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Asymbiotic culture of *Cattleya intermedia* Graham (Orchidaceae): the influence of macronutrient salts and sucrose concentrations on survival and development of plantlets

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ABSTRACT

Cattleya intermedia is an Atlantic Forest species endemic to Brazil that is classed as vulnerable on the list of threatened species. In this study, *C. intermedia* plantlets were micropropagated in an asymbiotic culture and the influence of different concentrations of sucrose (15, 30, 45 and 60 g L^{-1}, plus a zero sucrose medium) and macronutrient salts (complete Murashige and Skoog (MS) medium and half MS medium (with half-strength macronutrients)) on survival and development of the plantlets was evaluated. In all treatments 100% plantlet survival was achieved. The integrated analysis of height of aerial part, number of leaves per plantlet, fresh mass, number of roots per plantlet and length of the longest root showed that the plantlets exhibited greatest development at the half-strength macronutrient concentrations with 45 or 60 g L^{-1} of sucrose, as well as at the complete macronutrient concentration with 60 g L^{-1} of sucrose. Plantlets acclimatized and reintroduced to an environment in which the species occurs naturally exhibited 98.6% survival. The results obtained in this study allowed the establishment of optimal conditions for asymbiotic micropropagation, which is a requisite for future studies focused on conservation of *C. intermedia*.

Keywords: Atlantic Forest, carbohydrate, conservation, *in vitro* culture, orchids, propagation

Introduction

The Orchidaceae form a group of around 26,567 species distributed across all continents (World Checklist of Monocotyledons 2011), 70% of which have epiphytic habits (APG 2014). In Brazil, 236 genera and 2,524 species have been described (Barros *et al.* 2014) and they account for 45.8% of vascular epiphyte species in the Atlantic Forest (Kersten 2010). In addition to depletion by extraction, destruction and fragmentation of habitats are also threats to Orchidaceae populations (Neto *et al.* 2013), considering that just 8.5% of the Atlantic Forest biome is currently consisting of forest remnants larger than 100 hectares (Fundação SOS Mata Atlântica & INPE 2011).

Cattleya intermedia is an epiphytic Orchidaceae, endemic to Brazil, occurring in the south and southeast regions of the country, in Atlantic Forest environments. While the species prefers sites with altitudes between 0 and 50 m, it can also be found at elevations of up to 300 meters (Neto *et al.* 2013). The species grows up to 35 cm tall, has bifoliate, erect and cylindrical pseudobulbs, with oblong leaves that lie horizontal or are semi-erect. Inflorescences comprise from two to five pink flowers (Buzatto *et al.* 2010). The high ornamental value placed on the flowers has motivated intense extraction activities, causing a decline in natural populations (Cruz *et al.* 2003). *Cattleya intermedia* is currently classed as vulnerable both in the state of Rio Grande do Sul's list of threatened species of flora (Rio Grande do Sul 2014) and in the red book of Brazilian flora (Martinelli & Moraes 2013).

Germination of orchids is extremely restricted in their natural environments (Pedroso-de-Moraes *et al.* 2012) because they have very small seeds that do not contain sufficient nutrient reserves (Arditti 1992). Under natural conditions, a symbiotic interaction with fungi stimulates germination of seeds (Dearnaley 2007), providing carbon sources on which the embryos are dependent (Dressler 1981), although some species do have the capacity to germinate in the absence of fungi (APG 2014).

Both *in situ* and *ex situ* conservation strategies are of fundamental importance to preservation of threatened species such as *Cattleya intermedia*. One important tool is *in vitro* tissue culture from seeds (Grattapaglia & Machado 1998), since high germination rates are possible (Pedroso-de-Moraes *et al.* 2009) and the method enables the genetic variability of plantlets to be maintained (Benson 1999; Pinto

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et al. 2010), making them suitable for studies of reintroduction into natural environments (Rubluo *et al*. 1993; Decruse *et al*. 2003; Aggarwal *et al*. 2012).

One of the requisites of successful *in vitro* cultures is a nutrient medium that provides the substances plantlets need to develop (Besson *et al*. 2010). The Murashige and Skoog (MS) medium (Murashige & Skoog 1962) has been used effectively for propagation of a wide range of vegetable species (Grattapaglia & Machado 1998). However, it contains high concentrations of salts which can prove prejudicial to the morphological processes of the plantlets of certain species (Sakuta 1987). Sucrose is the most widely used source of carbon in nutrient mediums, responsible for providing the plant with metabolic energy and carbon skeletons, but high concentrations may reduce the photosynthetic capacity of tissues (Yamada & Sato 1978).

The objective of this study was to establish the optimal conditions for asymbiotic micropropagation of *Cattleya intermedia* by evaluating the influence of different concentrations of macronutrient salts and sucrose on the survival and development of plantlets. The hypothesis tested was that higher salt and carbon source concentrations would improve plantlet development.

Materials and Methods

Cattleya intermedia Graham capsules were collected from a population occurring in a forest fragment located within the municipal district of Novo Hamburgo, Rio Grande do Sul, Brazil. After washing in running water with a commercial liquid detergent and rinsing three times in distilled water, the capsules were placed in a laminar flow chamber and sterilized for 30 seconds in 70% ethanol and immersed in 2% sodium hypochlorite with Tween 20 for 10 minutes. The capsules were washed three times in sterilized distilled water and opened with a scalpel to remove the seeds.

The seeds were inoculated into flasks (with a volume of 200 mL) containing 30 mL of MS medium (Murashige & Skoog 1962) with 50% of the original formulation of macronutrient salts that had been supplemented with 30 g L^{-1} of sucrose and 10 g L^{-1} of activated charcoal, solidified with 6 g L^{-1} of agar, and pH-adjusted to 5.7, before sterilization in an autoclave (Unemoto *et al*. 2007). Cultures were maintained under controlled conditions, with light intensity of 100 µmol m^{-2}/s, photoperiod of 12 hours and temperature of 26±1°C.

After seed germination and protocorm development for 90 days, plantlets were submitted to standardization, based on Soares *et al*. (2008). Therefore, five plants were transferred to each flask (with a volume of 200 mL) containing 30 mL of the same medium used in the previous stage, for 90 days, until they reached a height of 1.5 to 2.0 cm. The plantlets were then transferred to flasks (with a volume of 200 mL) containing 30 mL of MS medium with the same concentrations of activated charcoal and agar and the same pH as for the initial stage of culturing. Two different concentrations of the original MS medium macronutrient salts formula (50 and 100%) and four different concentrations of sucrose (15, 30, 45 and 60 g L^{-1}) plus zero sucrose were evaluated. Ten repetitions of four plantlets per flask were prepared for each combination of salts and sucrose concentrations, making a total of 400 plantlets and 10 different treatments.

After a further 180 days under the same conditions of light intensity and temperature as the initial stage of culturing, plantlets were removed from the flasks and washed under running water. The following parameters were then determined for each plantlet: survival, height of aerial part, number of leaves, number of roots, length of longest root and fresh mass. These parameters were measured with the aid of a pachymeter and a high-precision balance.

Considering the results obtained from the different treatments, plantlets grown in media with 50 and 100% macronutrient salts concentrations combined with 45 and 60 g L^{-1} sucrose were chosen for *ex vitro* acclimatization, according to Sasamori *et al*. (2014). After five months, the survival of plantlets was assessed, when they were used to start a reintroduction study in a fragment of the Atlantic Forest within the lower stretch of the Sinos River Basin in which the species occurs.

Data obtained on survival were transformed into percentages. Data on height of the aerial part, number of leaves, number of roots, longest root length, and fresh mass were transformed into natural logarithms (ln). Means for sucrose concentrations in each salt concentration were subjected to analysis of variance (ANOVA) followed by Tukey's test, with a probability level of 5%. Means for salt concentrations in each sucrose concentration were compared using Student's *t* test with a probability level of 5%. Linear regression analysis was used to estimate the relationships between biotic parameters and sucrose concentrations in each salt concentration. Treatments were grouped on the basis of Euclidean distances using hierarchical cluster analysis with variable standardization. The variables used for this analysis were height of the aerial part, number of leaves, number of roots, longest root length, and fresh mass. Cluster analysis was conducted using BioEstat version 5.3 and all other analyzes were performed using SPSS version 20.

Results and Discussion

Survival of *Cattleya intermedia* was not influenced by concentration of macronutrient salts or of sucrose in the MS medium, since 100% of plantlets survived in all treatments. These results are considered especially important because in general the photosynthetic capacity of plantlets cultivated *in vitro* is reduced, making them at least partially dependent on an external source of carbohydrates (Yamada & Sato 1978), to the extent that necrosis of tissues can generally be observed at sucrose concentrations below 20 g L^{-1} (Torres *et al*. 1998), resulting in death. For example, plantlets of

Caularthron bicornutum, which is an epiphytic orchid native to Brazil, exhibited 30 to 47% survival rates when cultivated in MS medium with different sucrose concentrations (0, 10, 20, 30 40 and 50 g L^{-1}) and supplemented with half of the original macronutrients, and the highest percentage of live plantlets was observed in the medium containing 20 g L^{-1} of the carbohydrate (Pivetta *et al*. 2010). However, the fact that there are plants capable of producing carbohydrates in sufficient quantities to survive *in vitro* (Grout 1988), as observed in this study, does not necessarily mean that the following developmental stages are assured.

The development of the *Cattleya intermedia* plantlets was influenced by the different concentrations of macronutrient salts and sucrose in the culture medium. Both the height of the aerial part and the fresh mass gradually increased as the sucrose concentration increased (Tab. 1). The linear regression coefficients revealed that 94 and 95% of variation in the height of the aerial part and that 88 and 96% of variation of fresh mass were explained by the increase in sucrose concentration in the media with 50 and 100% of the original macronutrient salts concentrations, respectively (Tab. 2). Plantlets cultivated in the medium with 100% of the salts plus 60 g L^{-1} of sucrose had the significantly highest means for height of aerial part and fresh mass (6.6 cm and 2.0 g), followed by the plantlets in the treatments with 50% of the original macronutrient salts with 60 g L^{-1} of sucrose (5.5 cm and 1.3 g) and with 45 g L^{-1} of sucrose (5.4 cm and 1.4 g) (Tab. 1).

There was no gradual increase in number of leaves on plantlets propagated *in vitro* in proportion to increases in sucrose concentration (Tab. 1). When combined with 100% of the macronutrient salts, sucrose allowed formation of leaves in numbers that were statistically equal at all concentrations tested, with means varying from 8.2 to 9.2, and only the medium with zero sucrose produced plantlets with significantly lower numbers of leaves (5.8). In media with 50% of the macronutrient salts, plantlets growing with 15 and 45 g L^{-1} of sucrose had higher numbers of leaves (means of 8.7 and 8.2) than plantlets grown with zero sucrose. The numbers of leaves at concentrations of 30 and 60 g L^{-1} of sucrose were intermediate (Tab. 1). A study of *in vitro* culture of *Miltonia flavescens* did not detect an influence from different sucrose, glucose and maltose concentrations either (15, 30, 45 and 60 g L^{-1}), since plantlets presented a mean of 10.5 to 14.9 leaves in all carbohydrates tested (Besson *et al.* 2010).

In common with height of aerial part and fresh mass, the root system of *Cattleya intermedia* plantlets also benefited from increased sucrose concentrations. The regression coefficients revealed that 90 and 80% of variation in length of longest root and that 93 and 88% of variation in number of roots were explained by increases in sucrose, for media with 50 and 100% of macronutrient salts respectively (Tab. 2). When plantlets were cultivated in 100% of the original concentrations of salts, the highest mean numbers of roots (mean of 15.0) and greatest lengths of longest root (mean of 4.1 cm) were observed for the medium with 60 g L^{-1} of sucrose (Tab. 1). In the media with 50% of the macronutrient salts, the highest numbers of roots and the greatest lengths of longest root were observed for treatments with 45 g L^{-1} of sucrose (means of 14.9 and 4.5 cm, respectively) and 60 g L^{-1} of sucrose (means of 15.4 and 4.3 cm, respectively), which were not significantly different from each other. Of all of the sucrose concentrations tested, only the treatment with 45 g L^{-1} was significantly more beneficial with 50% of the macronutrient salts than with 100% of these nutrients (Tab. 1). Formation of a well-developed root system is of fundamental importance to conferring the greatest probability of survival during *ex vitro* acclimatization of the plantlets (Besson *et al*. 2010).

The benefits for the parameters assessed observed in this study as a result of increasing the sucrose concentration in the culture medium are as would be expected from the primordial function of carbohydrates of stimulating growth and root formation (George & Sherrington 1984) and, consequently, of increasing the biomass of plantlets by incorporation of carbon (Riek *et al*. 1997). Although there are reports in the literature that high concentrations of sugar can be prejudicial to micropropagated plantlets, because they can change the water potential of the medium (Paiva-Neto & Otoni 2003) and cause effects such as reduced absorption of water and mineral salts (Fráguas *et al*. 2003; Besson *et al*. 2010), and even inhibit the photosynthesis process (Yamada & Sato 1978; Cappelades *et al*. 1991; Hdider & Desjardins 1994; Kozai 1991), this condition is not true for all species. In general, plantlets propagated *in vitro* are considered semi-autotrophic, since there is inadequate light intensity for metabolic activities (Rolland *et al*. 2002). This characteristic means that such plantlets do not have the metabolic conditions necessary to supply the carbohydrates they need to develop (Yamada & Sato 1978; Barz & Hüsemann 1982). The consequence of this is that their cells need an exogenous carbohydrate supply (Besson *et al*. 2010; Hazarika 2003), because carbohydrates are essential for the respiration process and are the precursors for biosynthesis of structural and functional components such as oligosaccharides, amino acids and other molecules needed for growth (Caldas *et al*. 1998). Furthermore, a supply of carbohydrates is recognized as an important requirement for successful acclimatization *ex vitro*, because it enables stocks of starch and sucrose to be built up in cells (Capellades *et al*. 1991; Hazarika 2003).

In common with the present study, there are reports showing that MS medium with 100% or 50% of the original macronutrient salts concentrations, supplemented with sucrose, also has a positive influence on the development of other species of Orchidaceae. The height of the aerial portion, the number of leaves and the fresh mass of roots were all superior in *Cattleya granulosa* plantlets micropropagated in an MS medium containing 45 g L^{-1} of sucrose,

Table 1. Values (mean ± standard deviation) regarding height of the aerial part, number of leaves, longest root length, number of roots, and fresh mass in *Cattleya intermedia* propagated in MS medium with different concentrations of macronutrient salts and sucrose, after 180 days. Mean values followed by the same letter in the line do not differ significantly by the Tukey's test ($p<0.05$). *Indicates significant difference between the concentrations of macronutrient salts according to the Student t test ($p<0.05$).

Salts MS	Sucrose concentration (g L^{-1})					F	p
	0	15	30	45	60		
			Height of the aerial part (cm)				
50 % MS	3.24 ± 0.81d	3.82 ± 0.69c	4.59 ± 0.73b	5.37 ± 0.85a	5.51 ± 0.99a*	56.502	< 0.001
100 % MS	3.08 ± 0.64d	4.07 ± 0.65c	4.72 ± 0.86b	5.14 ± 0.92b	6.58 ± 1.31a	87.173	< 0.001
t	0.818	-1.63	-0.669	1.171	-4.06		
p	0.416	0.107	0.505	0.245	<0.001		
			Number of leaves				
50 % MS	6.7 ± 2.5b	8.7 ± 2.3a	7.8 ± 2.8ab	8.2 ± 2.5a	7.6 ± 2.6ab	4.642	0.001
100 % MS	5.8 ± 2.1b	8.9 ± 3.6a	9.2 ± 3.5a*	8.2 ± 2.8a	8.8 ± 3.4a	9.73	< 0.001
t	1.944	0.037	-2.09	0.064	-1.608		
p	0.056	0.971	0.04	0.949	0.112		
			Longest root lenght (cm)				
50 % MS	2.05 ± 0.77d	2.64 ± 1.18c	3.36 ± 1.62b	4.48 ± 1.24a*	4.29 ± 1.18a	35.909	< 0.001
100 % MS	1.78 ± 0.95c	2.63 ± 0.92b	3.42 ± 1.22ab	3.20 ± 0.98ab	4.14 ± 1.27a	28.415	< 0.001
t	1.733	-0.262	-0.514	5.229	0.58		
p	0.087	0.794	0.609	< 0.001	0.563		
			Number of roots				
50 % MS	5.7 ± 2.3c*	7.6 ± 2.5b	9.9 ± 4.7b	14.9 ± 4.8a*	15.4 ± 5.4a	46.477	< 0.001
100 % MS	4.3 ± 2.2d	7,9 ± 3.2c	9,7 ± 3.2bc	11,7 ± 3.6ab	15.0 ± 4.9a	56.869	< 0.001
t	2.407	-0.401	0.086	3.376	0.06		
p	0.019	0.690	0.931	0.001	0.952		
			Fresh mass (g)				
50 % MS	0.50 ± 0.25c	0.69 ± 0.34b	0.81 ± 0.38b	1.41 ± 0.51a	1.33 ± 0.55a	40.373	< 0.001
100 % MS	0.43 ± 0.19d	0.77 ± 0.37c	1.05 ± 0.47b*	1.26 ± 0.53b	2.01 ± 1.21a*	54.669	< 0.001
t	1.109	-0.976	-2.518	1.384	-3.13		
p	0.271	0.332	0.014	0.17	0.002		

Table 2. Linear regression (R^2) for morphological parameters of *Cattleya intermedia* plantlets and sucrose concentrations after 180 days growing in the MS medium.

Medium	Adjusted R^2	p	Regression Equation
		Height of the aerial part	
50 % MS	0.936	0.004	y = 1.179 + 0.010X
100 % MS	0.953	0.003	y = 1.149 + 0.012x
		Longest root lenght	
50 % MS	0.902	0.009	y = 0.680 + 0.014x
100 % MS	0.799	0.026	y = 0.568 + 0.014x
		Number of roots	
50 % MS	0.933	0.005	y =1.679 + 0.018x
100 % MS	0.884	0.011	y = 1.495 + 0.021x
		Fresh mass	
50 % MS	0.879	0.012	y = - 0.767 + 0.018x
100 % MS	0.962	0.002	y = - 0.823 + 0.023x

when compared with plantlets grown with 15 and 30 g L^{-1} or zero sucrose (Pinto *et al*. 2010). Species belonging to other genera of Orchidaceae also exhibited increased development *in vitro* in the presence of higher concentrations of sucrose. *Dendrobium nobile* plantlets cultured *in vitro* in a medium with half the concentration of macronutrients plus 60 g L^{-1} of sucrose exhibited greater mean heights of the aerial parts, greater fresh mass and high multiplication rates compared with plantlets in media with lower concentrations of sucrose (0 to 30 g L^{-1}) (Faria *et al*. 2004). At the same sucrose and macronutrient salts concentrations, plantlets of *Oncidium varicosum* cultivated *in vitro* also exhibited greater mean heights of the aerial part, greater fresh mass, higher numbers of roots and greater lengths of roots (Rego-Oliveira *et al*. 2003). *Oncidium baueri* plantlets cultivated in a medium with half the macronutrient salts concentrations and 40 g L^{-1} of sucrose exhibited higher means for the height of the aerial part and fresh mass and greater root system development (Sorace *et al*. 2008). Plantlets of *Anathallis adenochila* showed greatest height of aerial part and length of the longest root when grown in culture media with 50% of macronutrient salts and 30 or 60 g L^{-1} of sucrose (Endres-Júnior *et al*. 2014).

However, in contrast to results observed for *Cattleya intermedia*, lower sucrose concentrations can have a positive impact on the regeneration of certain orchids *in vitro*, and this phenomenon has even been described with relation to other species of *Cattleya*, providing evidence that each species exhibits specific behavior. Although propagation of *Cattleya loddigesii* plantlets in a medium with 100% of the macronutrients and 60 g L^{-1} of sucrose stimulated root development, development of the aerial part and gain in fresh mass were both superior in sucrose concentrations from 16 to 30 g L^{-1} with the addition of the growth regulator gibberellic acid (Rezende *et al*. 2009). In a further study, plantlets of the same species exhibited higher means for length of the aerial part, length of the largest leaf, number of leaves, number of roots, length of roots and fresh mass when cultivated in MS medium supplemented with 20 g L^{-1} of sucrose, and the plantlets' growth and development reduced in line with increasing concentrations of the carbohydrate (30 and 40 g L^{-1}) (Galdiano *et al*. 2013a). In cultures of *Cattleya violacea*, the greatest values for height of the aerial part, number of leaves, fresh mass, and number and length of roots were observed for plantlets grown in media with 50% of the macronutrient salts and 20 and 30 g L^{-1} of sucrose (Galdiano *et al*. 2013b). For *Miltonia flavescens*, the greatest height of the aerial part, the greatest gain of fresh mass and the largest number of roots were observed when plantlets were cultivated in MS medium with half of the concentrations of macronutrients and micronutrients, supplemented with 30 g L^{-1} of sucrose (Besson *et al*. 2010). Sucrose concentrations of 13 to 29 g L^{-1} proved beneficial for development of the root system and length of the aerial part of *Caularthron bicornutum* (Pivetta *et al*. 2010).

In the present study, *Cattleya intermedia* plantlets grown in MS medium with 60 g L^{-1} of sucrose exhibited greater height of the aerial part and greater fresh mass in a medium containing 100% of the original concentration of macronutrient salts, when compared with a medium with 50% of these salts (Tab. 1). The original MS medium is considered to be a nutrient substrate that is rich in salts, and its formula contains nitrogen in high concentrations, which can be absorbed in the form of nitrate (NO_3^-) or ammonium (NH_4^+) (Sakuta *et al*. 1987). Nitrogen can contribute to cell metabolism and acts as a buffer agent, controlling pH and contributing to absorption of other nutrients present in the medium (Nagao *et al*. 1994). There are reports that *Cattleya harrisoniana* var. *alba* (Beer) plantlets achieved greater development in media containing 100% of the macronutrient salts of the original MS, with the difference that in the study in question the medium also contained 2.5 g L^{-1} of activated charcoal (Schneiders *et al*. 2012). In contrast, for *Cattleya forbesii*, *Cyrtopodium paranaenese* and *Laelia cinnabarina* micropropagated plantlets, best results were recorded when media with half of the MS concentration of macronutrient salts were used (Unemoto *et al*. 2007; Rego-Oliveira & Faria 2005; Stancato & Faria 1996), whereas for *Laelia lundii*, *Miltonia flavescens* and *Catasetum fimbriatum*, no differences were observed between 50 and 100% salts concentrations (Unemoto *et al*. 2007; Muller *et al*. 2007; Rego-Oliveira & Faria 2005). These variable results highlight the importance of studies designed to investigate germination and development of orchid plantlets, since the genetic characteristics of each species are linked with their physiological peculiarities (Kozay *et al*. 1997; Fortes & Pereira 2001).

Although the results showed that *Cattleya intermedia* plantlets grown from seeds germinated *in vitro* exhibited 100% survival in MS media with all of the different concentrations of macronutrient salts and sucrose that were tested, in general, and after conducting a cluster analysis with all the plantlet parameters, it is possible to delineate two different groups of treatments (Fig. 1). The combinations of 100% of the MS macronutrient salts and 60 g L^{-1} of sucrose; 50% of the macronutrients and 60 g L^{-1} of sucrose; and 50% of salts with 45 g L^{-1} of sucrose all had similar influences on the plant responses. All of the remaining combinations of macronutrient salts and sucrose formed one large group, within which the two treatments with zero sucrose clustered together and differed from the others, being the treatments which had the least positive influence on the development of the plantlets. The subset formed by the treatments with 100 and 50% of the salts combined with 15 and 30 g L^{-1} of sucrose plus the treatment with 100% of the macronutrients and 45 g L^{-1} of sucrose also contained a further subdivision by which the two treatments with only 15 g L^{-1} of sucrose were less beneficial to the plants than those with higher carbohydrate concentrations.

In the present study, *Cattleya intermedia* plantlets grown in an asymbiotic culture and acclimatized in a coconut-

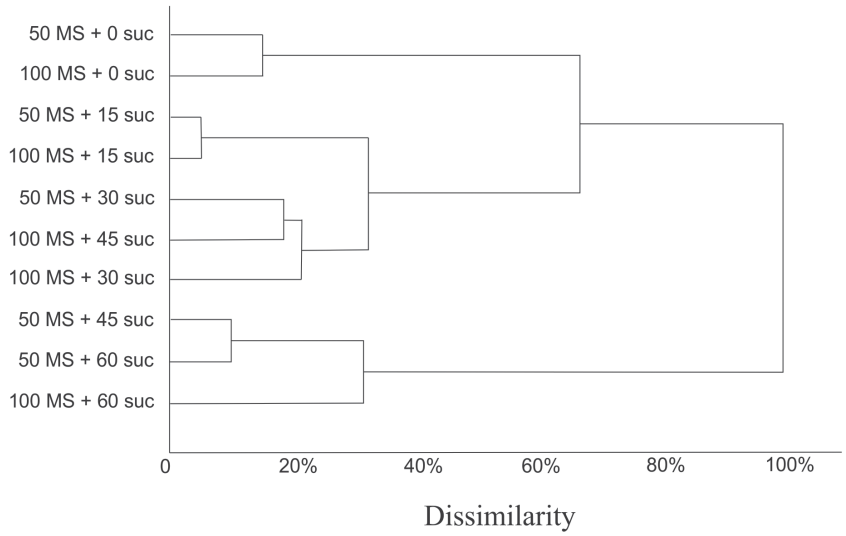

Figure 1. Dendrogram obtained from hierarchical cluster analysis of culture media treatments with variable standardization, utilizing the index of dissimilarity based on Euclidean distance. MS = macronutrient salts percentages of MS medium; suc = concentration of sucrose (g L^{-1}).

fiber-based substrate exhibited 96% survival after 5 months, confirming results reported for the same species by Sasamori *et al*. (2014), who recorded a mean survival rate of 94% for plantlets acclimatized in coconut fiber supplemented with other substrates. In the natural environment to which the plants were reintroduced, percentage survival observed after 12 months was 98.6%, which is comparatively superior to survival rates that have been reported for the same species (Dorneles & Trevelin 2011) and *Vanda coerulea* (Aggarwal *et al*. 2012). These plants are still being monitored to enable analysis of their qualitative and quantitative characteristics and their interaction with the typical biotic and abiotic variables of the natural environment.

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