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 epífita 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 asymbiotic 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...
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 cermua* 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 disinfested 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 1 N 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 µL 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*) = 12.47A₆₆₅ - 3.62A₆₄₉; chlorophyll *b* (*Chlb*) = 25.06A₆₄₉ - 6.5A₆₆₅; and carotenoids (*Car*) = (1000A₄₈₀ - 1.29 Chla – 53.78 Chlb)/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 (sqrt (*x* + 1)). Plant survival in the treatments was compared by the Kruskal-Wallis test, followed by the Student-Newman-Keuls test at 5% probability. The analyses were performed using Biostat, version 5.3, and SPSS, version 20.0.
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

| Components          | 100MS (mg L\(^{-1}\)) | 50MS | 25MS |
|---------------------|-----------------------|------|------|
| NH\(_4\)NO\(_3\)    | 1650.0                | 825.0| 412.5|
| KNO\(_3\)           | 1900.0                | 950.0| 475.0|
| CaCl\(_2\) 2H\(_2\)O| 440.0                 | 220.0| 110.0|
| MgSO\(_4\) 7H\(_2\)O| 370.0                 | 185.0| 92.5 |
| KH\(_2\)PO\(_4\)    | 170.0                 | 85.0 | 42.5 |

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) | 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      |      |      |      |
| p < 0.001       |      |      |      |
| **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 ± 2.3 Ab | 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      |      |      |      |
| p < 0.001       |      |      |      |
| **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      |      |      |      |
| p < 0.001       |      |      |      |
| **Longest root length (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      |      |      |      |
| p < 0.001       |      |      |      |
| **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       |      |      |      |
| p = 0.164       |      |      |      |

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. *MS* = concentration of macronutrient salts of the MS medium (25, 50 and 100%). * 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 combined 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. (Ramirez-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
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

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**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\(^{-1}\)) | Chlorophyll *a* (mg g\(^{-1}\)) | Chlorophyll *b* (mg g\(^{-1}\)) | Carotenoids (mg g\(^{-1}\)) |
|---------|----------------|-------------------------|-------------------------|------------------|
|         | 10  | 30  | 60  | 90  | 10  | 30  | 60  | 90  | 10  | 30  | 60  | 90  | 10  | 30  | 60  | 90  |
| 25MS    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 50MS    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 100MS   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

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.
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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 (Mosalceyanon 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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