Proline levels, oxidative metabolism and photosynthetic pigments during *in vitro* growth and acclimatization of *Pitcairnia encholirioides* L.B. Sm. (Bromeliaceae)

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Abstract

This study aimed to evaluate the variation in the levels of proline, oxidative metabolism and photosynthetic pigments in plants of *Pitcairnia encholirioides* grown *in vitro* under different conditions and after acclimatization. The analyses were performed after 150 days of *in vitro* cultivation in MS media supplemented with 10 µM GA_3 or 0.2 µM NAA, sucrose at 15 or 30 g L^{-1}, in test tubes which allowed gas exchange or in a hermetically sealed system, and 180 days after acclimatization. The *in vitro* maintenance in hermetically sealed flasks, with GA_3 and 15 g L^{-1} sucrose had adverse metabolic effects, which was demonstrated by the lower proline and photosynthetic pigments accumulation and by the increase in antioxidant enzymes activities. After acclimatization, differences for proline and photosynthetic pigments were no longer found and the enzymatic activities ranged unevenly. The results suggest that the *in vitro* cultivation in media with 0.2 µM NAA and 30 g L^{-1} sucrose, in test tubes capped with closures which allowed gas exchange, is more suitable for micropropagation of *P. encholirioides*, providing a prolonged maintenance of *in vitro* cultures and plantlets with superior quality for *ex vitro* development.

Keywords: enzymatic activity, oxidative stress, micropropagation, proline.

Niveis de prolina, do metabolismo oxidativo e dos pigmentos fotossintéticos durante o crescimento *in vitro* e aclimatização de *Pitcairnia encholirioides* L.B. Sm. (Bromeliaceae)

Resumo

Este trabalho objetivou avaliar a contribuição da prolina, do metabolismo oxidativo e dos pigmentos fotossintéticos na propagação *in vitro* e aclimatização de *Pitcairnia encholirioides*, uma bromélia criticamente ameaçada de extinção. As análises foram realizadas após 150 dias de cultivo *in vitro* em meio MS suplementado com 10 µM de GA_3 ou 0,2 µM de ANA, 15 ou 30 g L^{-1} de sacarose, em tubos de ensaio que permitiam trocas gasosas ou em sistema hermeticamente vedado, e também 180 dias após aclimatização. A manutenção *in vitro* em frascos hermeticamente fechados, com GA_3 e 15 g L^{-1} de sacarose apresentou efeito metabólico adverso, demonstrado pelo menor acúmulo de prolina e pigmentos fotossintéticos e também pelo aumento das atividades de enzimas antioxidantes. Após aclimatização, as diferenças para prolina e pigmentos fotossintéticos não foram mais encontradas e as atividades enzimáticas variaram de maneira desuniforme. Os resultados sugerem que o cultivo *in vitro* em meio com 0,2 µM de ANA e 30 g L^{-1} de sacarose, em tubos fechados com tampas que permitem trocas gasosas, é mais adequado para a micropropagação de *P. encholirioides*, proporcionando uma manutenção prolongada das culturas *in vitro* e plântulas com qualidade superior para o desenvolvimento *ex vitro*.

Palavras-chave: atividade enzimática, estresse oxidativo, micropropagação, prolina.

1. Introduction

In Brazil, bromeliads are popular ornamental plants often used in public and private gardens (Versieux and Wendt, 2007). Extractivism is one of the most important sources for supplying the consumer market (Coffani-Nunes and Forzza, 2000), contributing to the reduction of natural populations and extinction of some species (Pereira et al., 2008). *Pitcairnia encholirioides* L.B. Sm. is a quite rare bromeliad. For 70 years, it has only been recognized by the herbarium collection-type. However, in 2004, a population of around 900 plants was found on a much degraded rocky outcrop, in an area subject to fire and trampling by animals, located in Santa Maria Madalena, RJ, Brazil, suggesting that this species was among the most threatened in Rio de Janeiro (Martinelli and Forzza, 2006). Therefore, this species was included by the Biodiversitas Foundation in the critically endangered category (FUNDAÇÃO BIODIVERSITAS, 2005).

Micropropagation allows mass proliferation of threatened plants (Sarasan et al., 2006). As far as bromeliads are concerned, this biotechnology method provides an efficient commercial tool for the reintroduction of the plants, a fact which is extremely important for endangered species (Dal Vesco et al., 2011). In the last two decades, several studies regarding bromeliads have been published (Silveira et al., 2009; Santa-Rosa et al., 2013). After *in vitro* propagation, different systems are used in plantlet acclimatization (Deb and Imchen, 2010), a critical micropropagation phase which represents a limiting factor for its success (Chandra et al., 2010). Plant survival in field conditions depends on the quality of *in vitro* cultures (Faisal and Anis, 2009). The growth regulator, flask closure system and sucrose concentration in the culture medium affect plantlet survival in acclimatization, since these factors may affect the metabolism, modifying the morphological and physiological characteristics of the plants (Lucchesini and Mensuali-Sodi, 2004; Sáez et al., 2012). In addition to the modifications of *in vitro* culture conditions, several acclimatization procedures can be used, especially those which prevent water loss and biochemical disorders (Aragón et al., 2012).

Morphophysiological changes caused by *in vitro* culture, by light and due to hydric and osmotic stresses are the most important factors limiting plant acclimatization under field conditions, as they stimulate the oxidative metabolism by increasing the production of reactive oxygen species (ROS), especially the superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) (Baťková et al., 2008; Varshney and Anis, 2012). The $O_2^{\cdot-}$ spontaneously turns into H_2O_2, but this process is more efficient when catalyzed by superoxide dismutase (SOD). In the reaction, the H_2O_2 produced is scavenged by catalase (CAT) and peroxidase (POD), reducing the formation of hydroxyl radicals ($OH^{\cdot}$) (Scandalios, 1993). Finally, polyphenoloxidase (PPO) catalyses the O_2-dependent oxidation of catechols in quinones, and may synergistically act with POD, promoting its activation through the generation of H_2O_2 by the oxidation of phenolic compounds (Krishna et al., 2008). Although less studied, PPO is an abundant plant enzyme (Agrawal and Purohit, 2012).

In acclimatization, several metabolic changes can be activated in an attempt to neutralize the damages caused by hydric stress, including the accumulation of different compatible solutes (Hoekstra et al., 2001; Mohammadkhani and Heidari, 2008). Proline is the primary accumulated metabolite in different stress conditions, which is essential for plant establishment and tolerance (Molinari et al., 2007; Liang et al., 2013).

Although there are several studies addressing the morphophysiological and biochemical changes which occur during plant acclimatization, to our knowledge, there have been no reports concerning the endangered bromeliad species. This study aimed to evaluate the variation in the levels of proline, oxidative metabolism and photosynthetic pigments in *in vitro* and *ex vitro* cultured *P. encholirioides*, seeking to increase the survival of these plants in the acclimatization procedures.

2. Material and Methods

2.1. Plant material

Cultures of *Pitcairnia encholirioides* L.B. Sm. previously established *in vitro* from seeds collected in the natural environment in Santa Maria Madalena, RJ, Brazil, under license from regulatory agencies, were used in the studies.

2.2. Cultivation conditions

The seedlings were grown in test tubes (2.5 × 15 cm) in MS medium (Murashige and Skoog, 1962), supplemented with 30 g L^{-1} of sucrose (Sigma®), 7 g L^{-1} of agar (Sigma®) and with 10 μM gibberellic acid (GA_3) or 0.2 μM α-naphthalene-acetic acid (NAA), since both growth regulators promoted suitable developmental responses in previous micropropagation studies (data not shown). The culture medium pH was adjusted to 5.7 ± 0.1 before the autoclaving, performed for 20 min, at 120 °C and 1 atm.

In addition to the effects of these growth regulators, the effects of two sucrose concentrations (15 or 30 g L^{-1}) and two different closure systems for the test tubes were assessed, both by using polypropylene disposable closures, one of which permitted gas exchange (KAP-UTS (K25), Bellco®, 25 mm) and the other one (Sigma-Aldrich®, 25 mm) sealed with a stretchable self-adherent, 15-μm thick PVC film (Vitaspenser, Goodyear®), preventing ventilation by hermetically sealing the flask. The plants were kept for 150 days in a growth chamber under controlled temperature (26 ± 1 °C), photoperiod (16-8 h) and light intensity (40 μmol m^{-2} s^{-1}), without subculturing. After *in vitro* cultivation in those different conditions, the plantlets were subjected to acclimatization in polystyrene trays of 128 cells, filled with Plantmax Hortaliças HT (Eucatex®), moistened and covered with transparent plastic foil and kept under shade in a greenhouse, with humidity near 100% and a temperature of 27 ± 3 °C. After 35 days, the plants were moved to a greenhouse with a programmed misting system under plastic and shade (Sombrite® 75%). After 150 days

of *in vitro* cultivation and 180 days after transplantation to *ex vitro* conditions, leaf samples of the plantlets were obtained and subjected to different biochemical analysis and the plant development was visually compared among the treatments.

2.3. Biochemical analysis

In order to determine the proline contents, 0.3 g of fresh leaf tissues were weighed and immediately powdered in liquid nitrogen, followed by addition of 10 mL of sulfosalicylic acid at 3% (w/v) and subsequent purification through Whatman Nº 2 filter. The proline content was determined according to Bates et al. (1973). For the extraction of photosynthetic pigments, 0.1 g of fresh leaf was powdered in liquid nitrogen. Then, 25 mL of 80% acetone (v/v) was added, followed by filtration. The contents of photosynthetic pigments were determined according to Lichtenthaler (1987).

2.4. Enzymatic analysis

The extracts for determination of total protein levels and activities of SOD, CAT, POD and PPO were obtained by powdering 0.3 g of fresh leaf in liquid nitrogen, followed by addition of 10 mL of potassium phosphate buffer 0.1 M, pH 6.8, EDTA 0.1 mM and PMSF 1 mM, filtration through four layers of cheesecloth and centrifugation at 10,000 g for 15 minutes at 4 °C. The protein contents were analyzed by the enzymatic method of Lowry et al. (1951). SOD activity was measured according to Del Longo et al. (1993). The enzyme catalysis was carried out in a chamber illuminated by a 15 W fluorescent lamp for 3 min (Giannopolitis and Ries, 1977). Photoreduction of NBT to blue formazan was measured by the increase of absorbance at 560 nm. One unit of SOD is defined as the amount of enzyme necessary to inhibit NBT photoreduction by 50% (Beauchamp and Fridovich, 1971). POD activity was measured according to Kar and Mishra (1976), using a molar extinction coefficient of 2.47 mM^{-1} cm^{-1} (Chance and Maehly, 1955). PPO activity was measured as described for POD (Kar and Mishra, 1976). CAT activity was measured according to Havir and McHale (1987), as the absorbance decreased at 240 nm, assuming a molar extinction coefficient of 36 M^{-1} cm^{-1} (Anderson et al., 1995).

All the experiments were carried out in a completely randomized design with nine replications in a factorial 2 × 2 (sucrose at 15 or 30 g L^{-1} x closures caps unsealed or hermetically sealed with PVC film), totaling 4 treatments for each growth regulator (GA_3 or NAA). The data were processed and analyzed using ANOVA. The means were compared by the Scott-Knott test at 5% probability. The statistical analysis were performed using the SAEG program (System for Statistical Analysis, version 9.1, UFV, Brazil).

3. Results

In qualitative analysis, performed after 150 days of *in vitro* culture, it was observed that plantlets grown in culture medium with NAA and capped with closures allowing gas exchange showed best growth and morphological development (Figure 1a). In this micropropagation stage, there was considerable loss of explants due to senescence (57.6% on average). However, during acclimatization, the plantlets mortality was very low (11.3% on average). After transfer to *ex vitro* conditions, the *Pitcairnia encholirioides* plantlets showed typical development, without morphological alterations (Figure 1b).

In the analysis performed with the leaves of the *in vitro* plantlets, the proline contents oscillated specifically for each growth regulator (NAA or GA_3) in response to sucrose concentrations (Table 1). Significant differences were found only in materials from plantlets maintained in culture media supplemented with NAA, with high proline contents in tissues kept in the presence of 30 g L^{-1} sucrose, regardless the type of closure. In this concentration, in hermetically sealed culture tubes, proline accumulation was 32% higher than in the presence of 15 g L^{-1} sucrose. However, in culture media containing GA_3, this high proline content was not found in the presence of 30 g L^{-1} sucrose. After acclimatization, despite the use of different growth regulators and sucrose concentrations during the *in vitro* stage, significant differences in proline contents were not found among the treatments (Tables 1 and 2).

In general, the enzymatic activities of SOD, CAT and PPO were higher in plantlets cultured in media containing 15 g L^{-1} sucrose, regardless of the growth regulator and the closure system, except for the PPO in the presence of GA_3 (Table 1). For POD, however, an opposite tendency was found. In acclimatized plantlets from culture media supplemented with NAA, in general, the CAT, POD and PPO activities were significantly lower in tissues from plantlets cultured in media containing 30 g L^{-1} sucrose, except for POD and PPO in plantlets maintained in unsealed tubes (Table 2). For SOD, an opposite tendency was found, but the differences were not significant, regardless of the closure system. In plantlets from culture media supplemented with GA_3, a tendency for the SOD, CAT and POD activities to increase was observed in tissues from culture media containing the highest sucrose concentration, although, in some treatments, this increase was not significant. For PPO, an opposite tendency was found, but there were no significant differences. In this study, we notice the distinct responses of enzymatic activity to the sucrose concentrations when materials previously cultured in presence of NAA or GA_3 were evaluated.

Regarding to the content of photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoids), significant differences were found in response to the closure systems used *in vitro*, with lower pigment accumulation in plantlets cultured in hermetically sealed tubes, especially in culture medium containing NAA (Table 3). In media with GA_3, there was a lower pigment accumulation in hermetically sealed tubes containing 15 g L^{-1} sucrose. In media supplemented with 30 g L^{-1}, most of the results showed no significant differences.

Concerning the sucrose effects, lower pigment accumulation was observed in tissues from culture

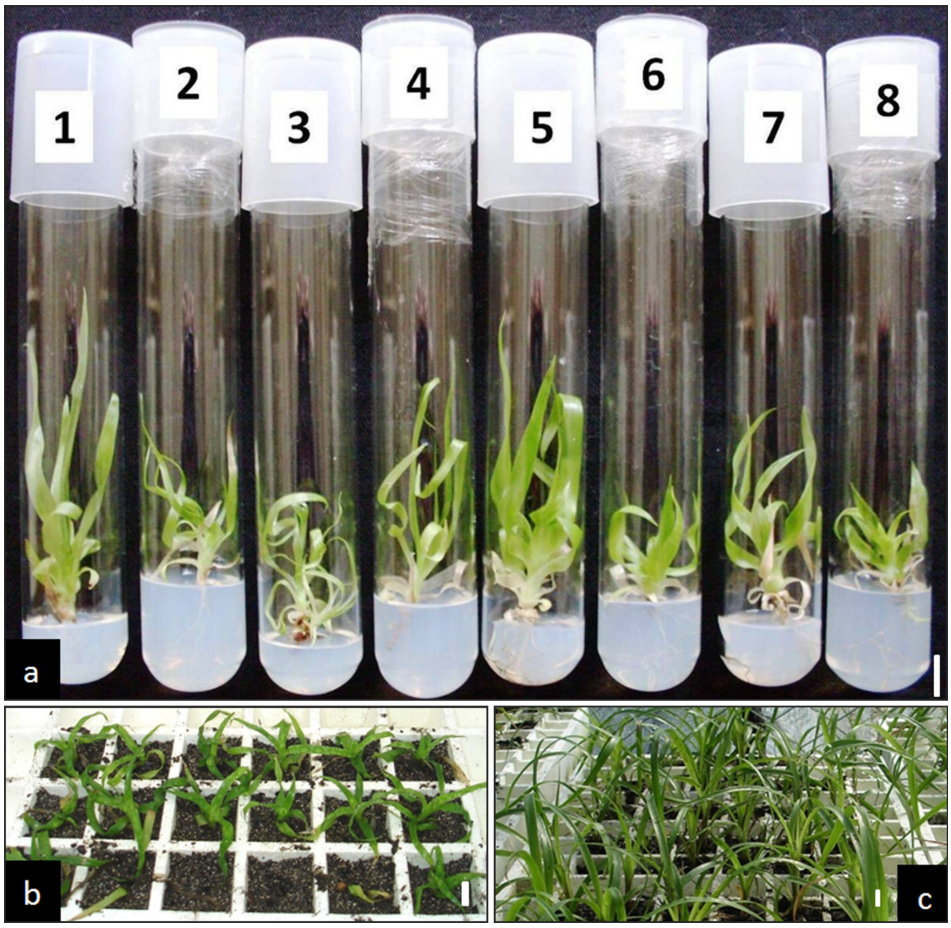

Figure 1. *Pitcairnia encholirioides* L.B. Sm. plantlets achieved after 150 d of *in vitro* culture (a), after 50 d (b) and 120 d (c) of acclimatization in a greenhouse. Treatments: **(1)** 10 µM GA_3, 15 g L^{-1} sucrose, unsealed closure; **(2)** 10 µM GA_3, 15 g L^{-1} sucrose, sealed closure; **(3)** 10 µM GA_3, 30 g L^{-1} sucrose, unsealed closure; **(4)** 10 µM GA3, 30 g L–1 sucrose, sealed closure; **(5)** 0.2 µM NAA, 15 g L^{-1} sucrose, unsealed closure; **(6)** 0.2 µM NAA, 15 g L^{-1} sucrose, sealed closure; **(7)** 0.2 µM NAA, 30 g L^{-1} sucrose, unsealed closure; and **8)** 0.2 µM NAA, 30 g L^{-1} sucrose, sealed closure. Scale bar: 1 cm.

Table 1. Proline [µmol g^{-1} (f.m.)] contents and SOD [U mg^{-1} (prot.)], CAT [mmol mg^{-1} (prot.) min^{-1}], POD [µmol mg^{-1} (prot.) min^{-1}] and PPO [µmol mg^{-1} (prot.) min^{-1}] activities in leaf tissues of *Pitcairnia encholirioides* after 150 days of *in vitro* culture in presence of GA_3 or NAA, in two sucrose concentrations (15 or 30 g L^{-1}) and under two types of closure (unsealed or sealed closure - US or S).

Treatment	Proline	SOD	CAT	POD	PPO
GA_3 US 15	1.200 Aa[1]	304.22 Aa	0.744 Aa	81.236 Ba	25.157 Aa
GA_3 US 30	1.124 Aa	189.09 Bb	0.583 Ba	97.658 Aa	26.523 Aa
GA_3 S 15	1.027 Ab	353.92 Aa	0.785 Aa	84.905 Ba	20.483 Ab
GA_3 S 30	0.925 Ab	257.83 Ba	0.627 Ba	93.248 Aa	20.000 Ab
NAA US 15	1.008 Ba	261.33 Ab	0.911 Ab	68.303 Ba	17.321 Aa
NAA US 30	1.166 Ab	200.57 Ba	0.683 Bb	91.902 Aa	11.432 Ba
NAA S 15	1.093 Ba	358.77 Aa	1.160 Aa	74.779 Aa	13.815 Ab
NAA S 30	1.447 Aa	222.81 Ba	0.822 Ba	81.874 Ab	10.140 Bb

[1]In each growth regulator (GA_3 or NAA), means identified by capital letters compare the effects of sucrose concentration for each type of closure, and means identified by lower case letters compare the effects of the type of closure used *in vitro* at each sucrose concentration. Means identified by the same uppercase or lowercase letters do not differ significantly by the Scott-Knott test at 5% probability.

Table 2. Proline [µmol g^{-1} (f.m.)] contents and SOD [U mg^{-1} (prot.)], CAT [mmol mg^{-1} (prot.) min^{-1}], POD [µmol mg^{-1} (prot.) min^{-1}] and PPO [µmol mg^{-1} (prot.) min^{-1}] activities in leaf tissues of *Pitcairnia encholirioides*, initially cultured *in vitro* for 150 d in the presence of GA_3 or NAA, in two sucrose concentrations (15 or 30 g L^{-1}) and under two types of closure (unsealed or sealed closure - US or S), 180 d after acclimatization.

Treatment	Proline	SOD	CAT	POD	PPO
GA_3 US 15	1.425 A[1]	132.04 A	0.994 A	79.383 B	34.842 A
GA_3 US 30	1.309 A	240.21 A	1.231 A	95.207 A	32.562 A
GA_3 S 15	1.193 A	291.43 A	1.232 B	112.959 A	52.356 A
GA_3 S 30	0.914 A	312.75 A	1.962 A	126.952 A	44.273 A
NAA US 15	0.990 A	285.36 A	1.939 A	98.739 A	35.759 A
NAA US 30	1.375 A	350.14 A	1.509 B	101.602 A	35.185 A
NAA S 15	0.845 A	241.28 A	2.504 A	145.465 A	50.327 A
NAA S 30	1.147 A	301.27 A	1.568 B	95.525 B	41.461 B

[1]Means identified by the same letters (comparing the effects of sucrose concentration, in each growth regulator, for each type of closure) do not differ significantly by the Scott-Knott test at 5% probability.

Table 3. Chlorophyll *a* [mg g^{-1} (f.m.)], chlorophyll *b* [mg g^{-1} (f.m.)], chlorophyll *a* + *b* [mg g^{-1} (f.m.)] and carotenoids [mg g^{-1} (f.m.)] contents, chlorophyll *a*/*b* ratio and chlorophyll *a* + *b*/carotenoids ratio in leaf tissues of *Pitcairnia encholirioides* after 150 d of *in vitro* culture in presence of GA_3 or NAA, in two sucrose concentrations (15 or 30 g L^{-1}) and under two types of closure (unsealed closure or sealed - US or S).

Treatment	Chl *a*	Chl *b*	Chl *a* + *b*	Carot	Chl *a*/Chl *b*	Chl *a* + *b*/ Carot
GA_3 US 15	0.912 Aa[1]	0.343 Aa	1.256 Aa	0.284 Aa	2.651 Aa	4.415 Aa
GA_3 US 30	1.034 Aa	0.354 Ab	1.388 Aa	0.333 Aa	2.999 Aa	4.200 Ab
GA_3 S 15	0.671 Bb	0.254 Ba	0.957 Bb	0.203 Bb	2.645 Aa	4.560 Aa
GA_3 S 30	1.222 Aa	0.490 Aa	1.713 Aa	0.359 Aa	2.506 Aa	4.765 Aa
NAA US 15	0.678 Aa	0.281 Aa	0.960 Aa	0.186 Aa	2.407 Aa	5.141 Aa
NAA US 30	0.408 Ba	0.212 Ba	0.621 Ba	0.128 Ba	1.922 Bb	4.843 Bb
NAA S 15	0.298 Ab	0.220 Ab	0.737 Ab	0.144 Ab	2.345 Aa	5.122 Aa
NAA S 30	0.273 Bb	0.133 Bb	0.407 Bb	0.079 Bb	2.042 Ba	5.135 Aa

[1]In each growth regulator (GA_3 or NAA), means identified by capital letters compare the effects of sucrose concentration for each type of closure, and means identified by lower case letters compare the effects of the type of closure used *in vitro* at each sucrose concentration. Means identified by the same uppercase or lowercase letters do not differ significantly by the Scott-Knott test at 5% probability.

media supplemented with 30 g L^{-1} sucrose and NAA, regardless the closure type. From plants grown in culture media supplemented with GA_3, the opposite effects were found, with lower pigments accumulation in tissues from plants cultured on media containing 15 g L^{-1} sucrose, in hermetically sealed vials (Table 3).

In the analysis performed on tissues from *in vitro* plantlets, significant effects in the chlorophyll *a*/*b* ratio (chl *a*/chl *b*) were found only in the plantlets maintained in presence of NAA, having lower ratios in tissues cultured in media with 30 g L^{-1} sucrose, regardless of the closure system (Table 3). When the closure systems effects were evaluated, differences were observed only for the chl *a*/chl *b* ratio in the presence of 30 g L^{-1} sucrose and NAA, with higher values found in the tissues from plants cultured in hermetically sealed tubes. For the chlorophyll *a* + *b*/carotenoids ratio (chl *a* + *b*/carot), differences were observed in response to the sucrose concentrations in plantlets from culture media containing NAA and kept in unsealed tubes, with higher values in the lower sucrose concentration. Regarding the closure system, irrespective of the growth regulator, significant differences were found in the culture media supplemented with 30 g L^{-1} sucrose, with higher values for chl *a* + *b*/carot in materials from hermetically closed tubes (Table 3). In this study, differences in the photosynthetic pigment contents and in these ratios were no longer found after acclimatization (Table 4).

When the photosynthetic pigments of acclimatized plantlets were matched with pigments of *in vitro* plantlets, lower contents for chl *a* were observed in all media, except for NAA, 15 and 30 g L^{-1} sucrose and sealed tubes. For chl *b*, they were lower in all cases, except for NAA, 30 g L^{-1} sucrose and sealed tubes.

Table 4. Chlorophyll *a* [mg g^{-1} (f.m.)], chlorophyll *b* [mg g^{-1} (f.m.)], chlorophyll *a* + *b* [mg g^{-1} (f.m.)] and carotenoids [mg g^{-1} (f.m.)] contents, chlorophyll *a*/*b* ratio and chlorophyll *a* + *b*/carotenoids ratio in leaf tissues of *Pitcairnia encholirioides*, initially cultured *in vitro* for 150 d in presence of GA_3 or NAA, in two sucrose concentrations (15 or 30 g L^{-1}) and under two types of closure (unsealed closure or sealed - US or S), 180 d after acclimatization.

Treatment	Chl *a*	Chl *b*	Chl *a* + *b*	Carot	Chl *a*/Chl *b*	Chl *a* + *b*/ Carot
GA_3 US 15	0.385 A[1]	0.141 A	0.527 A	0.144 A	2.723 A	3.652 A
GA_3 US 30	0.354 A	0.126 A	0.480 A	0.139 A	2.822 A	3.457 A
GA_3 S 15	0.356 A	0.130 A	0.486 A	0.139 A	2.794 A	3.450 A
GA_3 S 30	0.293 A	0.107 A	0.400 A	0.108 A	2.711 A	3.718 A
NAA US 15	0.454 A	0.161 A	0.616 A	0.171 A	2.812 B	3.595 A
NAA US 30	0.360 A	0.107 A	0.468 A	0.132 A	3.448 A	3.535 A
NAA S 15	0.398 A	0.132 A	0.531 A	0.145 A	3.022 A	3.630 A
NAA S 30	0.368 A	0.138 A	0.507 A	0.151 A	2.677 A	3.344 A

[1]Means identified by the same letters (comparing the effects of sucrose concentration, in each growth regulator, for each type of closure) do not differ significantly by the Scott-Knott test at 5% probability.

4. Discussion

High levels of sucrose increased the osmotic pressure in the culture media, which may result in cell water stress. The osmotic adjustment is an important response to harmful conditions, which occurs through the accumulation of compatible solutes (Hoekstra et al., 2001; Evers et al., 2010), molecules that maintain the water absorptive capacity and improve the drought tolerance (Kishor et al., 1995). Proline accumulation occurs due to the increase in synthesis and reduction in degradation, being one of the most frequent physiological responses induced by osmotic stress in plants (Zhang et al., 2013).

In this study, the differences found in plants maintained in culture media supplemented with NAA, with high proline contents in tissues kept with 30 g L^{-1} sucrose, regardless the type of closure seems to be a result of the osmotic adjustment in response to hydric stress caused by the high sucrose level in culture media. The increase in proline synthesis in transgenic tobacco (*Nicotiana tabacum*) plants over-producing proline by elimination of feedback inhibition of P5CS, the enzyme that catalyzes the proline biosynthesis, provides higher tolerance to hyperosmotic stress, giving substantial evidence of the relationship between this amino acid and drought tolerance (Kishor et al., 1995; Hong et al., 2000). Carvalho et al. (2013) found that proline modifies the expression of genes related to the plant responses to water deficit. In addition to the effects as osmotic adjustment mediator, proline also contributes as a stabilizer for subcellular structures, free radical scavenger and redox buffer, being the most important structural protein constituent of cell wall (Reddy et al., 2004; Molinari et al., 2007; Verslues and Sharma, 2010). Al-Khayri and Al-Bahrany (2002) found similar results to those obtained in the present study for *in vitro* callus of rice (*Oryza sativa*), in which increases in proline level in response to high sucrose levels were also observed.

After acclimatization, no significant differences in proline contents were found among the treatments. This is an important evidence of *P. encholirioides* acclimatization competence, regardless of *in vitro* culture conditions. These results suggest that proline is more important to the osmotic metabolism in *in vitro* culture of *P. encholirioides* than after plantlets adaptation to *ex vitro* conditions.

The SOD is the first enzymatic barrier against the harmful effects of oxidative stress, converting the $O_2^{\cdot-}$ into H_2O_2 (Alscher et al., 2002). In parallel, the CAT acts to reduce the H_2O_2 to H_2O and O_2, preventing the generation of more reactive radicals such as $OH^{\cdot}$ (Perl-Treves and Perl, 2002; Kibinza et al., 2011). CAT and SOD are the most efficient enzymes of antioxidant metabolism, since their combined actions convert hazardous molecules into H_2O and O_2, avoiding damage to cellular components (Scandalios, 1993). The results found in this study suggest a high ROS production in plantlets kept *in vitro* in presence of 15 g L^{-1} sucrose, which was probably a result of the hyperhydricity symptoms visually recognized, like a water-soaked appearance of the tissues, developed in the tissues of seedlings maintained in this condition, which required a high antioxidant activity for ROS neutralization. The inverse relationship between proline contents and POD activities, found by Kravić et al. (2013) in maize plants under osmotic stress, was not found in our study. In general, higher SOD and CAT activities were observed in materials kept in hermetically sealed tubes, where the relative humidity is close to 100%, a condition which promotes the hyperhydricity, hypoxia and, consequently, oxidative stress. The higher SOD and CAT activities found in tissues from plantlets grown *in vitro* in hermetically sealed tubes suggest that the impaired gas exchange is harmful to oxidative plant metabolism and could promote increases in ROS generation, requiring increased antioxidant enzyme activity.

During acclimatization, the plantlets are subjected to several stresses in response to changes in environmental conditions, which occurs due to impaired stomata function and reduced cuticle deposition on cells, resulting in dehydration and increase in ROS production (Baťková et al., 2008).

Despite that, after the transference to *ex vitro* conditions, the plantlets of *P. encholirioides* showed typical development, without morphological alterations, probably due to rustic bromeliad photosynthetic crassulacean acid metabolism, which is usually associated with improved drought tolerance and acclimatization capacity.

According to Gour et al. (2007), the chlorophyll content is one of the most important parameters for evaluation of the plant hardening after acclimatization. Carotenoids are also important in this phase, since they are required to protect the photosynthetic apparatus against damages caused by excess of radiant energy and contribute to non-enzymatic antioxidant defense systems (Perl-Treves and Perl, 2002; Pospíšilová et al., 2009). The results found in this work, with lower pigment accumulation in plantlets cultured in hermetically sealed tubes, especially in culture medium containing NAA, reinforce the hypothesis that the hermetic sealing of the vials could be detrimental to the development of autotrophic plants, reducing the efficiency and protection of the photosynthetic apparatus. Similar results were found by Chanemougasoundharam et al. (2004), with *Solanum tuberosum*, and by Mohamed and Alsadon (2010), for potato plants, for which the ventilation of the culture flasks contributed positively to the chlorophyll *b* content, not interfering, however, on levels of chlorophyll *a*.

The lower pigment accumulation observed in tissues from the culture media supplemented with 30 g L^{-1} sucrose and NAA, regardless the closure type, is a typical response found in the literature, being attributed to the effects of sucrose on biosynthesis of photosynthetic pigments, which result in inhibition of chlorophyll biosynthesis and *in vitro* photosynthesis (Eckstein et al., 2012). Plantlets grown without sucrose show a higher photosynthetic rate because this is the only route to carbon fixation (Iarema et al., 2012).

For plants grown in the presence of GA_3, the opposite effects was found, with lower pigments accumulation in tissues from plants cultured in media added with 15 g L^{-1} sucrose, in hermetically sealed vials. These results suggest that in this circumstance, the plantlets were subjected to more severe stressful conditions than in response to NAA. Since gibberellin synthesis inhibitors prevent the occurrence of hyperhydricity (Ziv, 1992), GA_3 can be associated with this morphologic disorder, which is enhanced by low osmotic pressure conditioned by the reduced sucrose concentration in the culture medium, which increases the water availability to the tissues. In this condition, the hyperhydricity is stimulated, being further enhanced by the use of closures which prevent the occurrence of gas exchange (Lai et al., 2005).

After acclimatization, differences in the photosynthetic pigment contents and in these ratios disappeared. These results are in agreement with those found by Kadleček et al. (2001), with tobacco, and indicate that the effects of *in vitro* conditions on the pigment contents may vanish after acclimatization and thus, for *P. encholirioides*, the alterations found *in vitro* would have a slight influence on photosynthesis after acclimatization. According to Pospíšilová et al. (2009), the chlorophyll contents could be higher or lower in acclimatized plants when compared with *in vitro* plantlets. The differences could be attributed to irradiation conditions, culture medium composition and *in vitro* CO_2 concentration. In addition, the tissues sources also affect the results, since persistent leaves generally exhibit reduction in pigment contents after acclimatization, and, in contrast, younger leaves tend to accumulate more chlorophylls and carotenoids (Van Huylenbroeck et al., 2000; Carvalho et al., 2001; Dias et al., 2013). Faisal and Anis (2009), working with *Rauvolfia tetraphylla* found increases in chlorophylls *a* and *b* in young leaves developed after acclimatization. Borghezan et al. (2003), studying some genotypes of grapevine (*Vitis* spp.), observed in two of them a higher total chlorophylls content in plantlets from *in vitro* tissues than from acclimatized plantlets. However, the opposite was found in four other genotypes.

In this study, the acclimatization was carried out in protected conditions for 180 days, which may have contributed to the partial recovery of the plantlets through acclimatization. If the analysis had been performed as soon as the plantlets were removed from *in vitro* condition, the results might have been different. Although the recovery for photosynthetic pigments and proline has been observed after acclimatization, most of the results found in this study suggest that the maintenance of plantlets in hermetically sealed vials containing culture media supplemented with GA_3 and 15 g L^{-1} sucrose is harmful for their further growth and development.

5. Conclusions

The results of this study suggest that plantlets grown in culture medium supplemented with GA_3, at a low sucrose concentration and kept in hermetically sealed tubes were subjected to a higher oxidative stress than those maintained in the presence of NAA, higher sucrose level and kept in flasks that allowed gaseous exchange. This resulted from the increase in CAT and SOD activities and, conversely, by the reduction of the proline and photosynthetic pigment contents in response to the first treatment. After acclimatization with adequate moisture, temperature and shading conditions, differences among the tissues for proline and photosynthetic pigments disappeared and the enzyme activities ranged unevenly, so that there was no generalization about their remaining effects in the *in vitro* phase. It is believed, however, that if these plantlets were established directly in a natural environment, without a careful acclimatization period, they would have a lower chance of survival.

It is further assumed that *Pitcairnia encholirioides* plantlets kept in culture media containing 0.2 μM NAA and 30 g L^{-1} sucrose, in test tubes capped with closures allowing a gas exchange, were morphophysiologically more suitable, thus could exhibit a higher survival in response to successive subcultures. These conditions allow a longer *in vitro* maintenance and a supply of physiologically improved plants for *ex vitro* development, which could contribute to conservation and reduce the threat of extinction of this species.

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