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Original Paper

Optimal conditions for *in vitro* culture of *Cattleya cernua*, a small orchid native of Atlantic Forest and Cerrado

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Abstract

Cattleya cernua is an epiphytic orchid native of the Atlantic Forest, Cerrado, Caatinga and Pampa. Aiming at the development of an *in vitro* conservation technology, plants were micropropagated through asymbiotic culture and the influence of different concentrations of sucrose (10, 30, 60 and 90 g L^{-1}) and macronutrients (25, 50 and 100% MS) on survival and development was evaluated. Plant survival ranged between 47 and 100%. The interaction between macronutrients and sucrose influenced plant development. The aerial system of the plants was higher in 100% MS medium combined with 30 or 60 g L^{-1} of sucrose. The number of roots was higher with reduced macronutrients, combined with 30 or 60 g L^{-1} of sucrose. The length of the largest root was also higher when macronutrients were reduced but combined with 10 or 30 g L^{-1} of sucrose. The greatest mass was recorded when 30 g L^{-1} of sucrose was added to the three salt concentrations. Chlorophyll did not differ between plants grown with 30 or 90 g L^{-1} of sucrose. We recommend cultivating the plants in MS medium with 30 g L^{-1} of sucrose for better development of the aerial system. *C. cernua* can be asymbiotically micropropagated, facilitating *ex vitro* conservation strategies.

Key words: carbon source, conservation, *in vitro* culture, nutrients, orchid.

Resumo

Cattleya cernua é uma orquídea epífitica nativa da Floresta Atlântica, do Cerrado, da Caatinga e do Pampa. Visando ao desenvolvimento de uma ferramenta tecnológica *in vitro* para a conservação, plantas foram micropropagadas por meio da cultura assimbiótica, e a influência de diferentes concentrações de sacarose (10, 30, 60 e 90 g L^{-1}) e macronutrientes (25, 50 e 100% MS) sobre a sobrevivência e o desenvolvimento das plantas foi avaliada. A sobrevivência das plantas variou entre 47 e 100%. A interação entre macronutrientes e sacarose influenciou o desenvolvimento das plantas. O sistema aéreo das plantas foi superior no meio 100% MS, combinado com 30 ou 60 g L^{-1} de sacarose. O número de raízes foi superior com macronutrientes reduzidos, combinados com 30 ou 60 g L^{-1} de sacarose. O comprimento da maior raiz também foi superior quando os macronutrientes foram reduzidos, mas combinados com 10 ou 30 g L^{-1} de sacarose. A maior massa foi registrada quando 30 g L^{-1} de sacarose foram adicionados às três concentrações de sais. A clorofila não diferiu entre plantas crescidas com 30 ou 90 g L^{-1} de sacarose. Nós recomendamos cultivar as plantas em meio MS com 30 g L^{-1} de sacarose para melhor desenvolvimento do sistema aéreo. *C. cernua* pode ser micropropagada assimbioticamente, facilitando estratégias de conservação *ex vitro*.

Palavras-chave: fonte de carbono, conservação, cultura *in vitro*, nutrientes, orquídea.

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Introduction

Cattleya cernua (Lindl.) Van den Berg (basionym *Sophronitis cernua* (Lindl.) Lindl.) is a characteristic holoepiphytic orchid that reaches 15 cm in height and can occur both in the higher regions of trunks, in the primary and intermediate branches, and in areas more exposed to direct solar radiation (Cunha & Forzza 2007; Buzatto *et al*. 2010; Schinini 2010). During the reproductive period *C. cernua* presents a bright inflorescence of three to seven red-orange flowers (Buzatto *et al*. 2010), giving the species high ornamental value (Moreira *et al*. 2014). The tonality of the petals is the reason why the species is used to produce many interspecific and intergeneric hybrids (RHS 2016), which has led to intense extractivism, resulting in reduced natural populations (Moreira *et al*. 2014; Fig. 1a).

The species is native to South America, occurring in Paraguay (Schinini 2010), the northeast region of Argentina in Missiones (Johnson 2001), and in Brazilian states in the South, Southeast, and Center-West regions as well as the state of Bahia, in the Atlantic Forest, Cerrado, Caatinga and Pampa biomes (van den Berg 2020). Although the species is widely distributed, information on occurrence records is scarce and sparse (Moreira *et al*. 2014).

Biomes such as the Cerrado and Atlantic Forest are considered global biodiversity *hotspots* (Myers *et al*. 2000) that have been highly degraded. Among the plant formations of the world similar to savannas, the Cerrado is recognized as one of the richest in biological diversity. On the other hand, in spite of its biological importance, the Cerrado has the smallest protected area among Brazilian biomes (MMA 2017). Scientific information of Orchidaceae occurring in the Cerrado is scarce. The Atlantic Forest, another Brazilian biome with a high degree of biological diversity, is reduced to about 8% of its original area of approximately 1,315,460 km² (Fundação SOS Mata Atlântica 2019). More than 20 thousand plant species are found in the Atlantic Forest, 40% of which are considered endemic (Fundação SOS Mata Atlântica 2019).

Due to intense environmental degradation of Brazilian biomes, *in situ* and *ex situ* conservation strategies for plants are extremely important for the conservation of species, especially epiphytic Orchidaceae, since several species of the family are listed as endangered (Martinelli & Moraes 2013). One of the *ex situ* conservation strategies is performed through *in vitro* propagation through sexually-produced seeds (Sasamori *et al*. 2015), since *in vitro* germination enables the genetic variability of the plants to be maintained (Benson 1999; Pinto *et al*. 2010). The asymbiotic culture results in a high seed germination rate (Pedroso-de-Moraes *et al*. 2009; Pereira *et al*. 2015; Herrera *et al*. 2017) and rapid development of seedlings (Galdiano Júnior *et al*. 2013a), which are attributes not achieved by orchids in the natural environment. Moreover, it can be a highly efficient process for *in vitro* orchid propagation, since in laboratory conditions symbiotic seed germination requires previous isolation and culture of the fungi, and presents higher risk of contamination (Johnson *et al*. 2007; Abraham *et al*. 2012). After growth and acclimatization of the micropropagated orchid plants, individuals can be used in species conservation programs by reintroduction into natural habitats (Endres Júnior *et al*. 2015; Sasamori *et al*. 2015), or be marketed as ornamental plants, thus alleviating pressure on natural populations (Mercier & Nievola 2003).

In vitro propagation allows a high number of seedlings to be obtained from seeds, which contributes to the maintenance of the genetic characteristics and different physiological requirements of the plants (Pedroso-de-Moraes *et al*. 2009; Besson *et al*. 2010; Pinto *et al*. 2010). Although the technique of *in vitro* culture has been in use for years, studies prior to scale propagation are necessary for the establishment of a suitable culture medium and growing conditions, which will contribute to the ideal growth and development of the species in a short period of time (Endres Júnior *et al*. 2014; Sasamori *et al*. 2015, 2016). However, several studies have focused on the propagation of ornamental and commercial species, usually of artificial hybrid species (Faria *et al*. 2002; Pedroso-de-Moraes *et al*. 2012), and few are aimed at conservation of genetic diversity.

The abiotic conditions of *in vitro* culture need to be established for each orchid species in view of its peculiar physiological characteristics. The effect of different concentrations of macronutrients and sucrose on the *in vitro* development of *Cattleya* Lindl. species was assessed for *C. violacea* (Kunth) Rolfe (Galdiano Júnior *et al*. 2013b) *C. loddigesii* Lindl. (Rezende *et al*. 2009) *C. granulosa* Lindl. (Pinto *et al*. 2010) and *C. intermedia* Graham (Sasamori *et al*. 2015), each species showing distinct responses in relation to morphometric variables. Sucrose, the main organic product added to culture medium,

provides energy and carbon for the biosynthesis of structural and functional components (George *et al*. 2008a), since photosynthetically active radiation and CO_2 concentration are insufficient for the optimal functioning of photosynthesis (George *et al*. 2008a; Xiao *et al*. 2011). Excess or deficiency of mineral nutrients in culture medium may be detrimental to the development of *in vitro* cultured seedlings (George *et al*. 2008b), resulting in longer cultivation times for individuals and, consequently, increased costs, which can lead to greater sensitivity to external environmental stress during acclimatization and, therefore, to greater loss of micropropagated plants.

In order to obtain individuals for conservation purposes, the objective of the present study was to establish optimal conditions for asymbiotic *in vitro* propagation of *C. cernua* by evaluating the effect of different concentrations of macronutrients and sucrose on the survival and development of plants. It is expected that reduced mineral nutrients and increased sucrose concentration will contribute to increasing aerial and root systems of plants since the carbon source in the culture medium is

Figure 1 – a. *Cattleya cernua*; b. plants 8 months after sowing; c. individualization of plants and start of experiment with different concentrations of macronutrients and sucrose; d. plants after 180 days growing in MS medium with 50% concentration of macronutrients and 30 g L^{-1} of sucrose. Bars = 10 mm.

essential for the *in vitro* growth phase of individuals (George *et al*. 2008a; Besson *et al*. 2010). In addition, epiphytic plants are constantly exposed to the stress of nutritional deficiency in the natural environment (Benzing 2000) and may be adapted to a lesser requirement of mineral nutrients, and thus respond to lower mineral nutrients in *in vitro* culture medium.

Material and Methods

Mature capsules were collected from five plants of a *Cattleya cernua* population (one capsule per plant) in a forest fragment located in the municipality of Rolante, state of Rio Grande do Sul, Brazil, and taken to the laboratory. The capsules were washed in running water with commercial detergent, rinsed three times with sterilized distilled water and taken to a laminar flow chamber where they were disinfest for 30 seconds in 70% ethanol and then submerged in 2% sodium hypochlorite with Tween® 20 for 10 minutes. The capsules were then washed three times in sterile distilled water and opened with a scalpel to remove the seeds.

The seeds of all the capsules were pooled in a single sample and inoculated in vials (200 mL) containing 30 mL of MS medium (Murashige & Skoog 1962), supplemented with 30 g L^{-1} of sucrose and 10 g L^{-1} of activated charcoal, solidified with 6 g L^{-1} of agar (Kasvi). The medium was adjusted to pH 5.7 (Unemoto *et al*. 2007) with 1N HCl by a pH meter (HI 2221, Hanna Instruments). The cultures remained in the growing room under controlled conditions with a photosynthetically active radiation (PAR) of 60 µmol m^{-2} s^{-1}, a photoperiod of 12/12 h light/dark cycles and a temperature of 26 ± 1 °C.

After seed germination and protocorm development for eight months (Fig. 1b), the seedlings were transferred to vials (200 mL) containing 30 mL of the same medium used in the germination stage. Five seedlings were transferred to each vial, where they remained for additional 60 days, until attaining a height of about 1.1 ± 0.3 cm and roots of 3.0 ± 1.4 in length. The seedlings were then transferred to vials (200 mL; Fig. 1c) containing 30 mL of MS medium with the same concentrations of activated charcoal and agar and the same pH as the initial culture step. Combinations of three concentrations of the original macronutrient formula of MS (25, 50 and 100%; Tab. 1) and four concentrations of sucrose (10, 30, 60 and 90 g L^{-1}) were evaluated. Fourteen replicates were performed for each combination of macronutrients and sucrose with five seedlings per vial for a total of 840 seedlings in 12 treatments.

After a period of 180 days under the same conditions of light and temperature as the initial culture step, plant survival was evaluated, using each vial as a replicate. The plants were removed from the vials and washed in running water. The following variables were evaluated for each plant: height of aerial part (HAP), number of leaves (NL), number of roots (NR), length of the longest root (LLR) and fresh mass (FM). The variables were measured using a caliper and a precision analytical balance. In addition to the morphological variables, levels of chlorophyll (*a* and *b*) and carotenoids in the leaves of plants from each treatment were also determined (Fig. 1d).

To determine the levels of photosynthetic pigments, leaf samples (distal half) were collected from propagated plants. Nine plants were selected for each treatment, which were divided into groups of three individuals, and leaf tissue samples were taken from each plant for a total of 20 mg for each group. For each 20 mg of leaf tissue, 1 mL of DMSO was added, in which the leaf tissue remained immersed for 24 hours in a water bath at 65 °C. Next, 100 uL triplicates of each 1 mL sample were removed and placed in each well of a 96-well cell culture dish for a total of nine samples per treatment. Readings were performed with a spectrophotometer (Spectramax M3®) at wavelengths of 665 nm, 649 nm and 480 nm. Concentrations were calculated according to the equations proposed by Wellburn (1994): chlorophyll *a* (*Chla*) = $12.47A_{665} - 3.62A_{649}$; chlorophyll *b* (*Chlb*) = $25.06A_{649} - 6.5A_{665}$; and carotenoids (*Car*) = $(1000A_{480} - 1.29\ Chla - 53.78Chlb)/220$.

Plant survival data were transformed into percentages. Data for height of the aerial part, leaf number, root number and fresh mass were natural log transformed (ln (x+1)). Data for length of largest root were square root transformed (root (x+1)). Plant survival in the treatments was compared by the Kruskal-Wallis test, followed by the Student-Newman-Keuls test at 5% probability. Data for morphological variables, as well as for leaf chlorophyll content, were compared by analysis of variance (two-way ANOVA), followed by the Bonferroni test at 5% probability. The analyses were performed using Biostat, version 5.3, and SPSS, version 20.0.

Table 1 – Composition of macronutrients of MS medium (Murashige & Skoog 1962) for the *in vitro* propagation of *Cattleya cernua*. 100MS = complete formulation of macronutrients; 50MS = reduction to 50% of macronutrients; 25MS = reduction to 25% of macronutrients).

	Treatments		
Components	100MS	50MS	25MS
	Macronutrients (mg L^{-1})		
NH_4NO_3	1650.0	825.0	412.5
KNO_3	1900.0	950.0	475.0
$CaCl_2$ $2H_2O$	440.0	220.0	110.0
$MgSO_4$ $7H_2O$	370.0	185.0	92.5
KH_2PO_4	170.0	85.0	42.5

Results

In vitro propagation of *Cattleya cernua* with different concentrations of macronutrients and sucrose resulted in plant survival between 47 and 100% (Fig. 2). In general, the addition of sucrose combined with reduced macronutrients provided a survival percentage greater than 85%. On the other hand, treatments in which high concentrations of sucrose (60 and 90 g L^{-1}) were combined with the complete concentration of macronutrients of MS medium (100% MS), proved detrimental to survival (Fig. 2).

In general, the different concentrations of macronutrients and sucrose significantly influenced the growth of plants of *C. cernua* (Tab. 2). Increased macronutrient concentration in the culture medium at the different concentrations of sucrose (10, 30, 60 and 90 g L^{-1}) generally contributed to a greater HAP. Thus, plants grown in treatments containing 100% of macronutrients had a significantly higher mean HAP (Tab. 2). Within each macronutrient concentration, the highest means for HAP were recorded for treatments containing 30 and 60 g L^{-1} of sucrose, while treatments containing 10 and 90 g L^{-1} of sucrose had significantly lower means for HAP (Tab. 2). Although there was a significant influence of sucrose and macronutrient concentration on HAP, this was not observed for the interaction between the concentration of both components (Tab. 2).

The NL of micropropagated plants of *C. cernua* was influenced by macronutrient concentration, sucrose concentration and the interaction between these treatments (Tab. 2). The lowest means for NL were observed in the presence of 60 and 90 g L^{-1} of sucrose when combined with the lowest concentration of MS salts (25MS). In addition, treatment with 30 g L^{-1} of sucrose also provided a significantly lower mean NL when combined with 25 and 50% of salts (Tab. 2). The different concentrations of sucrose, on the other hand, did not produce significant differences in NL in the treatments with 50 and 100% of macronutrients. When only 25% of the macronutrients of the MS medium were added, the lowest sucrose

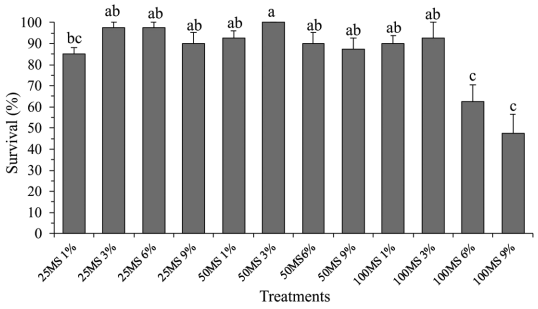

Figure 2 – Survival (mean ± standard error) of plants of *Cattleya cernua* propagated *in vitro* for 180 days at different concentrations of macronutrients and sucrose. 25MS and 50MS = 25% and 50% concentration of macronutrients, respectively; 100MS = complete concentration of macronutrients; 1, 3, 6 and 9% indicate concentrations of 10, 30, 60 and 90 g L^{-1} of sucrose, respectively. Different letters indicate significant differences between treatments according to Student-Newman-Keuls test at 5% probability. Kruskal-Wallis: $H = 46.059$; $p < 0.001$.

Table 2 – Values (mean ± standard deviation) of height of the aerial part, number of leaves, number of roots, length of longest root and fresh mass of plants of *Cattleya cernua* micropropagated for 180 days in MS medium with different concentrations of macronutrients and sucrose.

	Sucrose (g L^{-1})					
MS[1]	**10**	**30**	**60**	**90**		
			Height of the aerial part (cm)			
25MS	1.4 ± 0.3 Bc	1.7 ± 0.4 Ac	1.8 ± 0.7 Ac	1.5 ± 0.4 Bc	F = 18.140	p < 0.001
50MS	1.6 ± 0.3 Bb	1.9 ± 0.4 Ab	1.8 ± 0.4 Ab	1.7 ± 0.4 Bb		
100MS	1.8 ± 0.4 Ba	2.4 ± 0.6 Aa	2.1 ± 0.4 Aa	1.8 ± 0.4 Ba		
		F = 33.448			macronutrients * sucrose[2]	
		p < 0.001			F = 1.944	p = 0.073
			Number of leaves			
25MS	6.0 ± 2.1 Aa	4.1 ± 2.0 Bb	3.9 ± 1.7 Bb	4.1 ± 1.7 Bb	F = 8.781	p < 0.001
50MS	6.0 ± 2.4 Aa	4.8 ± 2.3Ab	5.6 ± 2.2 Aa	5.0 ± 2.3 Aab		
100MS	7.6 ± 3.5 Aa	7.6 ± 4.0 Aa	6.6 ± 3.1 Aa	6.2 ± 3.4 Aa		
		F = 30.420			macronutrients * sucrose	
		p < 0.001			F = 2.227	p = 0.040
			Number of roots			
25MS	5.8 ± 2.1 Ba	9.4 ± 3.3 Aa	11.3 ± 4.4 Aa	9.2 ± 3.3 Aa	F = 5.083	p < 0.001
50MS	6.6 ± 3.4 Ca	9.4 ± 6.3 Ba	12.6 ± 5.0 Aa	8.8 ± 4.0 BCa		
100MS	4.3 ± 1.9 Bb	8.0 ± 3.4 Aa	6.9 ± 4.4 Ab	7.6 ± 4.1 Aa		
		F = 22.661			macronutrients * sucrose	
		p < 0.001			F = 3.079	p = 0.006
			Longest root lenght (cm)			
25MS	5.3 ± 2.3 ABa	6.5 ± 2.6 Aa	4.3 ± 1.7 BCa	3.9 ± 1.8 Ca	F = 16.817	p < 0.001
50MS	5.2 ± 1.9 Aa	5.0 ± 2.0 Ab	4.4 ± 1.9 Aa	3.3 ± 1.7 Ba		
100MS	2.1 ± 1.1 Bb	3.4 ± 1.5 Ac	2.7 ± 1.4 ABb	2.1 ± 1.0 Bb		
		F = 67.766			macronutrients * sucrose	
		p < 0.001			F = 3.061	p = 0.006
			Fresh mass (mg)			
25MS	477 ± 289 Ba	742 ± 366 Aa	485 ± 284 Ba	260 ± 151 Ca	F = 22.227	p < 0.001
50MS	475 ± 275 Aa	625 ± 543 Aa	508 ± 331 Aa	341 ± 241 Ba		
100MS	286 ± 143 Ba	605 ± 350 Aa	437 ± 378 Aa	388 ± 285 Ba		
		F = 1.814			macronutrients * sucrose	
		p = 0.164			F = 2.369	p = 0.029

Means followed by different letters in a row (upper case) and column (lower case) indicate a significant difference according to the Bonferroni test at 5% probability. [1]MS = concentration of macronutrient salts of the MS medium (25, 50 and 100%). [2] = Indicates the interaction between macronutrient and sucrose concentrations.

concentration (10 g L^{-1}) was found to be beneficial for leaf production, with a significantly higher NL.

The root system of plants was also influenced by macronutrient concentration, sucrose concentration and the interaction between these treatments (Tab. 2). In general, the highest mean NR was observed when macronutrients were reduced in the culture medium, although a statistical difference was not observed in some treatments, such as 60 and 90 g L^{-1} of sucrose. When plants of *C. cernua* were cultivated in 25 and 100% of macronutrients, concentrations of between 30 and 90 g L^{-1} of sucrose were shown to be beneficial for root production. For the treatment with 50% of salts, the combination with 60 g L^{-1} of sucrose provided significantly higher mean NR. Reduction of macronutrients also provided the highest averages for root length of cultivated plants. The LLR was significantly greater for plants cultivated with 10 and 30 g L^{-1} of sucrose combined with 25 and 50% of macronutrients (Tab. 2). The sucrose concentration of 90 g L^{-1} was shown to be detrimental to root growth of *C. cernua*, as well as the total macronutrient concentration of MS medium.

In general, there was no influence of macronutrient concentration in the culture medium on plant FM, since there was no statistical difference between the concentrations of salts within each sucrose treatment. Sucrose concentration, on the other hand, influenced the fresh mass of plants, as did the interaction with mineral nutrient concentration of the medium. The highest mean values of FM were recorded for plants grown in the treatment with 30 g L^{-1} of sucrose. In addition, when half of the salt concentration was used, the FM of plants was higher when 10 and 60 g L^{-1} of sucrose were added. The mean FM of plants was also significantly higher in the treatment with the complete concentration of macronutrients combined with 30 and 60 g L^{-1} of sucrose (Tab. 2).

The levels of chlorophyll *a*, chlorophyll *b* and carotenoids of plants of *C. cernua* did not statistically differ between the different concentrations of macronutrient salts of the MS medium (25, 50 and 100%). The concentration of sucrose, on the other hand, influenced the levels of photosynthetic pigments, and the interaction between salts and sucrose was significant for chlorophyll *a* and carotenoids (Tab. 3). For chlorophyll *a*, the treatments with 30, 60 and 90 g L^{-1} of sucrose provided higher levels, except for individuals of the treatment with 50MS and 60 g L^{-1} of sucrose, which had a significantly lower mean. For chlorophyll *b*, plants of treatments with 30 and 90 g L^{-1} of sucrose had significantly higher averages (Tab. 3). The level of carotenoids was also significantly higher in the treatment with the highest concentration of sucrose (90 g L^{-1} of sucrose). Moreover, when the concentration of macronutrient salts of the MS medium was reduced to 25%, treatments with 30 and 60 g L^{-1} of sucrose also provided higher carotenoid levels in the cultivated plants (Tab. 3).

Discussion

High survival rates of *Cattleya cernua* were observed in most combinations of MS macronutrients and sucrose concentrations, with the exception of 100% macronutrients combinated with 6 or 9% sucrose. The use of 20 and 30 g L^{-1} of carbohydrate have been generally recommended for tissue culture, although studies have shown that different species respond better, or to lower concentrations of sucrose (Moreira *et al*. 2007; Pivetta *et al*. 2010; Galdiano Júnior *et al*. 2013a,b; Martins *et al*. 2015; Koene *et al*. 2019), or to higher concentrations, between 40 and 60 g L^{-1} of the same carbohydrate (Rego-Oliveira *et al*. 2003; Besson *et al*. 2010; Endres *et al*. 2014; Sasamori *et al*. 2015). This trend has also been observed for macronutrients, with *C. cernua* benefitting from lower concentrations, as do *Anathallis adenochila* (Loefgr.) F. Barros (Endres *et al*. 2014), *Laelia anceps* Lindl. (Ramírez-Mosqueda *et al*. 2019) and *Vriesea incurvata* Gaudich. (Sasamori *et al*. 2016), while other species like *Miltonia flavescens* (Lindl.) Lindl., *Cattleya loddigesii* Lindl. and *C. intermedia* Graham *ex* Hook., require higher concentrations of nutrients (Müller *et al*. 2007; Soares *et al*. 2009; Sasamori *et al*. 2015).

No evidence of deficiency and/or toxicity was found in the leaves of the propagated individuals of *C. cernua* both at low concentrations of macronutrients and at the total concentration of the MS medium. When high concentrations of macronutrients are added to the culture medium, such compounds may become toxic to plants, leading to irregular development or death of individuals (George *et al*. 2008b). Likewise, low concentrations of nutrients in the culture medium can contribute to the formation of chlorosis and necrosis in leaves, since individuals experience deficiencies of nutrients that regulate metabolic processes (Marschner 2012). Because it is considered one of the most nutrient-rich media, the

Table 3 – Values (mean ± standard deviation) of chlorophyll (*a* and *b*) and carotenoid levels of plants of *Cattleya cernua* micropropagated for 180 days in MS medium with different concentrations of macronutrients and sucrose.

MS[1]	Sucrose (g L^{-1})					
	10	30	60	90		
		Chlorophyll *a* (mg g^{-1})				
25MS	86 ± 18 Ba	160 ± 25 Aa	150 ± 26 Aa	190 ± 32 Aa	F = 29.681	p < 0.001
50MS	110 ± 26 Ba	166 ± 26 Aa	96 ± 52 Ba	195 ± 27 Aa		
100MS	87 ± 26 Ba	151 ± 21 Aa	127 ± 29 ABa	153 ± 22 Aa		
		F = 2.096			macronutrients * sucrose[2]	
		p = 0.132			F = 2.830	p = 0.017
		Chlorophyll *b* (mg g^{-1})				
25MS	66 ± 31 Ba	160 ± 46 Aa	123 ± 51 Ba	173 ± 55 Aa	F = 12.151	p < 0.001
50MS	97 ± 55 Ba	171 ± 55 Aa	79 ± 52 Ba	167 ± 41 Aa		
100MS	79 ± 52 Ba	160 ± 51 Aa	105 ± 57 Ba	146 ± 42 Aa		
		F = 0.161			macronutrients * sucrose	
		p = 0.852			F = 0.706	p = 0.646
		Carotenoids (mg g^{-1})				
25MS	14 ± 02 Ba	24 ± 03 Aa	21 ± 05 Aa	20 ± 03 Aa	F = 60.998	p < 0.001
50MS	16 ± 02 Ca	22 ± 02 Ba	13 ± 03 Ca	34 ± 03 Aa		
100MS	14 ± 02 Ca	20 ± 02 Ba	22 ± 03 Ba	27 ± 03 Aa		
		F = 1.434			macronutrients * sucrose	
		p = 0.246			F = 18.576	p < 0.001

Means followed by different letters in a row (upper case) and column (lower case) indicate a significant difference according to the Bonferroni test at 5% probability. [1]MS = concentration of macronutrient salts of the MS medium (25, 50 and 100%). [2] = Indicates the interaction between macronutrient and sucrose concentrations.

use of MS may have contributed to the successful cultivation of *C. cernua* plants in the reduced nutrient treatments. Another important point to consider is the fact that the species possesses an epiphytic habit, thus allowing the micropropagated individuals to exhibit physiological adaptations to conditions of nutritional deficiency. For *in vitro* cultivation of *Laelia anceps*, the reduction of MS salts also did not harm its growth and development due to the low nutritional requirements since the species has an epiphytic habit (Ramírez-Mosqueda *et al*. 2019). However, even if nutrient shortage allows the species to develop slowly, both in the natural environment and in *in vitro* culture, such a condition is not of interest in the propagation process. Plants of *C. cernua* grown *in vitro* had greater development of the aerial system in the medium with the complete concentration of macronutrients, which is thus a determinant factor for *in vitro* mass culture. This has been the objective of searches for a culture medium composition that contributes to the production of vigorous plants in a short period of time (Galdiano Júnior *et al*. 2013a,b; Endres Júnior *et al*. 2015; Sasamori *et al*. 2015).

The treatment with the highest concentration of sucrose (90 g L^{-1}) was not beneficial for the development of plants, although increased sucrose in culture medium is essential as a carbon source. Carbohydrate added to the medium is used for the biosynthesis of structural and functional

components for growth, and its ideal concentration depends on the requirements of each plant species (George *et al*. 2008a). The adequate supply of carbohydrate increases the reserves of starch and sucrose in the leaves of micropropagated plants, which then act as energy storage organs that will supply the acclimatization stage for the growth of new leaves adapted to the *ex vitro* environment, thus improving the performance of acclimatization (Capellades *et al*. 1991; Hazarika 2003; Fuentes *et al*. 2006).

During micropropagation, cultivated plants can undergo stress conditions, leading to the formation of morphological (hyperhydricity) and physiological changes (photosynthesis and gas exchange) (Ziv 1991). When plants of *C. cernua* were removed from the vials, structures, such as leaves and roots, were broken during the handling of individuals, indicating the fragility of the plants grown in the treatment with 10 g L^{-1} of sucrose. Brittle organs, low cell wall strength and low chlorophyll content are characteristics of plants experiencing hyperhydricity (Franck *et al*. 2004; Kevers *et al*. 2004).

The morphological variables recorded for *C. cernua* corroborate the finding that macronutrient and sucrose concentrations are specific to the *in vitro* growth and development of various species, including orchids of the same genus. For the micropropagation of *C. violacea*, culture medium with 50% of macronutrient salts and between 20 and 30 g L^{-1} of sucrose provided greater values for aerial part height, number of leaves, fresh mass and the number and length of roots (Galdiano Júnior *et al*. 2013b). In the cultivation of *C. loddigesii*, medium with 100% macronutrients and 60 g L^{-1} of sucrose stimulated the development of the root system; however, for the aerial system, the best treatments were with the addition of between 16 and 30 g L^{-1} associated with the growth regulator gibberellic acid (Rezende *et al*. 2009). When plants of *C. granulosa* were micropropagated in MS containing 45 g L^{-1} of sucrose, aerial part height, number of leaves and fresh root mass were higher than for plants micropropagated in the presence of 15 and 30 g L^{-1} (Pinto *et al*. 2010). Plants of *C. intermedia* propagated in MS medium combined with 60 g L^{-1} of sucrose, as well as in medium with 50% macronutrients plus 45 or 60 g L^{-1} of sucrose, had the highest averages for both the aerial and root systems of individuals (Sasamori *et al*. 2015).

As in other micropropagation studies (Araújo *et al*. 2006; Tamaki *et al*. 2007; Unemoto *et al*. 2007; Martins *et al*. 2015; Sasamori *et al*. 2016), the development of the root system recorded for *C. cernua* confirms that low concentrations of macronutrients may be beneficial to plants during *in vitro* cultivation. The use of media with reduced concentrations of macronutrients can stimulate root formation and growth (George *et al*. 2008a), which is important for contact with the substrate (Zhang *et al*. 2010). Tamaki *et al*. (2007) suggested that low concentrations of nutrients in culture medium may induce the translocation of auxins, which are responsible for cell elongation, from leaves to roots (Marschner 2012), thus contributing to the development of the root system and, consequently, to higher plant survival during *ex vitro* acclimatization (Besson *et al*. 2010). On the other hand, very long branched roots are not recommended for *in vitro* culture because they make it difficult to wash and remove culture media adhered to the plants when they are removed from the vials. Short roots and/or root primordia can contribute to the rooting of plants in acclimatization, since they are still in a stage of active growth (Woodhead & Bird 1998; Costa *et al*. 2008).

In general, the morphological characteristics of orchid roots, including those of *C. cernua*, act directly on the biomass of propagated individuals through the incorporation of carbon (De Riek *et al*. 1997), which is obtained from the carbohydrate added to the medium. Sucrose added to media is the main source of carbon for the formation of structural skeletons and also serves to stimulate root growth and formation, with a concentration of 20 to 30 g L^{-1} of carbohydrate being recommended for rooting *in vitro* cultured plants (George *et al*. 2008a). Leaves and pseudobulbs of orchids have cells capable of storing large amounts of water and carbohydrates, while the roots possess a multiple epidermis (velamen) that acts as a "sponge" by storing moisture and nutrients (Benzing 1990; Silva & Milaneze-Gutierre 2004). Due to these morphological characteristics of orchid roots and aerial system, the fresh mass of cultivated plants was not influenced by the concentration of macronutrients in the medium.

High concentrations of sucrose in the culture medium may also be harmful to plants by making it difficult to absorb water and nutrients (Paiva Neto & Otoni 2003; Fráguas *et al*. 2003; Besson *et al*. 2010; Koene *et al*. 2019). Indeed, when plants of the treatment with 90 g L^{-1} of sucrose were removed from their vials, their leaves were observed to be slightly dehydrated, indicating a possible alteration

of the osmotic potential of the medium (Paiva Neto & Otoni 2003; George *et al*. 2008a). High concentrations of carbohydrates may also alter the structure of the photosynthetic apparatus, inhibiting chlorophyll synthesis and reducing the photosynthetic capacity of tissues (Silva 2004), which is quite low in the *in vitro* condition (Rolland *et al*. 2002). However, inhibition in the formation of chlorophyll *a* and *b* was not observed for plants of *C. cernua* in the treatments with higher concentrations of carbohydrate; in fact, there was significantly higher averages for the pigments in the treatments with high concentrations of sucrose. Reduced carbohydrate in the culture medium, on the other hand, can stimulate the plants to an autotrophic condition *in vitro* (Mosaleeyanon *et al*. 2004; Schmildt *et al.* 2014), an event that was not observed for the plants of *C. cernua* cultivated at the concentration of 10 g L^{-1} of sucrose, which had significantly lower values for photosynthetic pigments.

The data obtained in the present study allowed the description of efficient *in vitro* culture of *C. cernua*. Plant growth was influenced by the interaction between macronutrient and sucrose concentrations, with the exception of height of the aerial part and chlorophyll *b*. Although reduced concentrations were beneficial for the root system, the treatment "100MS + 30 g L^{-1} of sucrose" had the best results for most of the evaluated variables, being the most suitable for *in vitro* culture of the species. The lower root length in this combination of macronutrients and sucrose could serve as an ally in the rooting process during plant acclimatization. The *in vitro* propagation of *C. cernua* can contribute to the conservation of this epiphytic species native to South America through indirect actions such as the marketing of plants cultivated *in vitro*, which would alleviate pressures on natural populations, and/or their use for environmental education. It can also contribute directly to conservation by providing individuals for use in reintroduction programs for this species.

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