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In vitro propagation of *Anathallis adenochila* (Loefgr.) F. Barros (Orchidaceae), a species endemic to southern and southeastern Brazil

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ABSTRACT

Anathallis adenochila is a small epiphytic orchid, endemic to the Atlantic Forest in the southern and southeastern regions of Brazil. Information on the species is scarce and limited to distribution and occurrence data, with no reports about its development. In the present study, plantlets were propagated *in vitro*, and the influence of different concentrations of macronutrient salts and sucrose on the survival and development of the species was assessed. The analysis were performed on complete Murashige and Skoog (MS) medium and half MS medium (with half-strength macronutrients) containing 10, 30 or 60 g L^{-1} of sucrose. Plantlet survival, height of the aerial part, and the length of the longest root were significantly greater on half MS medium containing 30 or 60 g L^{-1} of sucrose. The number of leaves per plantlet was higher in the presence of 60 g L^{-1} of sucrose, regardless of macronutrient concentration, and the highest number of roots was observed in plantlets cultured on half MS medium with 60 g L^{-1} of sucrose. This first report of *Anathallis adenochila in vitro* propagation may contribute to future studies on the physiological and ecological aspects of the life cycle of this species.

Key words: conservation, carbon source, micropropagation, orchid, mineral salts

Introduction

The Atlantic Forest is in the third position on the global list of priority areas for the conservation of vascular plants (Myers *et al*. 2000), epiphytic plants standing out as one of most remarkable characteristic of this biome, with high levels of richness and diversity (Kersten 2010). Considered not only one of the most important global biodiversity hotspots (Myers *et al*. 2000) but also one of the most endangered biomes in the world, the Atlantic Forest in the state of Rio Grande do Sul, Brazil, has been reduced to 7.48% of its original area of approximately 1,360,000 km^2 (Fundação SOS Mata Atlântica and INPE 2011).

Epiphytes are essential for the maintenance of biodiversity and interactive balance of forest communities, positively influencing the ecological processes of ecosystems by providing nutritional resources and specialized microenvironments for the fauna in the canopy and by participating in nutrient cycling mechanisms (Lugo & Scatena 1992; Rocha *et al.* 2004). In humid tropical and subtropical forest formations, there is great diversity of Orchidaceae species, which account for 70% of all vascular epiphytes in such formations (Moraes *et al*. 2010). Specifically in the Atlantic Forest, Orchidaceae is represented by 176 genera and 1257 species, of which 719 are endemic to the biome (Barros *et al.* 2009). Representatives of Orchidaceae are included in the Red Data Book prepared by the International Union for Conservation of Nature and Natural Resources (IUCN 2013) and in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2013).

Anathallis adenochila (Loefgr.) F. Barros is an epiphytic orchid belonging to the subtribe Pleurothallidinae of the subfamily Epidendreae. The species is endemic to the southern and southeastern regions of Brazil, occurring in the Atlantic Forest (Barros *et al*. 2013). *Anathallis adenochila* plants are small, measuring only approximately 5 cm in height, and exhibit sympodial growth, as well as having no pseudobulbs. Its flowers are distributed in multi-flowered inflorescences, with sepals joined at the base (Buzzato *et al.* 2007). Information on the species is scarce and limited to distribution and occurrence data (Buzzato *et al.* 2007; Brustulin & Schmitt 2008).

Although orchids produce a large amount of seeds, that are fragile and do not have significant endosperm or nutrient material (Mitra 1971). In addition, under natural conditions, only a very limited number of seeds germinate (Stancato & Faria 1996; Pedroso-de-Moraes *et al*. 2012). This limits the success of the continuity of populations, especially in impacted areas. Associated with this limitation, the loss of habitats due to forest fragmentation makes

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species vulnerable (Tremblay *et al.* 2005) and, along with predatory collection, increases the pressure on natural populations.

In vitro tissue culture is an important tool for the genetic conservation of species and for the sustainable use of plants, through propagation, which increases specimen availability (Soares *et al.* 2009; Unemoto *et al.* 2007). The asymbiotic germination of orchid seeds under *in vitro* conditions enables high germination rates (Pedroso-de-Moraes *et al.* 2009) and the maintenance of the genetic variability of plants (Pinto *et al.* 2010). Although tissue culture has long been used, Orchidaceae species have different demands and tolerances with regard to abiotic conditions *in vitro* (Arditti 1967; Stancato & Faria 1996; Faria *et al.* 2004; Sorace *et al.* 2008). Most studies aim to improve *in vitro* culture conditions for species with ornamental and commercial value, which are often subjected to genetic improvement (Faria *et al.* 2002; Moraes *et al.* 2002; Faria *et al.* 2006; Pedroso-de-Moraes *et al.* 2012), and do not focus on native species that are less significant from an economic point of view (Arditti & Ernest 1992) but important for the conservation of genetic diversity and for the dynamics of the ecosystem.

It is important to understand the physiological demands of the species to be propagated, with the purpose of obtaining better quality plantlets in *in vitro* cultures. The Murashige and Skoog (MS) medium (Murashige & Skoog 1962) is one of the most widely used culture media for *in vitro* propagation of several plant species. Some studies have demonstrated that the concentrations of sucrose and macronutrient salts in MS culture medium have different effects on plantlet development and growth, depending on the species and type of explant (Sorace *et al.* 2008). Such effects are identified based on the evaluation of several parameters, like the development of leaves and roots, as well the biometry of these structures (Araújo *et al.* 2007; Muller *et al.* 2007).

The aim of the study was to propagate *Anathallis adenochila* plants using *in vitro* culture and evaluate the effect of different concentrations of sucrose and macronutrient salts on plantlet survival and development. To our knowledge, this represents the first attempt at *in vitro* propagation of the species. Our objective was to contribute to ontogenetic studies and to conservation programs based on the reintroduction of plants into their natural habitat.

Materials and methods

Anathallis adenochila capsules were collected at Henrique Luís Roessler Municipal Park (29°40'54"S; 51°06'56"W; elevation, 16.4 m), a Conservation Unit located in the urban area within the municipality of Novo Hamburgo, in the state of Rio Grande do Sul, Brazil. After being washed in running water with commercial detergent and rinsed in distilled water three times, the capsules were transferred to a laminar flow chamber, where they were washed in 70% ethyl alcohol for 30 s and submerged in 2% sodium hypochlorite for 10 min. The capsules were then washed in autoclaved distilled water four times and opened with a scalpel to remove the seeds. Seeds were placed in 200-ml flasks containing 50 ml of MS culture medium with 50% of the original formulation of macronutrient salts, containing 30 g L^{-1} of sucrose and 10 g L^{-1} of activated charcoal, solidified with 6 g L^{-1} of agar and with the pH adjusted to 5.7 before autoclave sterilization (Unemoto *et al.* 2007). We then added 1 ml of autoclaved distilled water to each flask (Arditti & Ernest 1992). For 12 months (the time required for obtaining plantlets ≥ 0.5 cm in height), the cultures were kept under controlled conditions: a luminous intensity of 100 µmol m^{-2}/s; a 12/12-h light/dark cycle; and a temperature of 26±1°C.

Plantlets measuring 0.5-0.7 cm in height were selected, 270 of which were cultured in 200-ml flasks containing 50 ml of MS medium, with the same concentrations of activated charcoal and agar and the same pH used in the initial culture stage. We analyzed combinations of two concentrations of the original formula of macronutrient salts in the MS medium (50% and 100%) and three concentrations of sucrose (10, 30 and 60 g L^{-1}). For each combination of salt and sucrose concentration, we cultured five plantlets per flask, totaling 11 flasks, which were kept for 240 days under the same conditions of luminosity and temperature used in the initial culture stage, and subculture was performed at 120 days.

After 240 days, plantlets were removed from the flasks and washed in running water. The following parameters were evaluated: survival; height of the aerial part; number of leaves; number of roots; longest root length; and fresh mass. Measurements were taken using a caliper. The survival data obtained were transformed into percentages. The survival data obtained for sucrose concentrations in each salt concentration were compared by the Student-Newman-Keuls test, with a probability level of 5%. The survival data obtained for salt concentrations in each sucrose concentration were compared by the Mann-Whitney test, with a probability level of 5%. The values for height of the aerial part, number of leaves, number of roots, longest root length and fresh mass were log (x+1) transformed (for fresh mass) or log ($\sqrt{}$(x+1)) transformed (for the remaining parameters). Means obtained for sucrose concentrations in each salt concentration were submitted to ANOVA followed by Tukey's test, with a probability level of 5%. Means of these parameters obtained for salt concentrations in each sucrose concentration were compared by Student's t-test, with a probability level of 5%.

Results and discussion

The higher percentages of survival were observed in plantlets treated with 30 or 60 g L^{-1} of sucrose associated with 50% of the original concentration of macronutrient salts (Fig. 1). In the presence of 10 g L^{-1} of sucrose combined with 50% or 100% of macronutrients, only 20% and 6% of

the plantlets survived, respectively. On media with lower percentages of sucrose, plantlet necrosis was preceded by chlorosis, which can occur in tissues cultured *in vitro* in the presence of sucrose concentrations lower than 20 g L^{-1} (Grattapaglia & Machado 1998). Cells do not find normal luminosity conditions and carbon dioxide concentration adequate for photosynthesis when in culture (Skrebsky *et al*. 2004), requiring a supply of carbohydrates as an energy source, although they can exhibit some degree of autotrophic growth (Pinto *et al*. 2010). The fact that the higher survival rates were observed for treatments in which macronutrients were at half of their original concentration may be related to nitrogen concentration in the MS medium and to the metabolic function of this element, because high concentrations of ammonium and nitrate may inhibit plantlet germination and growth, as observed for the orchid *Vanilla planifolia* Andrews (Pedroso-de-Moraes *et al*. 2012).

Morphological parameters showed the influence of macronutrient and sucrose concentrations on the growth and development of *Anathallis adenochila* plantlets. Height of the aerial part and longest root length were highest for the treatments with 50% of the original macronutrient concentration and 30 or 60 g L^{-1} of sucrose. The highest number of leaves per plantlet was obtained with the addition of 60 g L^{-1} of sucrose to the MS medium, at 100% and 50% of macronutrient concentration. However, the number of roots per plantlet was highest for the treatment with 60 g L^{-1} of sucrose and 50% of macronutrient salts (Tab. 1 and Fig. 2).

When using the original macronutrient formulation, plantlet fresh mass was significantly higher in 60 g L^{-1} of sucrose than in the lower concentrations tested. In the treatments with 50% of the original macronutrient concentration, higher fresh masses were observed in the presence of 30 and 60 g L^{-1} of sucrose (Tab. 1). It should be noted that, in the media with 60 g L^{-1} of sucrose, plantlet growth was visually remarkable (Fig. 2), and that the increase in the number of leaves and roots was reflected in the increase in fresh mass, which, in the media with 50% of the original macronutrient concentration, was three and ten times as high as fresh mass in the media with 30 and 10 g L^{-1} of sucrose, respectively (Tab. 1).

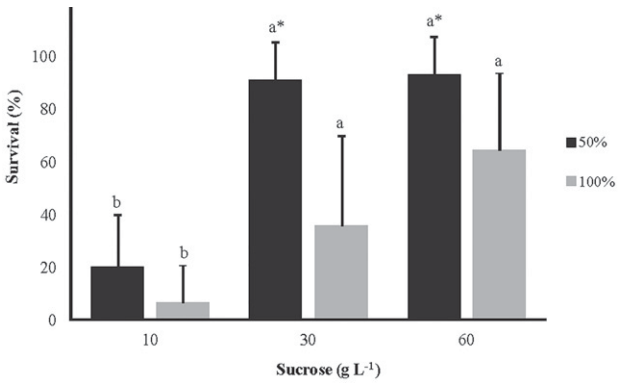

Figure 1. Survival of *Anathallis adenochila* plantlets propagated in different concentrations of MS macronutrient salts and sucrose after 240 days.
Mean values followed by the same letter comparing columns of the same color do not differ significantly by the Student-Newman-Keuls test ($p<0.05$).
*Significant difference between the concentrations of macronutrient salts in the same sucrose concentration, according to the Mann-Whitney test ($p<0.05$).

In general, the exogenous carbohydrate supply is essential for *in vitro* plant growth and development, because it provides carbons that will be used in respiration and that are precursors for the synthesis of structural and functional compounds (Caldas *et al*. 1998; Thorpe *et al*. 2008). Sucrose concentrations of 20-30 g L^{-1} are commonly used in *in vitro* culture studies of Orchidaceae (Arditti 1974). Although sucrose concentrations > 50 g L^{-1} could be considered harmful to the development of plantlets of some species, due to the excessive osmotic potential of the medium (Arditti & Ernst 1992; Paiva-Neto & Otoni 2003) and to the inhibition of the photosynthetic process (Yamada & Sato 1978; Capellades *et al*. 1991; Hdider & Desjardins 1994), there have been reports that the addition of 60 g L^{-1} of sucrose to the medium positively influences the development of epiphytic orchids. That has been observed in *Oncidium varicosum* Lindl., which showed significantly higher values for growth of the aerial part, number of roots, longest root length, and fresh mass in MS medium containing 50% of the original macronutrient concentration and 60 g L^{-1} of sucrose than in the same medium with sucrose concentrations of 0, 10, 20, 30 or 90 g L^{-1} (Rego-Oliveira *et al*. 2003). Faria *et al*. (2004) reported that *Dendrobium nobile* Lindl. plantlets developed in an MS medium with 50% of the original macronutrient formulation and 60 g L^{-1} of sucrose exhibit greater vertical growth, as well as high multiplication rate and fresh mass accumulation, when compared with plantlets treated with lower sucrose concentrations (0-30 g L^{-1}). The authors found that the different sucrose concentrations did not influence the formation of plantlet roots, except when there was no sucrose in the medium, a situation in which there was no root growth.

The increase in plantlet growth and development in the presence of high sucrose concentrations does not represent a standard behavior for Orchidaceae. Positive effects of relatively low concentrations of sucrose have also been reported for epiphytic orchids such as *Caularthron bicornutum* Raf., which showed a higher number of roots and greater height of the aerial part in MS media containing 10-30 g L^{-1} of sucrose (Pivetta *et al*. 2010). In *Oncidium baueri* Lindl., treatment at 50% of the macronutrient concentration with 40 g L^{-1} of sucrose has been shown to result in higher values for height of the aerial part, fresh mass and root length, in addition to benefiting root formation, when compared with treatments with 30 or 60 g L^{-1} of sucrose (Sorace *et al*. 2008). *Cattleya loddigesii* Lindl. plantlets showed greater height of the aerial part and fresh mass in 16-30 g L^{-1} of sucrose when associated with the growth regulator gibberellic acid. However, for the formation of roots, 60 g L^{-1} of sucrose has been shown to be the most beneficial concentration (Rezende *et al*. 2009). Similar concentrations of sucrose (20-30 g L^{-1}) have been found to

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 *Anathallis adenochila* propagated in MS medium with different concentrations of macronutrient salts and sucrose, after 240 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 ($p<0.05$).

	MS salts (%)	Sucrose concentrations (g L^{-1})			F	p
		10	30	60		
Height of the aerial part (cm)	100	0.6 ± 0.1 b	0.85 ± 0.31 ab	1.04 ± 0.28 a	5.704	0.006
	50	0.81 ± 0.20 b	1.44 ± 0.46 a*	1.43 ± 0.33 a*	13.011	<0.001
	t	1.700	5.000	5.620		
	p	0.117	<0.001	<0.001		
Number of leaves	100	3.67 ± 0.58 b	8.13 ± 5.45 b	20.94 ± 10.15 a	19.095	<0.001
	50	2.5 ± 2.72 c	10.04 ± 4.61 b	18.26 ± 11.66 a	28.338	<0.001
	t	-1.070	1.606	-1.311		
	p	0.308	0.113	0.193		
Longest root length (cm)	100	0.47 ± 0.35 b	1.14 ± 0.61 ab	1.39 ± 0.71 a	3.52	0.037
	50	0.88 ± 0.31 b	2.13 ± 0.95 a*	2.34 ± 0.91 a*	13.244	<0.001
	t	2.022	4.089	5.337		
	p	0.068	<0.001	<0.001		
Number of roots	100	2.33 ± 1.15 b	6.13 ± 4.27 ab	13.47 ± 7.64 a	10.56	<0.001
	50	3.4 ± 2.67 c	9.86 ± 3.78 b*	18.36 ± 9.54 a*	37.359	<0.001
	t	0.627	3.638	2.620		
	p	0.544	0.001	0.010		
Fresh mass (g)	100	0.03 ± 0.02 b	0.18 ± 0.14 b*	0.29 ± 0.25 a	20.453	<0.001
	50	0.03 ± 0.01 b	0.11 ± 0.11 a	0.33 ± 0.34 a	37.402	<0.001
	t	-0.393	2.564	-0.464		
	p	0.702	0.013	0.644		

increase growth in *Cattleya violacea* (Kunth) Rolfe (Galdiano Júnior *et al*. 2013). Hybrid plantlets of *Cattleya labiata* Lindl. x *Laelia itambana* Pabst and *Cattleya warneri* T. Moore x *Laelia purpurata* Lindl. & Paxton have been found to develop better in 20 g L^{-1} of sucrose than in other concentrations (Fráguas *et al*. 2003; Moreira *et al*. 2007).

The reduction in the concentration of macronutrient salts of the original formulation of the MS medium has a beneficial effect on several parameters of orchid development, as observed for *Cattleya cinnabarina* (Bateman ex Lindl.) Van der Berg (Stancato & Faria 1996), *Phalaenopsis* Blume (Griesbach 2002), *Catasetum fimbriatum* (E. Morren) Lindl. & Paxton (Rego-Oliveira & Faria 2005) and *Oncidium baueri* (Sorace *et al*. 2008). Pedroso-de-Moraes *et al*. (2012) demonstrated that the development of *Vanilla planifolia* is influenced by the availability of nitrogen salts. The authors reported higher germination percentages and velocity, as well as greater plantlet growth, at 25% of the original concentrations of ammonium nitrate and potassium nitrate in MS medium. Nitrogen is a constituent of molecules such as amino acids, enzymes and proteins, its bioavailability and absorption by tissues therefore being related to plant metabolism. High salt concentrations tend to inhibit rooting phases, especially the stage of root growth. Therefore, dilutions of macronutrient formulations have enabled more efficient root formation (Grattapaglia & Machado 1998), as found for the orchids *Anathallis adenochila* (in the present study), *Cattleya nobilior* Rchb.f. (Araújo *et al*. 2005), and *Vanilla planifolia* (Pedroso-de-Moraes *et al*. 2012), as well as for the bromeliads *Pitcairnia flammea* Lindl. and *Vriesea philippocoburgii* Wawra (Mercier & Kerbauy 1991).

Ex vitro plantlet acclimatization is an important stage, and its success depends on, among other factors, the physiological conditions of the plants. Because *in vitro* carbohydrate availability is a chemical condition very different from that found during acclimatization (Faria *et al*. 2004), plantlets that receive great amounts of sucrose as a source of energy for their metabolic activities may show reduced photosynthesis during acclimatization (Rolland *et al*. 2002) and often undergo necrosis. In the present study, plantlets were acclimatized and, at this writing, had been developing, exhibiting normal phenotypes, for more than 12 months. Plantlets grown on MS media with 50% of the macronutrient salts and containing 30 or 60 g L^{-1} of sucrose flourished even before acclimatization, in the *in vitro* culture phase. This first report of *in vitro* propagation of *Anathallis adenochila* may contribute to future studies on physiological and ecological aspects of the life cycle of this epiphytic species that is endemic to Brazil.

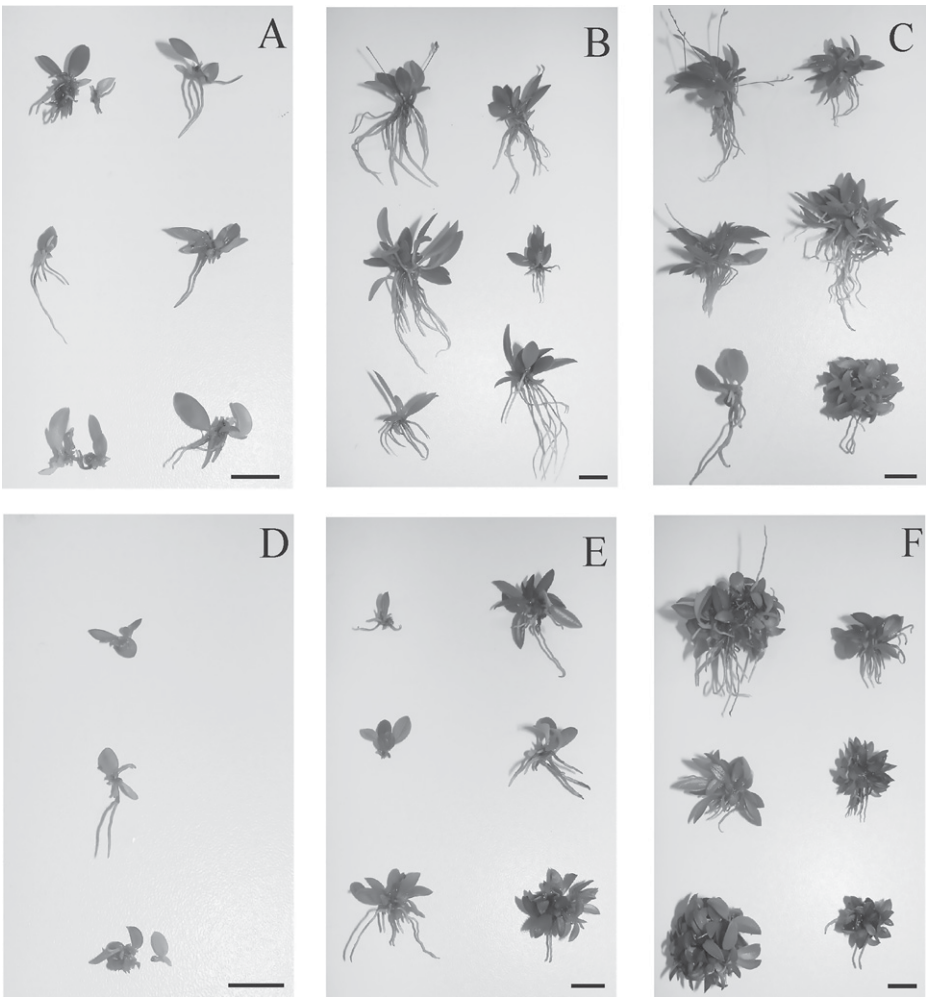

Figure 2. *Anathallis adenochila* plantlets obtained in different concentrations of MS macronutrient salts and sucrose after 240 days. **A-C**: 50% MS; 10, 30 and 60 g L^{-1} of sucrose, respectively; **D-F**: 100% MS; 10, 30 and 60 g L^{-1} of sucrose, respectively.
Bars = 2 cm.

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