$$

where: G_1, G_2, ... G_n= number of normal seedlings in the first, second, ..., nth observations.

N_1, N_2, ... N_n= number of days (or hours) after sowing. Unit: dimensionless.

d) Mean germination time (MGT):

Calculated by the formula of Labouriau (1983):

$$MGT = \left(\sum ni \times ti\right) / \sum ni$$

where: n_i = number of seeds germinated by day i.

t_i = incubation time.

i= 1 to 32 days. Unit: days.

e) Mean germination speed (MGS):

Calculated by the formula of Labouriau (1983):

$$\text{MGS} = \frac{1}{t}$$

where: **t**= mean germination time. Unit: days.

Statistics

The data were submitted to variance analysis, and the means compared by Tukey at 5% probability using the statistical software SISVAR (Ferreira, 2011).

Results

The result of the variance analysis indicates that there was a significant difference between the treatments used for almost all variables analyzed (Table 1) except for bacterial contamination that was not verified in any treatment.

Table 1. Summary of variance analysis for fungal -contaminated seeds (FC) (%), germination (G) (%), germination speed index (GSI), mean germination time (MGT) (days), mean germination speed (MGS) (days), percentage of perfect seedlings (P) and percentage of imperfect seedlings (I) from seeds pretreated with sodium hypochlorite for different periods of time and grown *in vitro.*

Variation factor	GL	FC (%)*	G (%)*	GSI*	MGT*	MGS*	P (%)*	I (%)*
Asepsis time	3	1580,22	122645	4428	1586	110,56	383719	162384
Residue	637	0,616	2,44	0,06	0,091	0,071	1,312	34,7
General mean	-	1,86	71,67	10,65	9,82	3,01	52,65	61,91
CV (%)	-	1,67	0,09	0,09	0,12	0,35	0,09	0,38

* significant to tukey test; $p<0,05$

For the FC, the time of 30 min was what allowed a greater asepsis of the seeds. In Fig. 2a-d it is possible to visualize plants contaminated by fungi in the different treatments performed. As for seed germination, its onset occurred from the third day after sowing, being the best result obtained for the time of 10 min of asepsis with 88.1% germination. As the immersion time increased, the percentage of G decreased (Table 2; Fig. 3a d).

Table 2. Result for fungal -contaminated seeds (FC) (%), germination (G) (%), germination speed index (GSI), mean germination time (MGT) (days), mean germination speed (MGS) (days), percentage of perfect seedlings (P) and percentage of imperfect seedlings (I) derived from seeds pretreated with sodium hypochlorite for different periods of time and grown *in vitro*.

Asepsis time	FC	G	GSI	MGT	MGS	Seedlings	
						P	I
min	%	%	-	Days		%	
10	$3{,}07^{B}$	$88{,}1^{A}$	$14{,}34^{A}$	$12{,}53^{A}$	$2{,}31^{D}$	$55{,}6^{B}$	$39{,}2^{C}$
20	$3{,}76^{A}$	$77{,}6^{B}$	$10{,}57^{C}$	$8{,}58^{D}$	$3{,}39^{A}$	$29{,}6^{C}$	$66{,}4^{B}$
30	$0{,}00^{D}$	$70{,}8^{C}$	$10{,}78^{B}$	$8{,}97^{C}$	$3{,}23^{B}$	$64{,}9^{A}$	$35{,}1^{D}$
40	$0{,}63^{C}$	$50{,}3^{D}$	$6{,}91^{D}$	$9{,}23^{B}$	$3{,}13^{C}$	$25{,}9^{D}$	$74{,}1^{A}$

Different letters (A-D) within the column for each parameter indicate significant differences between means (Tukey test; $p<0{,}05$).

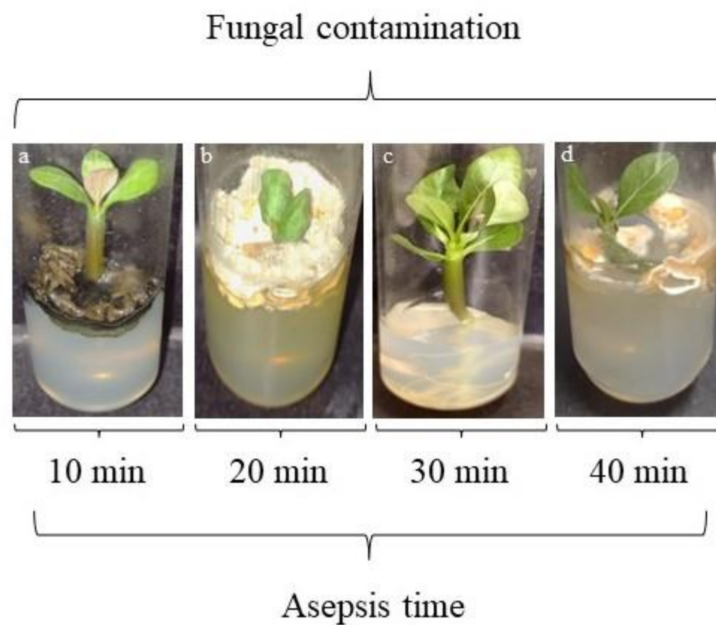

Fig. 2. Seedlings of *A. obesum*, with presence and absence of fungal contamination: 10 min (a), 20 min (b), 30 min (c), 40 min (d).

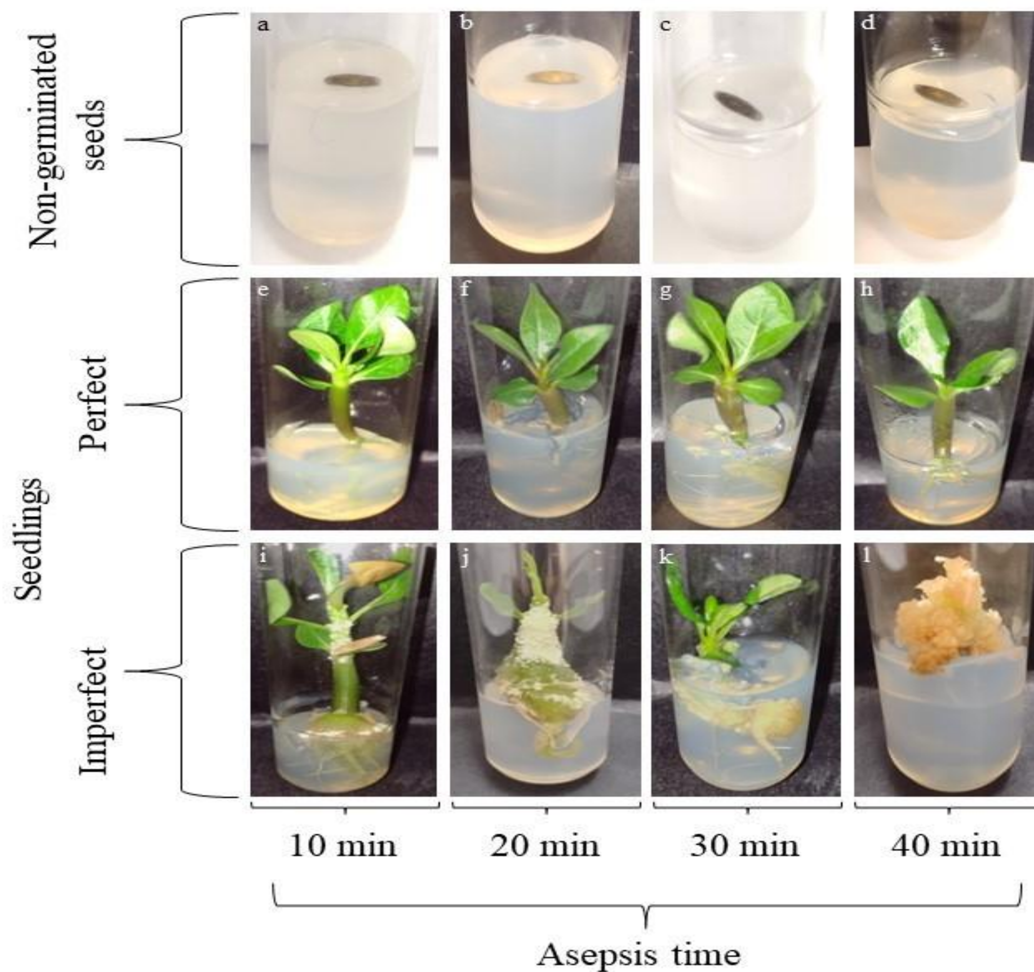

Fig. 3. Seeds and seedlings of *A. obesum*, propagated *in vitro*. Ungerminated seeds: 10 min (a), 20 min (b), 30 min (c), 40 min (d); Perfect seedlings: 10 min (e), 20 min (f), 30 min (g), 40 min (h) and; Imperfect seedlings: 10 min (i), 20 min (j), 30 min (k), 40 min (l).

The asepsis time of 10 min provided higher GSI (14.4) and MGT (12.53), presenting a large amount of perfect seedlings (55.6%) (Table 2) (Fig. 3e-h). For mgs, the best result (3.39) was obtained with a period of 20 min of immersion in sodium hypochlorite solution (Table 2), on the other hand, resulted in a high amount of imperfect seedlings (Fig. 3i-l). Seed immersion for 30 min was the one that provided the highest percentage of P (64.9%) and lower I percentage (35.1%) (Table 2).

Discussion

One of the biggest problems in *in vitro* germination of *A. obesum* is the contamination of the culture medium by fungi and bacteria, competing with the vegetable for nutrients, producing toxic substances and inhibiting development (Neto et al., 2014). Seeds may be contaminated endogenously or exogenously, which makes asepsis, for *in vitro* cultivation one of the most critical steps. Fungi and bacteria may be housed in the seed tegument, and may develop during the germination process in the culture medium (Scherwinski, Alterthum, 2010).

The reduction in the growth of microorganisms and obtaining healthy seedlings, free of fungi

and bacteria, can be obtained through seed asepsis methods, promoting the elimination and or superficial reduction of phytopathogens, reducing competition for nutrients in the culture medium. The absence of bacteria inside the tubes probably occurred due to disinfestation of the flow chamber, utensils and seeds, using 70% alcohol and sodium hypochlorite, which penetrates the cell wall of bacteria and disables the enzyme triosephosphate dihydrogenase, essential for the survival of microorganisms. Similarly, the low rate of fungal contamination probably occurred due to the use of sodium hypochlorite solution at different immersion times. The best combination of the ideal sanitizer concentration with more adequate immersion time allows obtaining decontaminated tissue without causing its death (Menegaes et al., 2019).

The best results obtained in the germination test of A. obesum may be directly related to the physiological maturity and respiratory activity of the seeds. Germination initiated through the process of instilling activates the Krebs cycle that depends on the moisture content of the seeds to produce energy that will help in the root emission process. The low moisture content in the seeds of this species (12%), allows a greater absorption of sodium hypochlorite solution 30% (2.5% of active chlorine) with the gradual increase in its exposure time, which may have negatively affected germination, consequently significantly reducing the final stand of seedlings, possibly as a result of damage suffered by embryo or reserve tissues, since sodium hypochlorite is a strong oxidizing agent (Neto et al., 2014).

The best result for GSI and MGT for the asepsis time of 10 min indicates a loss of vigor of the seeds as their exposure time to sanitizer increases, which may be directly related to the lower absorption of the disinfectant substance (Neto et al., 2014).

The use of sodium hypochlorite in the seed asepsis process is verified in studies carried out by: (Bevilacqua et al., 2011; Marigold); (Flores et al.,2012; Camu-Camuzeiro); (Oliveira et al., 2012; *Schizolobium amazonicum*); (Cavalcante et al., 2018; *Epidendrum secundum* Jacq); (Medeiros et al., 2015; *Caesalpinia ferrea*); (Pinheiro et al., 2016; forest species); (Menegaes et al., 2019; Safflower); (Santos et al., 2020; *Cenostigma tocantinum*); (Menegaes et al., 2021; Safflower); (Resende et al., 2021; Friar's Head).

The highest results for MGS indicate a reduction in seed germination power, influenced by asepsis time (Neto et al., 2014). The perfect seedlings present desirable characteristics in relation to the adult plant that will originate, especially: a) vigor; b) sanity; c) well-developed root; d) adequate height; e) healthy leaves; f) resistance to pests and diseases) (Scherwinski, Alterthum, 2010).

It is infered that the time of asepsis combined with genetic factors can cause higher rates of abnormal plants, causing irreversible damage in the seedlings of *A. obesum*, negatively affecting their development, impairing their commercial production.

Conclusion

The sodium hypochlorite solution (30%) for 30 min of immersion demonstrates a higher level

of efficiency in disinfestation, without any FC and BC, however it reduced the G in seeds of *A. obesum*, producing a large amount of perfect seedlings with reduction in the number of imperfect seedlings.

Author contribution statement

Elaboration of the research project, collection of data analysis and interpretation, preparation of the article, final approval of the version to be published.

Competing Declaration of Interest

No interest to declare.

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