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In vitro propagation of *Vriesea incurvata*: conservation of a bromeliad endemic to the Atlantic Forest

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ABSTRACT – *Vriesea incurvata* is an epiphytic bromeliad of the Atlantic Forest with ornamental attributes that encourage extractivism. This study assessed the influence of sucrose (10, 30 and 60 g L^{-1}) on the survival and development of *V. incurvata* plantlets cultivated for 180 days on MS medium and acclimatizated for 150 days under controlled conditions. Survival of 100% was recorded in all *in vitro* treatments. Sucrose at concentration of 60 g L^{-1} provided highest length of the aerial portion and of the longest root, greatest number of leaves and roots, highest fresh mass and lowest contents of chlorophylls and carotenoids. The higher sucrose concentration also positively influenced the development of plantlets *ex vitro,* allowing for increased averages for the parameters assessed. The results indicate micropropagation as an alternative by which *V. incurvata* plantlets may be provided for commercial means, reducing the extractivism from natural populations, or for reintroduction into their natural habitat.

Keywords: carbon source, chlorophyll, micropropagation, nutrition

RESUMO – **Propagação *in vitro* de *Vriesea incurvata*: conservação de uma bromélia endêmica da Floresta Atlântica**. *Vriesea incurvata* é uma bromélia epifítica da Floresta Atlântica, com atributos ornamentais que estimulam o extrativismo. Este estudo avaliou a influência da sacarose (10, 30 e 60 g L^{-1}) na sobrevivência e no desenvolvimento de plântulas de *V. incurvata* cultivadas por 180 dias em meio MS e aclimatizadas por 150 dias sob condições controladas. Sobrevivência de 100% foi observada em todos os tratamentos *in vitro*. Sacarose na concentração de 60 g L^{-1} propiciou maior comprimento da porção aérea e da raiz mais longa, maior número de folhas e raízes, maior massa fresca e conteúdos menores de clorofilas e carotenoides. A maior concentração de sacarose também influenciou positivamente o desenvolvimento das plântulas *ex vitro,* permitindo maiores médias para os parâmetros avaliados. Os resultados indicam a micropropagação como alternativa para a obtenção de plântulas de *V. incurvata* para fins comerciais, reduzindo o extrativismo de populações naturais, ou para reintrodução em habitat natural.

Palavras-chave: clorofila, fonte de carbono, micropropagação, nutrição

INTRODUCTION

Bromeliaceae Juss. has neotropical distribution with 58 genera and 3,248 species described (Luther 2010), of which 40% are found in Brazil (Martinelli *et al*. 2008). The highest number of endemic species is found in the Atlantic Forest, distributed along the coast of Brazil (Martinelli *et al.* 2008). Epiphytic "tank" bromeliads accumulate rainwater in their rosettes (Benzing 1990), forming different micro habitats for plant species and primarily for animals (Rocha *et al*. 1997). Bromeliads are also excellent at intercepting nutrients from the air (Oliveira & Coelho Neto 2001), and by incorporating these nutrients, they play an important role in the forest system (Oliveira 2004). However, the fact that the Atlantic Forest is suffering an increasing reduction in its plant cover (Fundação SOS Mata Atlântica & INPE 2014) has an especially significant impact on *Bromeliaceae,* of which the threat of extinction is aggravated due to its high ornamental potential (Forzza *et al*. 2013).

Vriesea incurvata Gaudich. is an epiphytic bromeliad endemic to Brazil, distributed throughout the Atlantic Forest biome in the states of Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, and Rio de Janeiro (Forzza *et al*. 2015). The specimens are a maximum of 50 cm in height, with smooth leaves and no thorns, arranged into rosettes to accumulate water. The inflorescence is sub-multifloral spike between 30 to 40 cm in height (Negrelle & Muraro 2006). In the state of Paraná, *V. incurvata* is among the bromeliads which are most gathered and sold illegally (Negrelle & Anacleto 2012). Although there is no record for the other States, the ornamental attributes of the species encourage the gathering of specimens in their reproductive stage, which contributes to the decline of natural populations.

In natural conditions or by means of conventional propagation, the seeds produced by epiphytic bromeliads possess low germination capability (Mercier & Kerbauy 1995), due to the need for specific, adequate conditions of abiotic factors and the substrate (Winkler *et al*. 2005). *In vitro* culture using seeds is recommended for the conservation of bromeliads, being that there exists little information regarding the biology of the reproduction

and the behavior of the seeds, and it contributes to the maintaining of genetic variability (Carneiro & Mansur 2004). Using *in vitro* propagation, high rates of seed germination can be reached and healthy plantlets can be obtained in a short period of time (Mercier & Kerbauy 1995). These plantlets may be used for commercialization, lowering pressures on the natural populations (Tamaki *et al*. 2011), or to provide programs for reintroduction into natural environments (Benson 1999).

Sucrose, the nutrient medium's main source of carbon, is considered one of the fundamental substances for *in vitro* plant morphogenesis (Grattapaglia & Machado 1998, Thorpe *et al*. 2008), responsible for supplying metabolic energy and carbon skeletons (Thorpe *et al*. 2008), and its concentration in the medium depends on what the species requires (Caldas *et al.* 1998, Besson *et al*. 2010). If a lack of sucrose can be harmful to the development of the specimens, high concentrations can inhibit the synthesis of chlorophyll and reduce the ability of tissues to photosynthesize (Yamada & Sato 1978, Rolland *et al*. 2002), despite the rate of *in vitro* photosynthesis being low (Thorpe *et al.* 2008). Despite the importance of this carbohydrate to the development of the specimens, little is understood of the effect of its concentration on the survival and the development of bromeliad plantlets *in vitro* and during acclimatization *ex vitro*.

The knowledge about the best nutritional conditions for the *in vitro* development of bromeliads is still scarce (Kanashiro *et al*. 2007, Aranda-Peres *et al*. 2009, Costa *et al*. 2012, Martins *et al*. 2015), and little is known about the effect of the culture medium on the acclimatization of plantlets (Kurita *et al*. 2014). Specifically for *V. incurvata*, no records are found in the literature. The present study assessed the influence of different concentrations of sucrose on the *in vitro* survival and development of *V. incurvata* plantlets and the *ex vitro* acclimatization, aiming the establishment of a protocol for the production of plants for conservation or commercial purposes. Our hypothesis was that a greater number of plantlets would survive and that they would present increased development in the presence of higher concentrations of sucrose during the *in vitro* and *ex vitro* stages.

MATERIAL AND METHODS

In vitro propagation of *V. incurvata*

Plantlets 1.0 ± 0.2 cm in height were germinated from seeds according to Droste *et al*. (2005), and the capsules were collected from specimens of a population established in a fragment of the Atlantic Forest in Rio Grande do Sul, Brazil. After 60 days in the germination medium, the plantlets were cultivated in flasks with 30 mL of MS medium (Murashige & Skoog 1962), supplemented with 4 g L^{-1} of Phytagel™, 5 g L^{-1} of activated charcoal, pH set to 6.4 before sterilization in autoclave. The treatments consisted of 10, 30 and 60 g L^{-1} of sucrose combined, respectively, with two compositions of the mineral salts of the MS medium: 25% of the original concentration of the macronutrients (25M) and 25% of the original concentrations of the nitrogenous salts (25N). These salts combinations allowed for increased development of *V. incurvata* plantlets in preliminary tests (Sasamori *et al*. 2016). Seventy specimens were grown for each of six treatments, distributed into groups of five in 14 flasks (200 mL volume) for a total of 420 plantlets. A subculture was taken every 60 days to a fresh medium of the same composition as the previous medium.

Each specimen was measured after 180 days for length of the aerial portion, the number of leaves, number of roots, length of the longest root, and fresh mass, using a pachymeter and a high-precision balance. The chlorophyll content of the plantlets from each treatment was also determined at this time. Leaf samples were collected from plantlets from each of the treatments, taking 20 mg triplicates of leaf tissue immersed in 1 mL of Dimethyl sulfoxide (DMSO) for 24 h in a 65 °C water bath. Spectrophotometer readings were taken (Spectramax® M3) using 96-well plates for cell cultures, having dispensed 100 µL aliquots into each well. For each 1 mL sample, triplicates of 100 µL were taken for readings, totaling nine samples per treatment. The spectrophotometer readings were taken at wavelengths 665 nm, 649 nm, and 480 nm. The concentrations were calculated according to the equations proposed by Wellburn (1994).

Ex vitro acclimatization of *V. incurvata*

After measuring the biometric data, 45 plantlets were randomly selected from each treatment and submitted to acclimatization, following the methodology of Sasamori *et al*. (2014), and planted in a commercial substrate (Carolina Soil®, with a base of peat, vermiculite, and carbonized rice hulls) in transparent plastic containers covered with lids (24 cm x 18 cm, 10 cm in height). A layer of crushed rock (granite) was placed at the bottom of each container to facilitate draining and aeration of the root system. Fifteen plantlets were planted into each container and kept at 26±1 °C, filtering out 70% of the natural light using a polypropylene screen.

Leaf fertilization with the commercial fertilizer Peters® (NPK 20-20-20) at a concentration of 1 g L^{-1} were added every two weeks. To keep the plantlets at high humidity, the containers were covered with transparent plastic lids for 60 days, after which the gradual removal of the lids began, exposing the specimens to the atmospheric air. Irrigation was done manually every two days or as need. The plantlets remained in acclimatization for 150 days, after which data was obtained in terms of the survival, length of the aerial portion, number of leaves, number of roots, length of the longest root, and fresh mass of each specimen. Following conclusion of acclimatization, leaf samples were randomly collected from plantlets from each of the treatments to measure the concentration of the contents of

photosynthetic pigments. The biometric parameters and the concentrations of pigments were measured as described previously for the *in vitro* stage.

Statistical analysis

The survival data were transformed into percentages. The biometric data obtained in the *in vitro* culture experiments and from the *ex vitro* acclimatization were transformed, respectively, into log (x+1) and square root (x) for data standardization. The biometric data were compared by analysis of variance (ANOVA), followed by the Tukey test at 5% probability, in addition to the Student t test at 5% probability. The Pearson correlation analysis was performed in order to verify the relationship between sucrose and the biotic parameters (length of the aerial portion, number of leaves, length of the longest root, number of roots and fresh mass) during the acclimatization stage. Analyses were performed using the statistics program SPSS, version 20 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

All culture media yielded the survival of 100% of the *V. incurvata* specimens. Even in the presence of the lowest concentration of sucrose, the plantlets did not present necrosis of the leaves, which generally occurs in tissues cultured in a medium with concentrations of this carbohydrate lower than 20 g L^{-1} (Grattapaglia & Machado 1998), and the result of which is a high plantlet mortality rate. Assessment of the initial development of the plantlets *ex vitro* is important, however, being that the amount of carbohydrate stored in their tissues may not be sufficient for the process of acclimatization and development, despite having allowed for the survival of the specimens during the *in vitro* culture (Grout 1988).

The development of the aerial portion was influenced by the concentration of sucrose, as its addition to the culture medium lead to a gradual increase in the length of the aerial portion and the number of leaves. The plantlets grown in the media supplemented with 60 g L^{-1} of sucrose presented, as a significantly mean, the highest averages of the length of the aerial portion and the number of leaves, yet upon comparing the treatments with 10 and 30 g L^{-1} of sucrose, the plantlets did not present a significant difference among themselves for the same parameters and in both media (25N and 25M) (Tab. 1; Fig. 1). In the micropropagation process, the addition of sucrose to the culture medium is essential as a source of carbon for the plantlets, as it is used for biosynthesis of structural and functional components of growth, although the ideal concentration depends on what each plant species requires (Caldas *et al*. 1998). Generally, supplying carbohydrates exogenously allows for the increase of the starch and sucrose reserves in the leaves of the micropropagated plantlets, acting as organs for the storage of energy, which will be used in the acclimatization stage for growing new leaves adapted to the *ex vitro* environment, improving the success of acclimatization (Capellades *et al*. 1991, Fuentes *et al*. 2006).

For the root system of the plantlets, the increase of sucrose concentration in the culture medium benefited the increase of the length of the longest root and of the number of roots. The 25N culture medium supplemented with 60 g L^{-1} of sucrose yielded the highest averages for both parameters, which, however, did not significantly differ from those in the medium with 30 g L^{-1} of sucrose, which showed medium values. The 25M medium yielded a significantly greater length of the longest root of the plantlets when supplemented with 30 and 60 g L^{-1} of sucrose, while no statistical difference between the concentrations of carbohydrate was found for the number of roots (Tab. 1; Fig. 1). The development of the root system of the *V. incurvata* plantlets was significantly greater for all concentrations of sucrose tested when only the nitrogen concentration of the medium was reduced (25N) (Tab. 1; Fig. 1). The reduction of the original macronutrients (25M) may have led to a lack of nutrients essential to the development of the root system of the plantlets (Grattapaglia & Machado 1998), which, when compared to the specimens grown in the 25N medium, showed lower averages. The formation and growth of roots during the *in vitro* culture is important to the subsequent acclimatization stage of the plantlets, allowing for the specimens' increased survival (Besson *et al*. 2010) and although carbohydrate concentrations between 2 and 3% are generally used for the rooting of plantlets grown *in vitro* (Grattapaglia & Machado 1998), the concentration of 60 g L^{-1} of sucrose was seen to be beneficial to the development of the root system of *V. incurvata*.

The addition of sucrose to the culture medium was even seen to be beneficial to the fresh mass increase of the propagated specimens of *V. incurvata*. The plantlets grown in media supplemented with 60 g L^{-1} of sucrose showed significantly the highest average for fresh mass, while in both media, 25N and 25M, the plantlets showed no significant difference among themselves when comparing the treatments with 10 and 30 g L^{-1} of sucrose (Tab. 1).

Just as for *V. incurvata*, plantlets of the orchids *Cattleya intermedia* Graham (Sasamori *et al*. 2015) and *Oncidium varicosum* Lindl. & Paxton (Rego-Oliveira *et al*. 2003) showed higher averages in the height of the aerial portion, fresh mass, number and length of roots in a medium with 60 g L^{-1} of sucrose, while *Dendrobium nobile* Lindl. (Faria *et al*. 2004) showed an increased height of the aerial portion and fresh mass in this sucrose concentration. However, elevated carbohydrate concentrations may cause negative effects such as a change in the water potential of the medium and a reduction in the absorption of water and salts by the plantlets (Besson *et al*. 2010), which is related to the fact that some species experience greater development in the presence of less carbohydrate, such as the bromeliad *Bilbergia zebrina* (Herb.) Lindl., which showed higher average values for the height of the aerial portion, length of the longest root, and fresh mass in a medium with 15 to 45 g L^{-1} of sucrose (Martins *et al*. 2015).

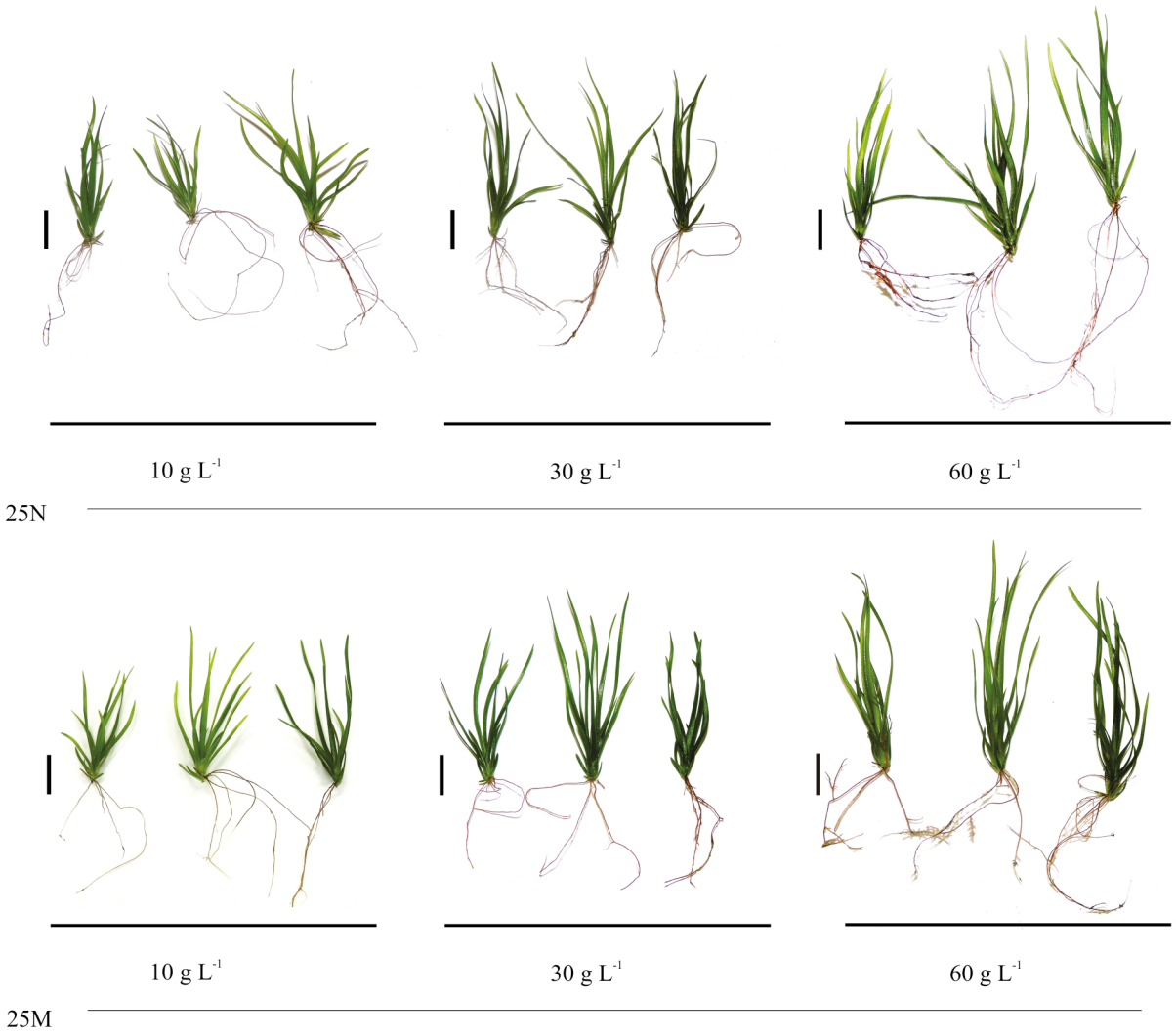

Fig. 1. *Vriesea incurvata* plantlets after 180 days in MS medium with different macronutrient combinations (25N and 25M) and sucrose concentrations (10, 30 and 60 g L^{-1}). 25M - 25% of the original concentrations of the macronutrients; 25N - 25% of the original concentrations of the nitrogenous salts. Scale bars = 1 cm.

The production of photosynthetic pigments by leaf tissue of the *V. incurvata* plantlets was influenced by the increase in sucrose concentration of the culture medium. In the 25N medium, the highest contents of chlorophylls *a* and *b* and of carotenoids were checked when the plantlets were grown in the presence of 30 g L^{-1} of sucrose, and lower averages were recorded in the treatment with 60 g L^{-1} of sucrose. In the 25M medium, the average contents of chlorophylls *a* and *b* in the presence of 10 and 30 g L^{-1} of sucrose were significantly higher than the average in the medium with 60 g L^{-1} of this carbohydrate (Tab. 1). In comparison, the contents of the three pigments in the leaf tissues were significantly lower in the 25M medium than in the plantlets in the 25N medium, except for the carotenoid content in the treatment with 60 g L^{-1} of sucrose (Tab. 1). The contents of chlorophylls *a* and *b*, and of carotenoids of the leaf tissue of *V. incurvata* corroborate observations in other species, for which high concentrations of carbohydrates in the culture medium leads to the inhibition of the chlorophyll content (Yamada & Sato 1978, Cappellades *et al*. 1991, Hdider & Desjardins 1994). The contents of photosynthetic pigments show a variation according to the plant species, and the molecules of chlorophyll *a* are generally most abundant, followed by chlorophyll *b* and carotenoids (Streit *et al*. 2005), corroborating the results observed for *V. incurvata*. Furthermore, plantlets grown *in vitro* show a low content of carotenoids due to the low intensity of light (Fuentes *et al*. 2006).

In the *in vitro* propagation, the plantlets are considered mixotrophic, as they show low photosynthetic activity (Rolland *et al*. 2002), which results in low capacity to produce carbohydrates necessary for development (Yamada & Sato 1978), rendering the plantlets dependent on an exogenous source of carbohydrates (Besson *et al*. 2010). In contrast, high concentrations of sucrose in the culture medium may specifically inhibit the production of chlorophyll and photosynthesis, making autotrophic growth less viable (Yamada & Sato 1978, George *et al*. 2008). The high concentration of sucrose in the culture medium

Table 1. Values (mean ± standard deviation) regarding of the parameters measured in *Vriesea incurvata* plantlets after 180 days in MS medium with different macronutrient combinations and sucrose concentrations.

Medium	Sucrose concentration (g L^{-1})				
	10	30	60	F	p
	Length of the aerial portion (cm)				
25N	3.8 ± 0.8 b	4.1 ± 0.8 b	4.8 ± 1.2 a	14.149	< 0.001
25M	3.6 ± 0.8 b	3.8 ± 0.7 b	4.6 ± 1.4 a	16.299	< 0.001
t	1.147	1.430	0.817	-	-
p	0.254	0.155	0.415	-	-
	Number of leaves				
25N	13.1 ± 2.3 b	13.8 ± 2.4 b*	14.8 ± 2.1 a	9.198	< 0.001
25M	13.8 ± 2.2 a	12.6 ± 1.9 b	14.3 ± 2.7 a	8.703	< 0.001
t	-1.750	2.968	1.490	-	-
p	0.083	0.004	0.139	-	-
	Longest root length (cm)				
25N	5.1 ± 1.4 b**	5.5 ± 2.0 ab*	5.9 ± 1.4 a**	3.892	0.022
25M	3.6 ± 1.1 b	4.4 ± 1.4 a	4.8 ± 1.5 a	12.641	< 0.001
t	6.459	3.060	4.490	-	-
p	< 0.001	0.003	< 0.001	-	-
	Number of roots				
25N	5.0 ± 1.0 b*	5.2 ± 1.4 ab*	5.9 ± 1.6 a*	5.021	0.008
25M	4.4 ± 0.9 a	4.5 ± 1.2 a	5.0 ± 1.7 a	2.065	0.130
t	3.330	3.131	3.104	-	-
p	0.001	0.002	0.002	-	-
	Fresh mass (mg)				
25N	103 ± 33 b*	109 ± 42 b*	181 ± 94 a	27.690	< 0.001
25M	88 ± 32 b	90 ± 34 b	164 ± 118 a	21.683	< 0.001
t	2.511	2.732	0.994	-	-
p	0.013	0.007	0.322	-	-
	Chlorophyll *a* (Chl *a*) content (mg g^{-1})				
25N	0.282 ± 0.017 b*	0.353 ± 0.040 a*	0.212 ± 0.044 c*	46.755	< 0.001
25M	0.247 ± 0.012 a	0.269 ± 0.064 a	0.135 ± 0.076 b	18.576	< 0.001
t	5.955	3.846	3.027	-	-
p	<0.001	0.001	0.006	-	-
	Chlorophyll *b* (Chl *b*) content (mg g^{-1})				
25N	0.132 ± 0.005 b*	0.162 ± 0.018 a*	0.116 ± 0.015 c*	35.689	< 0.001
25M	0.116 ± 0.008 a	0.124 ± 0.029 a	0.093 ± 0.017 b	8.139	0.001
t	5.621	3.933	3.531	-	-
p	< 0.001	0.001	0.002	-	-
	Carotenoid (Car) content (mg g^{-1})				
25N	0.049 ± 0.004 b*	0.060 ± 0.010 a*	0.040 ± 0.007 c	21.295	< 0.001
25M	0.045 ± 0.002 a	0.048 ± 0.009 a	0.045 ± 0.003 a	1.302	0.286
t	2.264	2.953	-2.139	-	-
p	0.039	0.007	0.050	-	-

Average values on the line followed by the same letter did not differ significantly according to the Tukey test at 5% probability (p = probability; F = statistics of the ANOVA test). *Indicate significant differences between the concentrations of macronutrients according to Student *t* test at 5% probability (p = probability; t = statistics of the Student *t* test). 25M - 25% of the original concentrations of the macronutrients; 25N - 25% of the original concentrations of the nitrogenous salts.

contributes to the accumulation of soluble sugars in the leaf tissues of the plantlets, which may lead to the reduction of Rubisco enzyme activity and the reduction of rate of regeneration of ribulose-1,5-biphosphate carboxylase (Capellades *et al*. 1991).

Low chlorophyll content may also be harmful during the plantlet acclimatization stage, with their metabolism changing from mixotrophic to autotrophic *ex vitro*, when the plantlets need to produce energy by means of photosynthesis in order to establish their development (Fuentes *et al*. 2006). Some species propagated *in vitro* take around two weeks or more after being transferred to the *ex vitro* environment to reach a positive rate of photosynthesis (Grout & Aston 1978).

During the acclimatization period, necrosis was found in two specimens, one from the 25N treatment with 30 g L^{-1} of sucrose and the other from the 25M medium with 60 g L^{-1} of sucrose. The other treatments resulted in 100% survival of plantlets in such a way that no delayed influence of the

Table 2. Values (mean ± standard deviation) regarding the parameters measured in *Vriesea incurvata* plantlets after 150 days acclimatization on substrate.

Medium	Sucrose concentration (g L^{-1})				
	10	30	60	F	p
			Length of the aerial portion (cm)		
25N	5.3 ± 1.4 b	5.8 ± 1.6 b	7.2 ± 1.9 a*	15.866	< 0.001
25M	5.6 ± 1.3 b	5.6 ± 1.6 b	6.4 ± 1.7 a	3.552	0.031
t	-1.272	0.431	2.106	-	-
p	0.207	0.667	0.038	-	-
			Number of leaves		
25N	16.3 ± 2.6 b	19.6 ± 3.8 a	18.8 ± 3.2 a	12.247	< 0.001
25M	20.7 ± 3.9 a**	18.4 ± 3.3 b	18.0 ± 4.2 b	7.275	0.001
t	-6.264	1.658	1.168	-	-
p	< 0.001	0.101	0.246	-	-
			Longest root length (cm)		
25N	5.1 ± 1.0 a*	5.6 ± 1.6 a*	5.9 ± 1.4 a*	2.276	0.107
25M	4.3 ± 1.4 a	4.4 ± 1.1 a	4.8 ± 1.2 a	2.378	0.097
t	3.166	4.418	3.061	-	-
p	0.003	< 0.001	0.003	-	-
			Number of roots		
25N	5.9 ± 1.3 b	6.6 ± 1.8 ab*	7.0 ± 2.0 a*	4.626	0.011
25M	5.7 ± 1.2 a	5.6 ± 1.5 a	6.2 ± 1.8 a	1.620	0.202
t	0.629	3.026	2.163	-	-
p	0.531	0.003	0.033	-	-
			Fresh mass (mg)		
25N	230 ± 121 b	249 ± 122 b	447 ± 271 a	17.906	< 0.001
25M	209 ± 84 b	230 ± 108 b	389 ± 315 a	10.803	< 0.001
t	0.714	0.681	1.226	-	-
p	0.477	0.498	0.224	-	-
			Chlorophyll *a* (Chl *a*) content (mg g^{-1})		
25N	0.258 ± 0.019 a	0.224 ± 0.047 a	0.236 ± 0.040 a	1.288	0.305
25M	0.206 ± 0.061 b	0.192 ± 0.047 b	0.384 ± 0.039 a*	27.481	< 0.001
t	1.982	1.171	-6.499	-	-
p	0.095	0.269	< 0.001	-	-
			Chlorophyll *b* (Chl *b*) content (mg g^{-1})		
25N	0.095 ± 0.023 a	0.114 ± 0.032 a	0.087 ± 0.038 a	1.153	0.342
25M	0.120 ± 0.065 b	0.071 ± 0.056 b	0.222 ± 0.034 a*	12.424	0.001
t	-0.879	1.618	-6.420	-	-
p	0.421	0.137	< 0.001	-	-
			Carotenoid (Car) content (mg g^{-1})		
25N	0.051 ± 0.004 a*	0.043 ± 0.007 b	0.043 ± 0.004 b	5.148	0.020
25M	0.036 ± 0.009 b	0.036 ± 0.008 b	0.058 ± 0.004 a*	19.997	< 0.001
t	3.923	1.727	-7.217	-	-
p	0.003	0.115	< 0.001	-	-

Average values on the line followed by the same letter did not differ significantly according to the Tukey test at 5% probability (p = probability; F = statistics of the ANOVA test). *Indicate significant differences between the concentrations of macronutrients according to Student *t* test at 5% probability (p = probability; t = statistics of the Student *t* test). 25M - 25% of the original concentrations of the macronutrients; 25N - 25% of the original concentrations of the nitrogenous salts.

sucrose concentration used prior in the *in vitro* propagation stage was observed. Maintaining the high humidity of the air surrounding the plantlets is a fundamental and positive factor for the survival of the specimens during acclimatization, being that water stress is indicated as one of the main causes of necrosis in the plantlets during this period (Grattapaglia & Machado 1998). The methodology used in the acclimatization of the plantlets was efficient for the *ex vitro* adaptation of the specimens from this study, which corroborates the results of *C. intermedia* plantlet acclimatization on transparent plastic trays, which maintain the humidity around the plantlets (Sasamori *et al*. 2014).

For the development of acclimatized plantlets, a positive influence was observed from adding the sucrose during the *in vitro* propagation. Generally, during the acclimatization of the plantlets, an average relative growth of 39.1 to 55.6% of the length of the aerial portion was observed, along with an average relative rate increase of 123.3 to 155.6% of the fresh mass. The highest averages for the length of the aerial portion and the fresh mass were observed in the specimens from media with 60 g L^{-1} of sucrose, significantly higher than the averages of these parameters

in plantlets from treatments with 10 and 30 g L^{-1} of this carbohydrate. The Pearson correlation coefficients showed that the growth of the aerial portion in the 25N medium (R = 0.810; p = 0.008), and that the increase of the fresh mass in media 25N (R = 0.714; p = 0.031) and 25M (R = 0.777; p = 0.014), respectively, were positively related to the increase in sucrose concentration during the *in vitro* cultivation. The number of leaves also increased during the acclimatization period, which generally presented an average rate of relative growth of between 24.4 to 50.0%. However, although a significant difference in the number of leaves was recorded between the sucrose concentrations use in the media 25N and 25M (Tab. 2), sucrose concentration was not observed to have any influence (25N – R = 0.448; p = 0.226; 25M – R = 0.590; p = 0.095).

Sucrose concentration was not observed to have any influence on the root system of *V. incurvata* acclimatized plantlets. In most treatments, there was no root growth during acclimatization, except in the plantlets from the treatments of 25N with 30 g L^{-1} of sucrose and 25M with 10 g L^{-1} of sucrose, which presented a low average rate of relative growth of 1.8 and 19.4%, respectively. During the acclimatization of the plantlets, no relationship was observed between the longest root length and the concentration of sucrose (25N – R = 0.431; p = 0.247; 25M – R = 0.518; p = 0.153). For number of roots, generally, an average rate of relative increase of between 18.0 and 29.5% was observed. Moreover, in the 25 N medium, the highest average number of roots was observed in the plantlets from the treatment with 60 g L^{-1} of sucrose, which differed from the average obtained in the medium with 10 g L^{-1} (Tab. 2). As with the case of the number of roots, no relationship was observed between the number of roots and the concentration of sucrose during acclimatization (25N – R = 0.619; p = 0.076; 25M – R = 0.485; p = 0.186).

No difference was observed in the contents of photosynthetic pigments in the acclimatized plantlets. Specimens from the media with 60 g L^{-1} of sucrose, in which the contents of chlorophylls *a* and *b in vitro* were lower, showed an increase of this content after five months in acclimatization, and the treatment of 25M and 60 g L^{-1} of sucrose yielded the highest increase of chlorophylls *a* and *b,* as well as carotenoids, by a significantly way (Tab. 2).

The morphological parameters measured indicated that the increase in sucrose present in the culture medium, in terms of the greatest extent of plantlet development, allowed energy to be reserved in the plantlet tissues. Since this reserve could be used during the *ex vitro* acclimatization, *V. incurvata* plantlets showed increased growth and development in this stage. A positive relation between survival and fresh mass during *ex vitro* acclimatization and sucrose concentrations *in vitro* was also reported for *Ananas comosus* (L.) Merr. plantlets (Scherer *et al*. 2015).

Prior to the acclimatization stage, it is possible to perform a pre-adaptation to stimulate the plant to become an autotrophic condition *in vitro* and to present a significant photosynthetic rate, by reducing or eliminating the source of carbohydrate of the medium (Grout & Aston 1978, Grattapaglia & Machado 1998). This factor may determine the success of acclimatization (Leifert *et al*. 1995, Leite *et al*. 2000). In contrast, the addition of sucrose to the culture medium, besides allowing for the development of the plantlets *in vitro,* also contributes to the accumulation of energy reserves, which can be used during acclimatization (Skrebsky *et al*. 2004). In the study on *in vitro* culture and acclimatization of the Brazilian ginseng (*Pfaffia glomerata* Spreng. Pedersen), Skrebsky *et al*. (2004) concluded that the growth of the plantlets with a medium containing sucrose allowed for better success than that of the plantlets induced *in vitro* for autotrophic nutrition.

The data obtained in this study indicated that the concentration of 60 g L^{-1} of sucrose was beneficial for the propagation of *V. incurvata*, being that it allowed for increased development of plantlets, both in the *in vitro* environment and in the *ex vitro* environment. It was observed that the reserving of accumulated energy during the *in vitro* culture, under high concentrations of sucrose, is important to the adaptation of the plantlets during acclimatization, allowing for the metabolism to maintain the plantlets until the recovery of the photosynthetic apparatus, which generally takes two to three weeks (Streit *et al*. 2005). The results indicated micropropagation as an alternative to the conservation of *V. incurvata*, by which the propagated specimens may be provided for commercial means, reducing the extractivism of natural populations, or used in programs for reintroduction into their natural habitat.

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