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RESPOSTAS FISIOLÓGICAS A DESSECAÇÃO E A RE-HIDRATAÇÃO EM QUATRO ESPÉCIES DE PTERIDÓFITAS EPIFÍTICAS

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Porto Alegre, Julho de 2012

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RESUMO

As plantas que possuem hábito epifítico estão mais sujeitas as variações ambientais do que as de hábito terrestre, principalmente em relação à disponibilidade hídrica. A água, então, destaca-se como um dos fatores restritivos mais importantes. Entretanto, algumas espécies desenvolveram a capacidade de tolerar a dessecação, apresentando uma significante vantagem adaptativa na ocupação de habitats. O objetivo deste estudo foi verificar o grau e os mecanismos de tolerância à dessecação de quatro espécies de pteridófitas epifíticas que comumente co-ocorrem sobre mesmo forófito. Frondes expandidas e hidratadas foram coletadas para a quantificação e comparação do conteúdo relativo de água, integridade de membrana, pigmentos fotossintéticos, flavonóides, fenóis, açúcares solúveis e fluorescência da clorofila ao longo dos processos de dessecação e re-hidratação. Estas avaliações fisiológicas nos permitiram inferir que as espécies com características de tolerância à dessecação apresentam mais adaptações fisiológicas durante períodos de dessecação e re-hidratação do que àquelas que não apresentam este comportamento. Os resultados obtidos também revelaram a existência de diferentes graus de tolerância nas três espécies consideradas tolerantes à dessecação. *Polypodium polypodioides* var. *minimum* foi a mais tolerante, seguida de *Pleopletis pleopeltifolia* e *Polypodium hirsutissimum*. Enquanto *Microgramma squamulosa* demonstrou evitar à dessecação. Assim, concluímos que estas espécies apresentam diferentes estratégias ecofisiológicas em relação ao estresse hídrico, minimizando possíveis danos irreversíveis às membranas celulares. Desta forma, otimizam a captura de luz durante períodos de re-hidratação, diminuem a competição inter-específica e facilitam a co-ocorrência destas quatro espécies.

PALAVRAS-CHAVE: tolerância à dessecação, Polypodiaceae, integridade de membrana, flavonóides totais, fenóis totais, açúcares solúveis totais, fluorescência da clorofila.

ABSTRACT

Epiphytic plants are more sensible to environmental variations than terrestrial plants, especially in relation to water availability. The water then stands out as one of the most important limiting factors. However, some species have evolved the ability to tolerate desiccation, showing a significant adaptive advantage in the occupation of habitats. The aim of this study was to determine the degree and mechanisms of drought tolerance of four species of epiphytic ferns that commonly co-occur on the same host tree. Expanded and hydrated fronds were collected for quantification and comparison the relative water content, membrane integrity, photosynthetic pigments, flavonoids, phenols, soluble sugars and chlorophyll fluorescence over the processes of desiccation and rehydration. The results of the present study allowed us to infer that the tolerant species have more physiological adaptations during periods of desiccation and rehydration. Thus, tolerant species showed distinct degrees of tolerance: *Polypodium polypodioides* var. *minimum* was the most tolerant, followed by *Pleopletis pleopeltifolia* and *Polypodium hirsutissimum*. While *Microgramma squamulosa* shown avoidance desiccation. We conclude that these species exhibit different ecophysiological strategies in relation to water stress, minimizing possible irreversible damage to cell membranes. Thus optimize capture of light during periods of rehydration, reduce inter-specific competition and facilitating the co-occurrence of these four species.

KEYWORDS: desiccation tolerance, Polypodiaceae, membrane integrity, total flavonoids, total phenols, total soluble sugars, chlorophyll fluorescecnce.

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INTRODUÇÃO GERAL

A vida somente é possível com a presença de água nas células (Hietz 2010), pois esta além de preservar a integridade estrutural das membranas, promove o adequado funcionamento celular. Os organismos terrestres, então, necessitam de um conjunto de adaptações para sobreviver e prosperar em um ambiente com restrição hídrica (Wood 2005), minimizando a perda d'água já que se encontram em desequilíbrio com a atmosfera que é mais seca. A grande maioria dos organismos não consegue viver em equilíbrio com baixa disponibilidade hídrica e tende a morrer se expostos a uma seca severa. Entretanto, existem espécies de diferentes formas de vida que resistem a esta situação e recuperam completamente seu funcionamento fisiológico após um estado de dormência (Alpert & Oliver 2002; Vicré, Farrant & Driouich 2004), seja em estruturas especializadas ou em tecidos vegetativos (Bewley 1979).

Na classificação de estratégias de resistência ao estresse de Levitt (1980) esta capacidade é denominada de tolerância à dessecação. O estresse devido à seca, segundo Larcher (2002), ao contrário dos outros tipos de estresse, não ocorre abruptamente, desenvolvendo-se lentamente e aumentando sua intensidade com o tempo de duração. As perspectivas de sobrevivência de uma planta exposta ao déficit hídrico são maiores, quanto mais lento for o processo (plantas que evitam a dessecação) e quanto mais o protoplasma puder secar sem se danificar (plantas que toleram a dessecação). De maneira geral, as plantas crescem e se reproduzem apenas quando o equilíbrio de seus tecidos fisiologicamente ativos com o ar pode ser evitado (Alpert & Oliver 2002).

A dessecação, ao contrário da desidratação, é um processo inevitável para alguns organismos (Alpert 2005), não sendo apenas a exposição ao ar seco, mas também, à perda completa da água livre dos tecidos. Conseqüentemente, as plantas capazes de tolerar a

dessecação, devem possuir mecanismos que minimizem os danos e que sejam capazes de recuperar as funções normais durante a re-hidratação (Bochicchio *et al.* 1998).

A tolerância à dessecação é considerada por muitos como o componente chave na evolução das plantas, o que teria permitido as algas de água doce colonizar com sucesso o ambiente terrestre (Oliver *et al.* 2000). Neste cenário, as plantas terrestres evoluíram para mecanismos mais eficientes de transporte interno de água, com a tolerância sendo aparentemente perdida nos tecidos vegetativos e retida nas estruturas reprodutivas (Proctor & Tuba 2002), como grãos de pólen e sementes. Com a diversificação das plantas vasculares, é aceita ainda a hipótese de que a tolerância à dessecação em tecidos vegetativos re-evoluiu de maneira independente várias vezes, dando origem às plantas revivescentes observadas nos dias de hoje (Oliver *et al.* 2000).

Estas plantas são encontradas na maioria dos grupos taxonômicos, desde pteridófitas até dicotiledôneas, com exceção das gimnospermas (Vicré, Farrant & Driouich 2004). Embora seja consideravelmente rara entre as plantas vasculares, muitas pteridófitas podem exibir traços de tolerância à dessecação (Meirelles *et al.* 1997). Conforme Bewley & Krochko (1982) essa característica aparece em mais de 100 espécies de 30 gêneros distribuídos em oito famílias de criptógamas vasculares. Pteridófitas revivescentes diferem das fanerógamas com esse tipo de adaptação pela variedade de ambientes onde são encontradas, podendo ocorrer como epifíticas, rupícolas ou ainda colonizar solos com fertilidade bastante reduzida.

Alguns trabalhos preliminares sugerem a presença de tolerância à dessecação em pteridófitas brasileiras, como por exemplo, espécies do gênero *Anemia* (Gaff 1987; Meirelles 1990), ocorrendo ao lado de outras tidas como intolerantes no mesmo tipo de substrato, mas exibindo ligeiras diferenças de habitat. Algumas modificações estruturais, como a estocagem de água em órgãos como rizomas, permitem a co-ocorrência destas espécies. Estes arranjos morfológicos fazem com que espécies de plantas submetidas a um mesmo grau de estresse

ambiental, mantenham relações distintas quanto à sua sensibilidade ao estresse, apresentando respostas peculiares com relação ao seu “status” hídrico (Schlindwein 2002).

A primeira e mais sensível conseqüência ao déficit hídrico é o fechamento estomático, seguido da diminuição do turgor celular, reduzindo as taxas de crescimento; do aumento da síntese de ácido abscísico nas raízes que serve de sinalizador para o fechamento estomático; da conversão do amido em carboidratos solúveis que auxiliam na preservação da integridade celular, reduzindo possíveis danos as membranas e organelas (Crowe *et al*. 1992; Smirnoff 1992; Müller *et al.* 1997; Potts 1999; Kocheva *et al.* 2004); da síntese de proteínas que protegem os componentes celulares durante o processo de dessecação (Gwozdz, Bewley & Tucker 1974; Oliver 1996) e que reparam os danos após a re-hidratação (Gray *et al*. 2007; Aubert *et al*. 2007); da produção de enzimas antioxidantes que neutralizam as espécies de oxigênio reativo (ROS) gerados durante a seca (Seel, Hendry & Lee 1992; Kranner *et al.* 2003; Ledford & Niyogi 2005; Weismann, Garty & Hochman 2005a); dos carotenóides, especialmente aqueles do ciclo das xantofilas, que dissipam o excesso de energia absorvida na forma de calor (Bukhov *et al.* 2001; Masojidek *et al*. 2004); da divisão do vacúolo central, dos tilacóides nos cloroplastos; e, por fim, a ruptura das membranas celulares (Bewley 1979; Crowe, Hoekstra & Crowe 1992; Torres-Franklin *et al*. 2007). Se a rehidratação ocorre antes da ruptura das membranas, algumas plantas são capazes de restaurar a integridade celular (Larcher 2002).

Embora tenha ocorrido algum progresso na investigação bioquímica de plantas tolerantes, o significado ecológico desta característica ainda permanece inexplorado, principalmente em pteridófitas. As chances de obtenção de alguma resposta a respeito das vantagens relacionadas com esta estratégia de tolerar à dessecação estão diretamente relacionadas ao estudo da ecologia comparativa de espécies que ocupam o mesmo tipo de hábitat e que exibem comportamento contrastante.

Esta tese visa, de uma maneira geral, caracterizar a potencialidade de tolerância à dessecação em quatro espécies de pteridófitas de hábito epifítico. Para tanto, diferentes aspectos ecofisiológicos relevantes à compreensão da presença ou ausência de tolerância à dessecação em *Polypodium polypodioides* var. *minimum*, *Polypodium hirsutissimum*, *Pleopeltis pleopeltifolia* e *Microgramma squamulosa* foram avaliados.

A tese está estruturada em dois artigos complementares que envolvem a exposição de frondes destacadas a processos de dessecação e re-hidratação. No processo de dessecação as frondes foram submetidas a três diferentes condições de umidade relativa (0%, 60-70% e 100%) e as mensurações ocorreram em cinco diferentes períodos de tempo (0, 12, 24, 36 e 48h). Já no processo de re-hidratação as frondes foram expostas a uma mesma condição e as mensurações ocorreram em seis diferentes períodos de tempo (0, 2, 4, 6, 8, 24h).

No primeiro artigo foram avaliados o conteúdo relativo de água (CRA), o percentual de integridade de membrana e os teores de clorofila, carotenóides e flavonóides. E no segundo artigo foram avaliados o CRA, os teores de fenóis, os açúcares solúveis e a fluorescência da clorofila.

Através destas mensurações foi possível verificar o grau de tolerância à dessecação e, principalmente, as diferenças ecofisiológicas existentes entre as quatro espécies estudadas que nos permitiram inferir sobre a co-ocorrência destas em um mesmo forófito.

ARTIGO 1

Physiological responses to desiccation and rehydration in four epiphytic species of pteridophytes

Artigo submetido ao Periódico Plant Biology

ARTIGO 1

Physiological responses to desiccation and rehydration in four epiphytic species of pteridophytes

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Short title: Tolerance and desiccation avoidance in epiphytic pteridophytes

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Keywords

Polypodiaceae; desiccation tolerance; desiccation avoidance; relative content of water; flavonoids; photosynthetic pigments; membrane integrity.

ABSTRACT

Plants are constantly exposed to the variations in availability of water in their environment, and are particularly prominent in those with epiphytic habit. Desiccation tolerance then becomes a survival alternative for many epiphytes, including the pteridophytes. The aim of this study was to characterize and compare metabolic changes that occur during the processes of desiccation and rehydration in four epiphytic pteridophytes: *Polypodium polypodioides* var. *minimum*, *Polypodium hirsutissimum* and *Pleopeltis pleopeltifolia,* species that present characteristics of desiccation tolerance, and *Microgramma squamulosa,* which demonstrates desiccation avoidance. Hydrated fronds were collected from the phorophytes for evaluation of relative water content (RWC), chlorophyll, carotenoid, and flavonoid concentrations, and membrane integrity. In contrast with the avoider species, the desiccation tolerant curls their fronds at low RWC, suffered less damage to the cell membranes and exhibited a greater increase in the concentration of flavonoids during the desiccation period. In rehydration, these physiological parameters responded in the opposite way, except for *M. squamulosa*, which increased the level of flavonoids in its fronds. *Polypodium polypodioides* was the species that presented the highest level of desiccation tolerance, followed by *Pl. pleopeltifolia* and *P. hirsutissimum*, with intermediate patterns. On the other hand, metabolic responses during the processes of desiccation and rehydration confirmed *M. squamulosa* to be the species of greatest avoidance, and explained the characteristic lack of curling of its fronds. In conclusion, the species showed different strategies in optimizing the capture of light during periods of high water availability, thus minimizing inter-specific competition and facilitating their co-occurrence.

INTRODUCTION

Most hypotheses regarding the distribution of plant species are based on their differential capacity for survival when faced with restrictive factors inherent to their environment. Water limitation is one of these factors. Despite its abundance on the surface of the earth, the availability of water is the resource that more strongly limits the distribution of plants on a global scale (Lambers *et al.* 2008), being one of the most important ecological and evolutionary factors for the life on earth (Alpert 2005). Plant species show different strategies in order to need minimum amounts or no water at all in at least one moment in their life cycle. Such fact can be observed in spores, seeds and pollens (Farmsworth 2004; Bartels 2005; Illing *et al.* 2005), in bryophytes and lichens (Proctor & Tuba 2002; Oliver *et al.* 2005) and, more rarely, in adult ferns and angiosperms (Gaff 1987; Porembski & Barthlott 2000), because the capacity of a mature tissue, such as those in roots and leafs, in tolerating dehydration is very rare (Scott 2000). Plants that present this behavior are denominated by many authors as being tolerant to desiccation, poikilohydrics or still "resurrection plants" (Scott 2000; Alpert 2005).

According to Alpert & Oliver (2002), the tolerance to desiccation can be conceptually defined as a reversible cessation of the metabolism in response to the loss of water. This process is immediately interrupted as the level of water becomes high enough to rehydrate the plant tissue, bringing back its metabolism. According to Bernacchia *et al*. (1996), the complete rehydration of the plant tissues may occur in a period of 24 hours, as observed for *Craterostigma plantagineum*, with minimum or inexistent damage to the tissues, due to the activation of protective mechanisms (Bochicchio *et al.* 1998).

Water shortness represents a multidimensional stress that affects plants in various levels of their organization (Yordanov *et al.*, 2000). Depending on the intensity of this stress, cell membranes may disrupt (Bewley 1979; Crowe, Hoekstra & Crowe 1992) and causes both chemical and oxidative damages (Weismann, Garty & Hochman 2005b). Photosynthetic

organisms display several physiological mechanisms to avoid such damages: reductions in chlorophyll concentration, to avoid photodamage (Hietz 2010); accumulation of carotenoids, which dissipate as heat the excessive energy absorbed by chlorophylls in terrestrial plants and algae (Bukhov *et al.* 2001); synthesis of flavonoids, which act as UV-filters, anti-oxidants and potent scavengers of reactive oxygen species, preventing peroxidation of lipids (Rozema *et al.* 2002; Tattini *et al.* 2004); synthesis of proteins that protect cell components during the process of dehydration (Oliver 1996); and leaf curling, to provide additional protection against possible damages caused by the high incidence of light (Hietz 2010).

According to Hietz (2010), there are many species of pteridophytes that show traits of desiccation tolerance. This feature appears in more than 100 species of 30 genera, distributed in eight families of vascular cryptogams (Bewley & Krochko 1982). In contrast to phanerogams, ferns with this kind of adaptation can be found on phorophytes, rocky outcrops and low-fertility soils. The environment, by its turn, influences the most appropriate protection mechanisms, causing changing in the physiology of the plant or increasing its ability to repair possible damages (Rothschild & Mancinelli 2001).

When water becomes limiting, the fronds of ferns that present desiccation tolerance curl and get a lifeless aspect (Lambers *et al.* 2008). This happens due to changes in the form of the cell walls, which promote the folding of the cells, thus reducing the mechanical stress during dehydration, not only in ferns (Thompson & Platt 1997), but also in angiosperms (Farrant *et al.* 1999; Vander Willigen *et al.* 2004). This behavior helps preserve the leaves until water availability gets higher, either due to a major rainfall event or due to a significant increase in the relative humidity of the atmosphere. This gives these tolerant species a great competitive advantage over the others in the process of occupation of certain ecological niches (Scott 2000).

This study compares the degree and mechanisms of desiccation tolerance of four epiphytic ferns that commonly occur on the same phorophyte, but that display distinct degrees of leaf curling under conditions of reduced water availability. By quantifying and comparing changes in leaf water content, membrane integrity and concentration of photosynthetic pigments and flavonoids that take place during desiccation and rehydration periods, we tested the hypothesis that those species that display leaf curling are more tolerant to desiccation than those that do not express this behavior.

MATERIALS AND METHODS

The selected species

The four species of pteridophytes used in this study belong to the family Polypodiaceae: *Polypodium polypodioides* var. *minimum* (Bory) Kuhlm & Kuhn, *Polypodium hirsutissimum* (Raddi) de la Sota, *Pleopeltis pleopeltifolia* (Raddi) Alston and *Microgramma squamulosa* (Kaulf.) de la Sota. These species commonly share the same tree host, but have distinct spatial distribution. *Polypodium polypodioides* preferably occurs on the tree bole, where it covers extensive areas. It is rarely found on the thicker and lower branches of the tree host. The other three species grow in the upper, terminal branches, but have different habits. *Polypodium hirsutissimum* and *Pl. pleopeltifolia* have short, while *M. squamulosa* has long runners, allowing for a larger phorophyte occupation of the latter. *Polypodium hirsutissimum* has large leaves, which are densely covered by trichomes, while *P. polypodioides* has the smallest leaves amongst the studies species. General characteristics of the selected species are presented in Table 1.

The species were identified by botanical specialists, and the *vouchers* were deposited in the Herbarium ICN of the Department of Botany, Institute of Biological Sciences, of the Federal University of Rio Grande do Sul (UFRGS), under the registry numbers: ICN 182858 - *Polypodium polypodioides* var. *minimum* (Bory) Kuhlm & Kuhn; ICN 182859 - *Polypodium hirsutissimum* (Raddi) de la Sota; ICN 182860 - *Pleopeltis pleopeltifolia* (Raddi) Alston and ICN 182861 - *Microgramma squamulosa* (Kaulf.) de la Sota.

Frond sampling procedure

Ligustrum lucidum W. T. Aiton (Oleaceae), *Melia azedarach* L. (Meliaceae) and *Jacaranda mimosaefolia* D. Don. (Bignoniaceae) were the tree host species from where pteridophytes were sampled. Fronds were collected in two different occasions in November/2010, from trees located in the city of Porto Alegre, RS, Brazil (30º03'23"S - 51º13'15"O). In the first collect fronds were used to desiccation and in the second collect they are used for the rehydration procedures. Only mature and well hydrated fronds of the four species were collected, totalizing 180 fronds for each species, except for *P. polypodioides*, for which the number of collected fronds was twice as much due to the small size of its leaves. Right after being taken from the host tree, the fronds were packed in plastic bags.

Local weather is classified as Cfa (Köppen 1948). During the summer the average temperature is higher than 22ºC and with rainfall over than 30mm per month (Golfiari *et al.* 1978).

Dehydration and rehydration procedures

In both processes, fronds were transferred from the plastic bags to paper envelopes. Each envelope contained about twenty fronds. Fronds (inside their envelopes) exposed to the process of dehydration were submitted to three different relative humidity (RH) conditions: 0%, 60-70%, and 100%. The 0% condition was obtained by placing the envelopes in a

Table 1. General characteristics of the sampled species. Observations and measurements we made during a desiccation period. ab = apical branches; bb = basal branches, Bo = bole. Values of leaf area are based on 10 individuals from each species.

Species	Location on host	Leaf curling	Trichomes	Leaf area (cm^2)
P. polypodioides var. *minimum*	Bo, BB	whole frond	-	2.64
P. hirsutissimum	bb, ab	Partial	+	28.97
Pl. pleopeltifolia	bb, ab	whole frond	-	22.77
M. squamulosa	Bo, bb, ab	None	-	6.67

desiccator containing silica gel, this desiccator being placed in a dry chamber (RH < 30%). The 60-70% condition was that prevailing in laboratory. In this case, the envelopes were kept on a laboratory bench. Fronds submitted to the 100% condition were kept inside a germinator chamber, with controlled temperature and humidity. The average temperature in all three environments was 25°C. Fronds were analyzed at different time intervals after dehydrations started: 0, 2, 24, 36 and 48 hours. Measurements at time zero were those made right after frond collection.

The fronds used in the rehydration procedure were initially exposed to 0% RH for 48 hours in the desiccator, thus simulating the conditions of those fronds that passed through the full process of dehydration. After this period, fronds were immersed in deionized water for increasing periods of time: 0, 2, 4, 6, 8 and 24 hours. Measurements at time zero corresponded to the full dehydration condition. At each of these periods, fronds underwent the proposed analysis.

Analyses performed in the fronds

The relative water content (RWC), the concentrations of chlorophyll, carotenoids, and flavonoids, as well as the index of membrane integrity were evaluated at those pre-established time intervals, during both the phases of dehydration and rehydration. For all evaluations, ten fronds were used for each species, RH (in the case of the dehydration procedure), and time interval, except for membrane integrity, for which only three fronds were used. Values of all these parameters are expressed as a percentage of the highest value. Only the middle third of the fronds of *P. hirsutissimum*, *Pl. pleopeltifolia* e *M. squamulosa* was used for the analysis, except for *P. polypodioides,* for which entire frond was used due to its reduced size.

Relative Water Content (RWC)

At each of the time intervals (t) during the dehydration and rehydration processes, the fresh weight (FW) of frond sections was measured, followed by their immersion in deionized water for 24 hours in order to determine the turgid weight (TW). They were then oven-dried (70°C/24h) for evaluation of the dry weight (DW). The RWC was calculated according to the equation (BARRS 1968): RWC (%) = {[(FW or FW_t) – DW] / (TW – DW)} x 100.

Concentrations of chlorophyll and carotenoids

At each time interval, frond sections were oven-dried (60°C/48h) and ground. About 50 mg of the tissue were placed in plastic tubes containing 2 ml of hidroxometil acetone tris (0.1 M) (80:20, vol:vol), adjusted to pH 7.8 with HCl. The tubes were wrapped with aluminum foil to avoid light exposure and pigment degradation. The solutions were stored for 48 hours in a refrigerator (4°C). After that, they were centrifuged for 3 min at 10.000 rpm (MSE Micro Centaur) and the supernatant diluted in 6 ml of the same extraction solution. The absorbance readings lectures were made in a spectrophotometer (SpectraMax Plus384 Absorbance Microplate Reader) in the following wavelengths: 470, 537, 647, and 663 nm. Pigment concentration was calculated according to the equations of Sims & Gamon (2002).

Concentration of flavonoids

At each time interval, frond sections were oven-dried (60°C/48h) and ground. The extraction procedure was that proposed by Zhishen *et al*. (1999). About 40 mg of the ground tissue was put into falcon tubes containing 1.5 ml of ethanol (95%). Tubes were sonicated for 30 min in the dark, centrifuged for 5 min at 7000 rpm (MSE Micro Centaur), and the supernatant recovered. Four milliliters of distilled water, 0.5 ml of ethanol (95%), 0.3 ml of NaNO2 (5%), and 0.3 ml of $AlCl_3$ (10%) were added to the supernatant. After shaking and incubating in

ambient temperature for 6 min, 2 ml of NaOH 1M were added, together with 1.4 ml of distilled water to obtain 10 ml of solution. The solution was shaked and the absorbance readings were taken in a spectrophotometer (SpectraMax Plus384 Absorbance Microplate Reader) in the wavelength of 510 nm. Quercetin was used as standard flavonoid for the calibration curve and the concentration express in $\mu g.g^{-1}$.

Membrane Integrity

Frond samples used in the dehydration and rehydration procedures had the release of electrolytes quantified in an electric conductivimeter (Digimed, DM-31) at each of the previously established time intervals of each procedure. Each time interval was represented by 3 samples of each species, and for the rehydration analyses, the same fronds were used throughout the entire process. Fronds were bottled in glass flasks containing 100 ml of deionized water, whose electric conductivity was previously measured (adapted from Krishnamani *et al.* 1984). Incubation of the fronds took place at a constant temperature of 25ºC and with constant agitation of the flasks. The release of electrolytes was evaluated by the increase in free electrical conductivity (FC). After each evaluation, flasks were taken to a water bath (100°C/1h), and the total conductivity was measured (TC, μSiemens). At the end of the evaluations, the plant material was oven-dried (70°C/24h), and values were expressed in μSiemens/g dry mass. The absolute integrity percentage (PI) was then calculated as follows: $PI = (1 - FC_i/FC_n) \times 100$, where FC_i refers to FC at time zero and FC_n to FC evaluated at each time interval. The percentage of damage was calculated through the index of injury (I), where $I = 100 - PI$ (adapted from Vasquez-Tello *et al*., 1990).

Statistical analysis

Data collected during the desiccation process were submitted to three-way ANOVA, for comparison of the four species at different periods of time (five levels) and relative humidity conditions (three levels). In process of rehydration was used two-way ANOVA, for comparison between the four species and six different periods of time. Fischer's test ($P \leq 0.05$) was used to mean separation procedure in the process of desiccation and rehydration. Statistical analyses were performed using Sigma Plot statistical program, version 11.0 (SPSS Inc.).

RESULTS

Patterns of reduction in relative water content (RWC) during the desiccation period varied among species and relative humidities to which fronds were exposed (Fig. 1). At time zero, most species had a RWC around 70-80%, except for *Pl. pleopeltifolia*, which started at values between 40-50% when dehydrated at RH of 60-70 and 100%. *Microgramma squamulosa* underwent the lowest dehydration at all RH, and its RWC did not drop much below 50%. *Polypodium polypodioides* followed the same pattern of dehydration as *M. squamulosa* at a RH of 100%. However its dehydration was much faster and more pronounced at lower RH, particularly at 0%, where it reached a minimum RWC of about 20%. *Polypodium hirsutissimum* exhibited a very fast initial dehydration, displayed similar patterns at all three values of RH, and reached minimum values of RWC between 30 and 40%. Finally, *Pl. pleopeltifolia* typically had the lowest RWC at all times and RH, attaining, together with *P. polypodioides*, minimum values of around 20% at the lowest RH. *Polypodium polypodioides* and *P. hirsutissimum* didn't differ statistically from each other

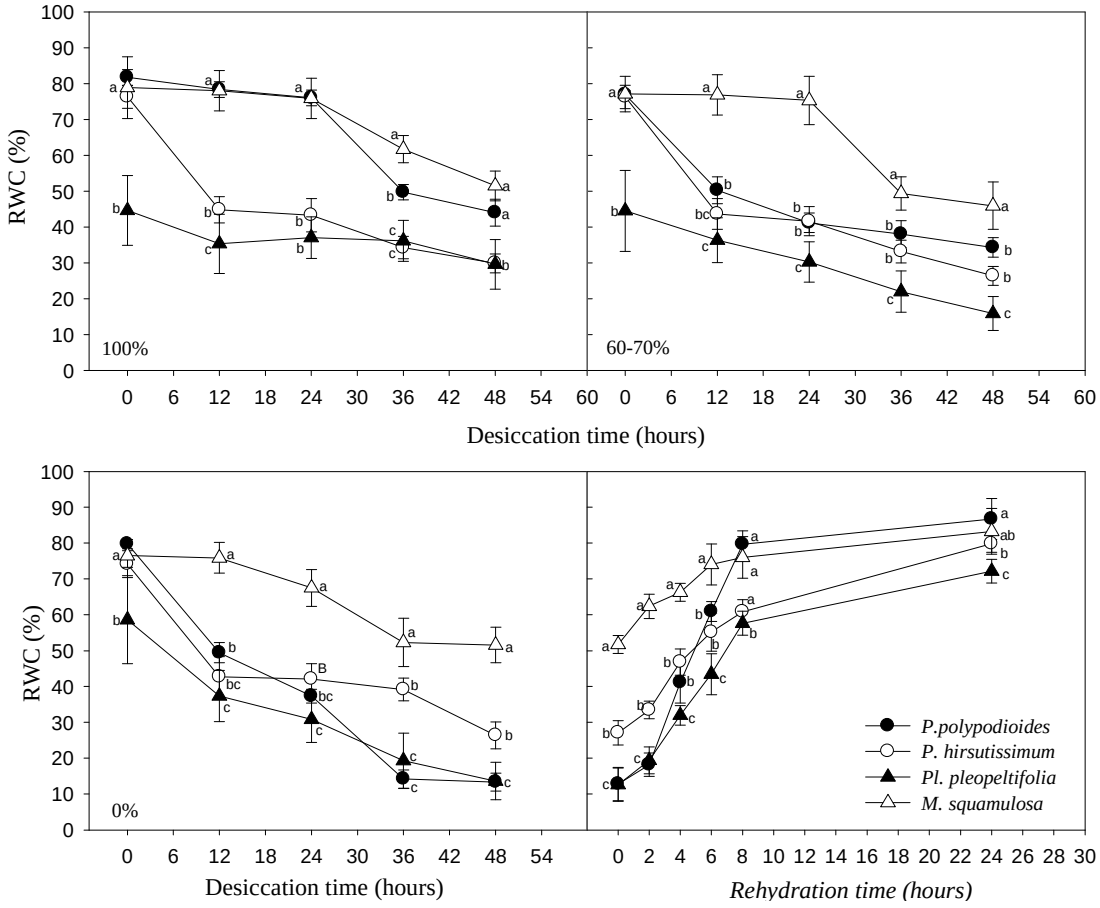

Fig. 1. Relative water content (RWC) in relation to desiccation time (hours) in three different conditions of relative humidity (0%, 60-70% and 100% RH) and in relation to *rehydration time (hours)* in fronds of the four species studied. Each point represents the average ± SE (standard error) of ten fronds. Different letters indicate significant differences between species means within the same gaps of time at $P<0.05$ (ANOVA; Fischer LSD test).

throughout the desiccation process in 60-70%RH had intermediate values of RWC% when compared to other species.

The increase in RWC during rehydration was more pronounced in the fronds of *P. polypodioides,* reaching values around 80% after 8 hours. *Microgramma squamulosa* didn't differ of *P. polypodioides* and neither *P. hirsutissimum* after 24 hours of desiccation. *Pleopeltis pleopeltifolia* fronds had values higher in final rehydration than those at the beginning of the desiccation process, 70% and 40-60% respectively.

There is a statistically significant interaction between species, different relative humidity and gaps of time ($P<0.05$) in relation to chlorophyll, carotenoids and flavonoids during desiccation and rehydration process (Table 2).

Chlorophyll concentration reduced for all species and all RH conditions during the dehydration process (Fig. 2 – the left column), and was more pronounced in *P. polypodioides*. The concentration in fronds *M. squamulosa* at the end of desiccation was maintained between 70% and 80% of the initial value, between 3 to 4 $mg.g^{-1}$ DW. Fronds of *P. hirsutissimum* exposed to RH of 0% and *Pl. pleopeltifolia* exposed to RH of 100% maintained chlorophyll levels above 80% of the initial value (~3 $mg.g^{-1}$ DW). The pattern of variation of the concentration of carotenoids during dehydration was quite variable and did not always exhibit a continuous decrease (Fig. 2 – central column). The greatest reductions in carotenoid concentrations was observed in *P. polypodioides,* with final concentrations around 0.3 $mg.g^{-1}$ DW, less than 50% of the initial values. In *Pl. pleopeltifolia*, final carotenoid concentrations to 60-70% and 100%RH were above the values of the other species. In *M. squamulosa,* initial decreases were followed by midway increases at RH of 0% and 60-70%, and final reductions did not go below 0.6 $mg.g^{-1}$ DW.

Table 2. Summary statistics for general linear models performed on fronds trait data of *P. polypodioides* var. *minimum*, *P. hirsutissimum*, *Pl. pleopeltifolia* and *M. squamulosa* during desiccation and rehydration process. Bold values denote significant differences ($P<0.05$). Where Sp = species, RH = relative humidity (0%, 60-70% and 100%) and GT = gaps of time (0, 12, 24, 36 and 48 hours).

Desiccation process							
		Chlorophyll		Carotenoids		Flavonoids	
Variables	DF	F	*P*	F	*P*	F	*P*
Sp	3	283.5	**<0.001**	610.4	**<0.001**	405.9	**<0.001**
RH	2	6.2	**0.003**	5.8	**0.004**	56.0	**<0.001**
GT	4	159.5	**<0.001**	398.2	**<0.001**	173.9	**<0.001**
Sp X RH	6	14.2	**<0.001**	41.8	**<0.001**	35.6	**<0.001**
Sp X GT	12	4.1	**<0.001**	12.8	**<0.001**	4.9	**<0.001**
RH X GT	8	1.6	0.122	6.5	**<0.001**	2.9	**0.005**
Sp X RH X GT	24	2.1	**0.005**	12.9	**<0.001**	4.4	**<0.001**
Rehydration Process							
		Chlorophyll		Carotenoids		Flavonoids	
Variables	df	F	*P*	F	*P*	F	*P*
Sp	3	138.4	**<0.001**	731.4	**<0.001**	345.5	**<0.001**
GT	5	37.8	**<0.001**	586.4	**<0.001**	12.3	**<0.001**
Sp X GT	15	1.4	0.185	23.5	**<0.001**	26.9	**<0.001**

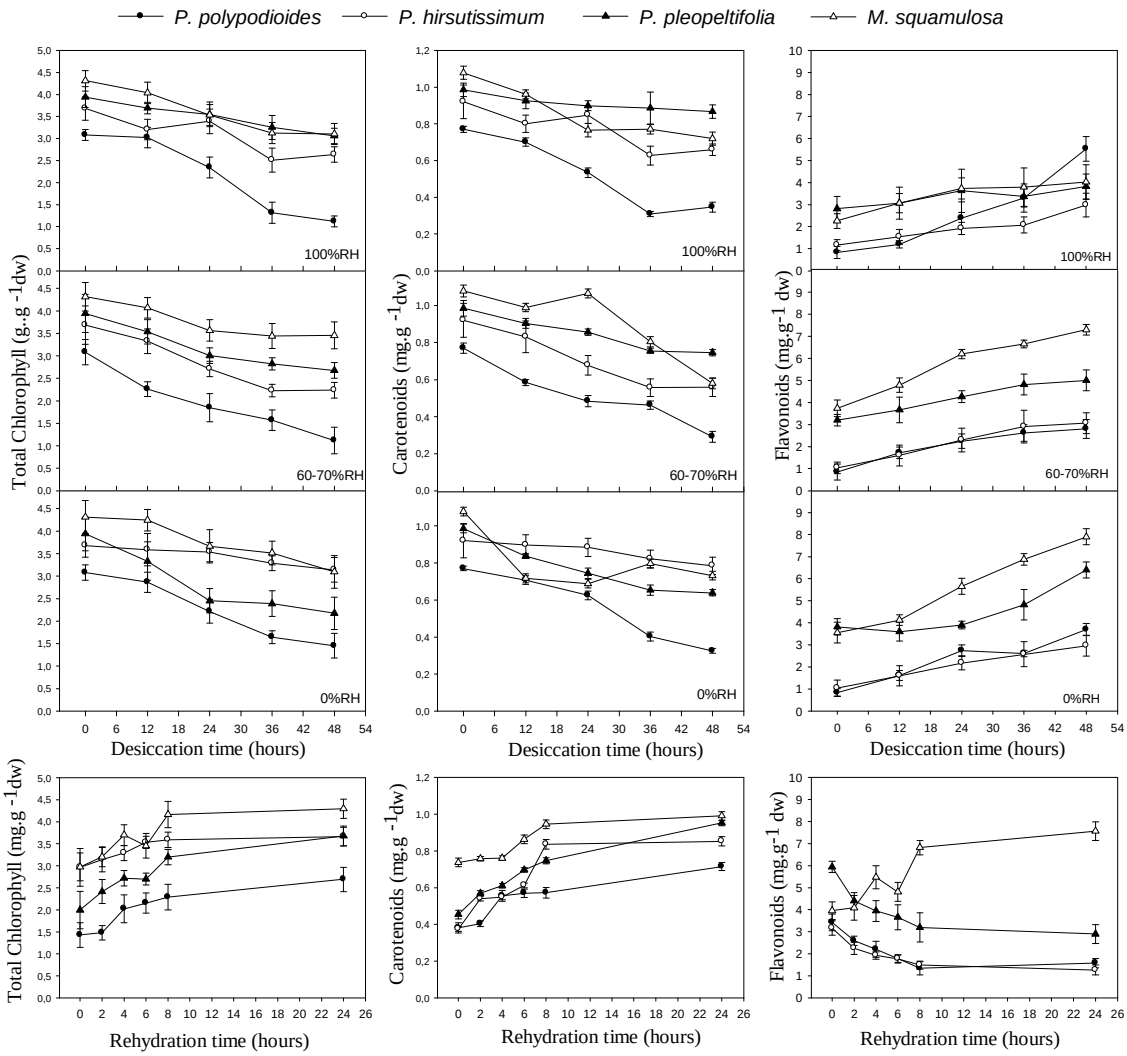

Fig. 2. Total content of chlorophyll, carotenoids and flavonoids in relation to desiccation time (hours) in three different conditions of relative humidity (0%, 60-70%, 100%) and in relation to rehydration time in fronds of *P. polypodioides* var. *minimum*, *P. hirsutissimum*, *Pl. pleopeltifolia* and *M. squamulosa*. Each point represents the average ± SE (standard error) of ten fronds.

The accumulation of flavonoids was observed for all four species throughout the desiccation process, regardless of humidity condition on which the fronds were exposed (Fig. 2 – the right column). *Microgramma squamulosa* accumulated the highest content of flavonoids in the end of the desiccation process in the relative humidity of 0% and 60-70%, with values above 7 $mg.g^{-1}$ DW, followed by *Pl. pleopeltifolia* with 6 and 5 $mg.g^{-1}$ DW respectively. Flavonoids concentration in *P. polypodioides* was higher than the others in the end of the desiccation process, with values greater than 5 $mg.g^{-1}$ DW.

Rehydration led to the foliage re-synthesizes chlorophyll and carotenoids that had been degraded during dehydration (Fig. 2). The fronds of *P. hirsutissimum* increased the carotenoid level more quickly, compared to *P. polypodioides* and *Pl. pleopeltifolia.* Already *M. squamulosa* presented a more stable pattern in this parameter. There was a decrease in flavonoid content during rehydration, except for *M. squamulosa* which showed accumulation end around 7 $mg.g^{-1}$ DW compared with the other below 3 $mg.g^{-1}$ DW.

There was an inverse linear relationship between the RWC and the content of flavonoids in dehydration demonstrated by the graphs regression (Fig. 3 and 4). The reduction in the values of RWC was accompanied by an increase in levels of flavonoids in the four species studied, regardless of RH that they were exposed. *Polypodium polypodioides* showed a low correlation between the content of flavonoids and RWC in fronds exposed to 100% RH, in contrast to the high correlation values in the other two moisture conditions. The fronds of *M. squamulosa* which were exposed to 60-70% RH had the highest correlation value compared to other species. Rehydration in the values of flavonoids decreased with the increase of the RWC for *P. polypodioides, P. hirsutissimum* and *Pl. pleopeltifolia* while in *M. squamulosa* these values increased during this process (Fig. 4).

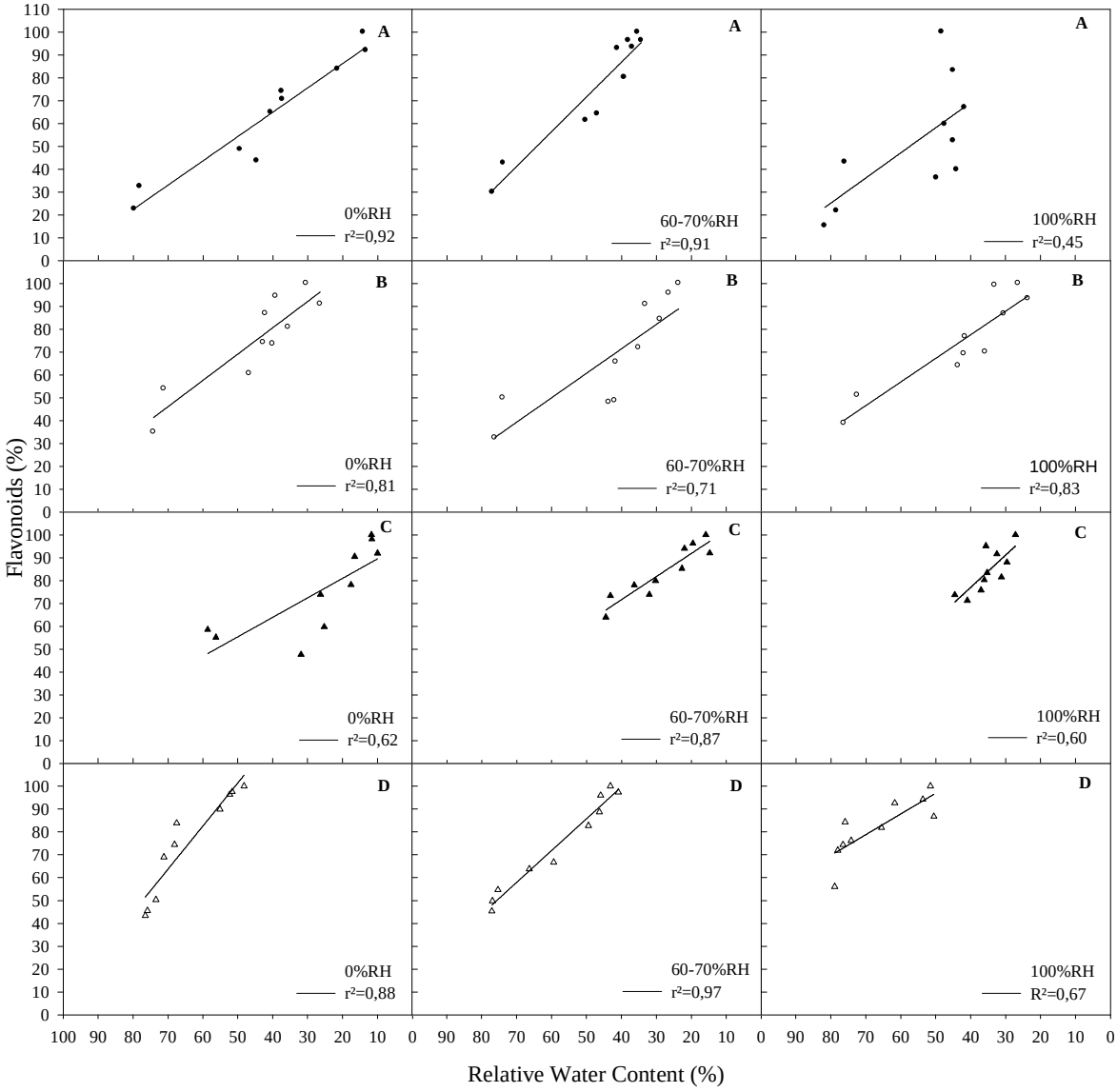

Fig. 3. Content of flavonoids (%) compared to the relative water content (%) during the desiccation process (0%, 60-70% e 100% RH) for *P. polypodioides* var. *minimum* (A), *P. hirsutissimum* (B), *Pl. pleopeltifolia* (C) and *M. squamulosa* (D). Each point represents the average of ten fronds as a percentage to the highest value obtained.

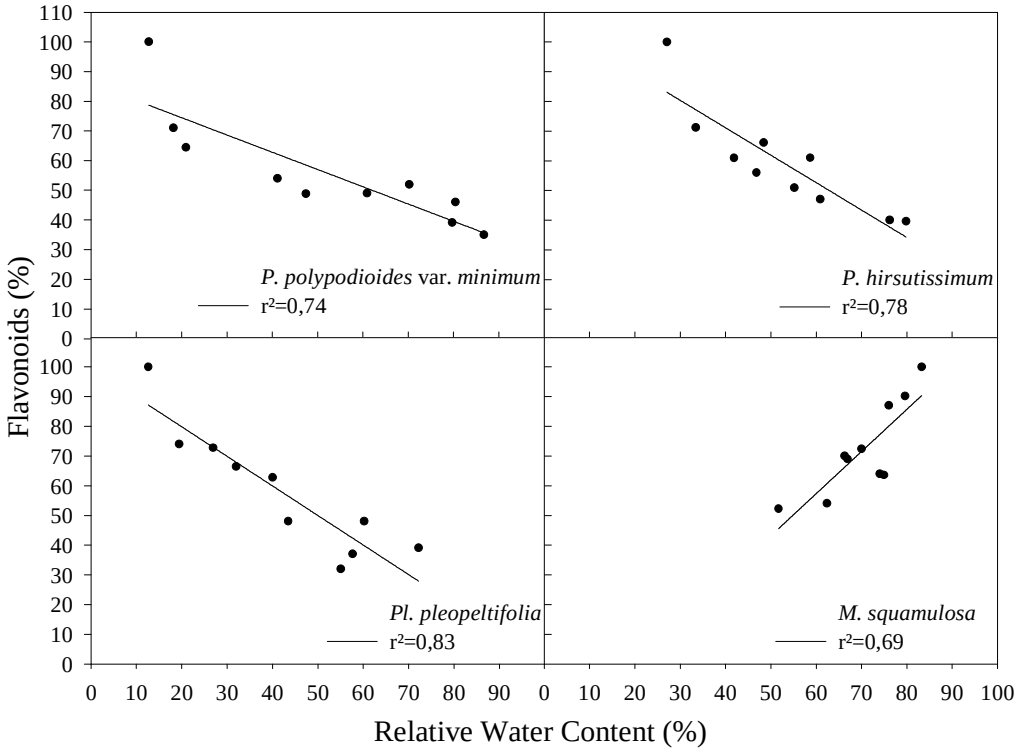

Fig. 4. Content of flavonoids (%) compared to the relative water content (%) during the rehydration process for each of the four species studied. Each point represents the average of ten fronds as a percentage to the highest value obtained.

The total conductivity in the desiccation and rehydration process was significantly higher than the free conductivity for all studied species, regardless of relative humidity or gaps of time (data not show). *Polypodium polypodioides* had the highest PI (71.99 to 89.89%) during desiccation at 0% RH (Table 3), while *M. squamulosa* had the lowest (38.25 a 62.98%). *Polypodium hirsutissimum* and *Pl. pleopeltifolia* showed intermediate PI values by 36 hours of desiccation, reaching values close to *P. polypodioides* (~80%) after 48 hours. *Polypodium polypodioides* and *Pl. pleopeltifolia* had the highest PI values in 60-70%RH, didn't differ statistically from each other in any of the time while *M. squamulosa* again showed the lowest values. Percentage of absolute integrity values in *P. polypodioides* in 100%RH was lower than those for the other two conditions of humidity. However, along with values *Pl. pleopeltifolia*, continued to be higher than in the other two species. *Microgramma squamulosa* presented the lowest values until 36h, however exceeded the value presented by *P. hirsutissimum* after 48 hours of desiccation.

Polypodium polypodioides and *Pl. pleopeltifolia* showed higher values of PI during the rehydration, followed by *M. squamulosa* and *P. hirsutissimum* (Table 3). Only there was no significant difference between *P. polypodioides* and *Pl. pleopeltifolia* after 24 hours of rehydration (P=0.069).

The percentage of damage to cell membranes was measured by the index of injury (Fig. 5). *Polypodium polypodioides* was the species that showed the lowest injury during dehydration, while *M. squamulosa* showed the highest, regardless of the condition of relative humidity. *Pleopeltis pleopeltifolia* showed a greater degree of injury to the cells when exposed to the lowest condition of relative humidity. *Polypodium polypodioides* and *Pl. pleopeltifolia* didn't differ in any gaps of time at intermediate relative humidity

Table 3. Membrane integrity in *P. polypodioides* var. *minimum* (*Pp*), *P. hirsutissimum* (*Ph*), *P. pleopeltifolia* (*Pl*) and *M. squamulosa* (*Ms*) during the dehydration process - in three different conditions of relative humidity (0%, 60-70% and 100% RH) - and rehydration process; where FC = free conductivity, TC = free conductivity (μSiemens) in each time interval and PI = percentage of absolute integrity [$PI(\%) = 1 - (FC_i/FC_n) * 100$].

PI (%) - *Desiccation time (hours)*					
RH	Species	***12h***	***24h***	***36h***	***48h***
0%	*Pp*	71.99[a]	82.80[a]	84.93[a]	89.89[a]
	Ph	61.65[b]	67.63[b]	66.44[b]	83.32[b]
	Pl	42.66[c]	56.72[c]	52.20[c]	80.44[c]
	Ms	38.25[d]	43.52[d]	52.06[c]	62.98[d]
60-70%	*Pp*	81.93[a]	86.08[a]	87.37[a]	91.48[a]
	Ph	71.83[c]	61.47[c]	76.25[b]	76.59[b]
	Pl	82.53[a]	88.30[a]	84.9[a]	89.86[a]
	Ms	56.46[b]	56.69[b]	45.49[c]	57.30[c]
100%	*Pp*	68.16[b]	75.76[b]	79.82[b]	82.38[b]
	Ph	52.52[c]	53.60[c]	52.74[c]	63.32[d]
	Pl	88.25[a]	90.12[a]	91.92[a]	93.51[a]
	Ms	36.09[d]	48.93[d]	49.12[d]	67.07[c]

PI (%) - *Rehydration time (hours)*					
Species	***2h***	***4h***	***6h***	***8h***	***24h***
Pp	81.30[b]	92.05[b]	95.32[b]	95.56[b]	97.22[a]
Ph	47.57[d]	44.12[d]	66.46[d]	55.53[d]	85.17[c]
Pl	94.50[a]	96.41[a]	97.34[a]	97.79[a]	98.84[a]
Ms	74.01[c]	82.50[c]	89.04[c]	91.50[c]	95.00[b]

* Different letters indicate significant differences between species means within the same gaps of time at $P<0.05$ (ANOVA; Fischer LSD test).

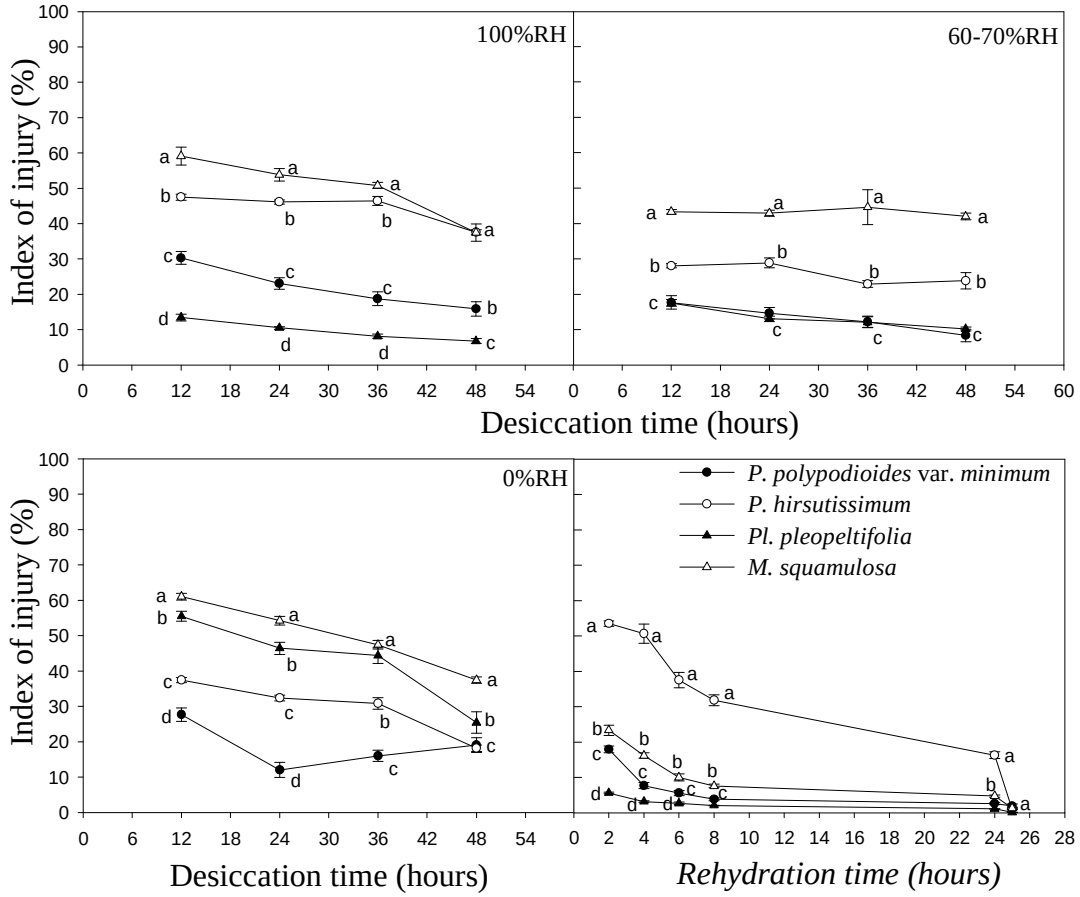

Fig. 5. Index of injury (*I = 100 – PI*) in relation to desiccation time (hours) in three different humidity conditions (0%, 60-70%, 100%) and in relation to rehydration time (hours) for each species studied. Each point represents the average of three fronds ± SE. Different letters indicate significant differences between species means within the same gaps of time at $P<0.05$ (ANOVA; Fischer LSD test).

(12h-*P*=0.954; 24h-*P*=0.438; 36h-*P*=0.966; 48h-*P*=0.360). The injury rates were higher for *P. hirsutissimum* during the rehydration. *Polypodium polypodioides*, *M. squamulosa* and *Pl. pleopeltifolia* showed a tendency towards stabilization of injuries to cell membranes after 8 hours of the dehydration process. Only there wasn't statistical difference between *P. polypodioides*, *Pl. pleopeltifolia* and *M. squamulosa* after 24 hours of rehydration.

DISCUSSION

The importance of spatial structures is widely recognized in ecological theory (Peterson & Parker 1998). Borcard *et al*. (2004) state that the interactions between communities and their physical environment, and between the organisms occur in temporal and spatial scales, causing spatial patterns that need to be evaluated to better understand the processes structuring these communities. These patterns are evident in the four species of epiphytic ferns in this study, which often co-occur in the same host tree. The competition between them is minimized by the occupation of different positions in the host tree, the type of winding of the fronds and the speed of response to variations in relative humidity of the surrounding air during the process of desiccation or rehydration.

In order to optimize the capture of light, the tiny fronds of *P. polypodioides* usually cover a large area of the host tree and as soon as there is increased moisture in the air, its leaves unfold to take advantage and capture light energy before they are shaded by other species of fronds with same behavior. Features such as the slope of the frond, the thickness of the blade, the smallest leaf area (due to the shape of the pinnate fronds) and shorter length of the frond can be understood as strategies to reduce the deleterious effects of light and water deficit according to Boeger *et al*. (2007), and minimize transpiration rates (Medri & Lleras 1980).

Although *P. hirsutissimum* provide the highest leaf area among studied species, their fronds can be protected against photo-oxidative damage by the presence of a large amount of trichomes (Watkins *et al*. 2006). According to Nobel (1991), trichomes reflect light, reduce leaf temperature and, consequently, reduce transpiration. In many ferns tolerant to desiccation, the trichomes also have the ability to absorb water, allowing rehydration so the plants keep wet in dry environments, avoiding the need to transport water from roots to the petiole (Hietz 2010).

This feature was noted by Stuart (1968) in specimens of *P. polypodioides* occurring in the southern United States, which did not respond directly to the supply of water placed on the rhizome, but rather the liquid water placed directly on the fronds or, then, to high humidity. This same behavior was also observed for *P. polypodioides* and *Pl. pleopeltifolia* in this study. From an intermediate degree of relative humidity, the species began the course of their fronds. This faster way of absorption of water by the species of epiphytic habit is very advantageous because it can take advantage of short events of water availability, as fast rain, morning dew, fog or simply increasing the relative humidity surrounding them, making it the so self-sufficient, since the water retention in rhytidome varies by host tree. According to Benzing (1990), the storage capacity of water is very low uncovered branches, and epiphytes that grow on them are exposed to frequent changes in water supply, what distinguishes those parts of most terrestrial habitats. More likely to minimize this disadvantage, it is quite a common occurrence for interspersed individuals of *P. polypodioides*, *Pl. pleopeltifolia* and *M. squamulosa* in the same branches of the host tree, providing an environment most likely to fluid retention.

Polypodium polypodioides, *P. hirsutissimum* and *Pl. pleopeltifolia* curl their fronds as soon as the relative humidity starts to reduce, getting a lifeless aspect. This adaptation is common in many ferns desiccation tolerant and is very advantageous because it reduces the

exposed leaf surface, reducing water loss and damages that may be caused by the light through the shade.

The way the fronds curl is pre-determined and specific to each species (Hietz 2010). Tryon & Tryon (1982) observed that the fronds of *Elaphoglossum calaguala* and *E. mathewsii* rolled longitudinally, while the fronds of *E. hirtum* wrapped in a spiral, a pattern also observed for the two species of the same genus in this study. *Polypodium hirsutissimum* scrolls along and *P. polypodioides* doubles the margin of their first fronds and after the peak of the foliage toward the base in a spiral. Since the *Pl. pleopeltifolia* fronds curl up and then inside, as also observed by Mehltreter (2008) for other species of the same genus, *Pl. furfuracea*. In some species of *Microgramma* the fronds curl up and not remain turgid during the dry season (Hietz 2010), as well as the fronds of *M. squamulosa*.

The capacity to curl the foliage according to Lebkuecher & Eickmeier (1993) is essential, because it prevents damage to recovery of the photosynthetic process during rehydration to protect and keep safe the photosynthetic apparatus from damage by light.

The speed at which dehydration occurs also directly influences the physiological responses of different species tolerant. According to Oliver (1996), plants tolerant to desiccation, requiring a slower drying suffer irreparable cell damage when they dry quickly. For this reason, besides the physiological and metabolic as well as anatomy, morphology and ultra-structural characteristics of leafs, effective in slowing the rate of water loss, may be particularly significant in the cells' ability to withstand extreme dehydration in its natural conditions (Dalla Vechia *et al*. 1998). Among the four species studied, *P. polypodioides* was the species that responded quickly to desiccation in low relative humidity and rehydration of their fronds, probably due to their smaller leaf area. According Helseth & Fischer (2005), the water uptake by plants is governed by the resurrection plant size and the area available for water flow. In their experiments, suggested that a small plant may rise faster than a large

plant, due to the fact that there is a longer delay in the redistribution of water in a larger plant. In a study also with *Polypodium*, Pessin (1924) and Stuart (1968) concluded that the trichomes did not facilitate the absorption of water, but allow water to spread evenly on the leaf surface. Trichomes on leafs of different species of *Cheilanthes* and *Ceterach* delay the capture of water for several hours because of air that is trapped between them (Gaff 1977). In leafs of *Ramonda mycony* was verified by Kappen & Valladares (2006) this same effect, where the spraying water on outstanding hairy leafs resulted in lower water absorption than those placed in immersion or those without pubescence. The delay resulting from the presence of trichomes and hairs suggests that rapid water uptake after desiccation could cause injury to cells of the leaves. This feature could explain the patterns of water uptake in *P. hirsutissimum*. The response of this species at three different humidity conditions during the dehydration process, possibly showed that the retention of air trapped between the trichomes and leaf epidermis made it hold an intermediate behavior to other species loss and water absorption. However, when exposed to rehydration, their fronds of the trichomes helped in absorbing water.

According to Georgieva *et al.* (2009), the physical properties of the photosynthetic apparatus are important during the desiccation process, since this period the probability of damage increases significantly and, therefore, should be maintained or repaired quickly after the onset of rehydration. The decrease in total chlorophyll content in *P. polypodioides* was more pronounced than in the others during the study period. The reduction of photosynthetic pigment during the time it spent dried, promoted a brown tint in the leaves, which is rapidly reversed to the green tint when hydrated. This same pattern was also observed by Jackson *et al.* (2006) for that species occurring in stems of *Quercus virginiana*. In *P. hirsutissimum* there was also the same change in color of the fronds, but the change in tone was not accompanied by a marked reduction as the chlorophyll content as it was for *P. polypodioides*. This may be

due to a conformational change and structural molecules of the photosynthetic apparatus during the desiccation process, a pattern also reported by Brugnoli & Bjorkman (1992) in protection against photo-inhibition in shade plants. The reduction in chlorophyll content to *Pl. pleopeltifolia* during the desiccation process was similar to those of *P. hirsutissimum*. However the change in the tone of the fronds was not observed, probably because of higher levels of carotenoids in their foliage throughout the desiccation process when compared to *P. hirsutissimum*, which confers protection. According to Hietz (2010), the main biochemical defenses against photo-inhibition are pigments and antioxidants, and carotenoids help to reduce the forms of chlorophyll excited and O_2 and also protect the thylakoid membranes.

Besides carotenoids, the presence of flavonoids during periods of dehydration is also very important. Its occurrence in the epidermal cells prevents the inner tissues of leaves and stems damaged by UV radiation, and are excellent scavengers of reactive oxygen species, preventing lipid peroxidation (Shirley 1996; Rozema *et al*. 1997) and act also as anti-oxidants in situations of excessive light (Rozema *et al.* 2002; Tattini *et al*. 2004). The high positive linear correlation found between the accumulation of flavonoids and relative water content for most the relative humidity conditions in the species studied possibly reflects the protective role of this pigment rather than reducing the levels of chlorophyll and carotenoids during desiccation. The protection afforded to the photosynthetic apparatus in *P. polypodioides*, due to the increased synthesis of flavonoids, possibly preserving the mechanisms involved in the rehydration phase, thus allowing a faster recovery of the levels of chlorophyll and carotenoids observed in this species. The synthesis of this pigment was not so pronounced in *Pl. pleopeltifolia*, probably due to the protection already afforded by larger amounts of carotenoids, compared to *P. polypodioides* during dehydration. The pattern in the accumulation of flavonoids in *P. hirsutissimum* was similar between the different conditions of relative humidity, probably due to the contribution of trichomes to protect their fronds

against the excessive incidence of light. Flavonoids also have an important role in protecting the photosynthetic apparatus in *M. squamulosa*, since this species preserved chlorophyll during dehydration, in the three relative humidity conditions. In *P. polypodioides*, *P. hirsutissimum* and *Pl. pleopeltifolia* the reduction in the amounts of flavonoids in rehydration revealed that its presence is actually associated with protecting cells of the fronds against the possible photo-oxidative damage (Gould *et al*. 2002; Rozema *et al*. 2002; Tattini *et al*. 2004) for the occurrence of stress. However, the increase of this pigment in *M. squamulosa* can be explained by the different strategy of this species due to the increasing water content of their tissues, since it is not tolerant to dehydration. In this case, flavonoids can act as a mechanism for the prevention of fungal contamination, normally used in excessively wet for higher plants are not tolerant to dehydration (Grayer & Harborne 1994).

The percentage of damage in cells of *P. polypodioides* during the dehydration process was, in the three conditions of relative humidity, the lowest among the studied species. This feature combined with others already discussed infer that this species is one that responds more effectively to natural processes and periodic dehydration and rehydration that their individuals are subjected in short periods of time, followed by *Pl. pleopeltifolia* and *P. hirsutissimum*. *Pleopeltis pleopeltifolia* showed little damage in the cells of the fronds in relative humidity above 60-70% and 100%, indicating that when exposed to a lower humidity, possibly their cells suffer irreversible damage.

The data obtained in relation to membrane integrity in this study are similar to the results of Mariano *et al.* (2009) with *Myracrodruon urundeuva*. They found that intermediate and basal leaves in these species were characterized as being more sensitive to high temperature treatment once had higher injury percentage. Pimentel *et al.* (2002) evaluated the release of electrolytes in leaf discs and found differences between the two cowpea genotypes subjected to drought for 10 and 17 days. The percentage of membrane integrity of stressed

plants was higher in the genotype that was considered to be the most tolerant because it showed better retention of electrolytes. Ben-Amor *et al*. (2006), working with two cultivars of the halophyte *Cakile maritima*, submitted to 0, 100, 200, 300 and 400 mM NaCl for 20 days, observed that electrolyte leakage increased in all treatments and was more sensitive significant genotype compared to the tolerance. The lowest values of injury percentage, with high values of percentage of absolute integrity, reflect greater membrane integrity during the leaves desiccation, due in part to the lower activity of hydrolytic enzymes protoplasmic and/or composition lipid membrane (Monteiro de Paula *et al.* 1990). *Polypodium polypodioides* in this study proved to be the species most adapted to withstand conditions with lower relative humidities, it showed the highest percentage of absolute integrity (PI) values and lowest index of injury at relative humidities of 0 and 60-70%. Unlike *Pl. Pleopeltifolia* that presented the highest values of PI and the lowest index of injury at relative humidities of 60-70 and 100%. *Microgramma squamulosa* has proved to be the most sensitive species, with lower PI values and the highest values of injury, regardless of moisture conditions.

During rehydration the cells of the fronds became more exposed to water and absorbed very quickly, causing a lot of damage to the membranes. The presence of trichomes on the fronds of *P. hirsutissimum* probably facilitated the absorption of water, making their fronds acquire an excessive amount, resulting in a higher percentage of damage the membranes in relation to other species of this study. The percentage of damage in *Pl. pleopeltifolia* was low throughout the entire process of rehydration, with higher values of PI and minor injury, possibly due to a slower and controlled absorption of the water available. Similar patterns were observed for *P. polypodioides* where the percentage of damage was slightly higher in the first hours of rehydration, probably due to its high absorption capacity.

Polypodium polypodioides was the species that showed a highest degree of tolerance to dehydration, followed by *Pl. pleopeltifolia* and *P. hirsutissimum* that showed intermediate

patterns. We suggest that this greater tolerance in *P. polypodioides* can be attributed to their greater geographic distribution compared to other species in this study.

In spite of the reduction in chlorophyll content during dehydration, there was no lack of this pigment in any species after the end of intervals assessments, classifying them as homeochlorophyllous. The increase in the concentration of flavonoids became important during dehydration, revealing its protective action at the photosynthetic apparatus in the four species during this period.

M. squamulosa confirmed it is a species with resistance to dehydration, either by their most apparent characteristic, by not curling up its fronds in the dehydration, and patterns over the metabolic processes of dehydration and rehydration. Thus, it is concluded that the four species of epiphytic pteridophytes studied have different strategies in optimizing the capture of light during periods of higher availability of water and minimize inter-specific competition, facilitating the co-occurrence.

To complement the standards of tolerance and resistance to dehydration of the four species of this study, future work could include measurements for the pattern of accumulation of phenolics, sugars such as sucrose, starch and chlorophyll fluorescence along the processes of dehydration and rehydration.

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ARTIGO 2

Chlorophyll fluorescence, phenols and sugar ratios in four species of epiphytic ferns during desiccation and rehydration processes

Artigo a ser submetido ao Periódico Flora

ARTIGO 2

Chlorophyll fluorescence, phenols and sugar ratios in four species of epiphytic ferns during desiccation and rehydration processes

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Short running title: Mechanisms of tolerance and resistance to desiccation in epiphytic ferns.

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Keywords: chlorophyll fluorescence, phenols, soluble sugars, desiccation tolerance, epiphytic ferns, Polypodiaceae.

ABSTRACT

Plants that are epiphytic habit are more subject to environmental variations than those of terrestrials, such as water availability and greater exposure to the changing of sunlight, wind, relative humidity and temperatures. Desiccation tolerance in many of these plants represents a major adaptive advantage in the occupation of these environments in relation to others that do not exhibit this behavior. The aim of this study was to compare the degree and mechanisms of desiccation tolerance of four epiphytic ferns that commonly occur on the same phorophyte: *Polypodium polypodioides* var. *minimum*, *Polypodium hirsutissimum*, *Pleopeltis pleopeltifolia* and *Microgramma squamulosa*. Expanded fronds were collected from the phorophytes for quantifying and comparing changes in relative water content (RWC), chlorophyll fluorescence, total phenols and soluble carbohydrates during desiccation and rehydration periods. Tolerant species curls their fronds at low RWC, reduced chlorophyll fluorescence and exhibited a greater increase in the concentration of phenols and soluble sugars during the desiccation period, while avoider species not present this behavior. In rehydration, these physiological parameters responded in the opposite way to tolerant species. *Polypodium polypodioides* var. *minimum* and *Pl. pleopeltifolia* were the species that presented the highest level of desiccation tolerance, followed by *P. hirsutissimum* with intermediate patterns and *M. squamulosa* that showed to be the species of greatest avoidance, explained the characteristic lack of curling of its fronds. In conclusion, the species showed different strategies to protect the integrity of their cells during periods of low water availability and different responses in optimizing the capture of light during rehydration periods, thereby minimizing inter-specific competition and facilitating their co-occurrence.

INTRODUCTION

Epiphytic plants are more often exposed to variations in humidity, temperature and light intensity in the canopy compared to terrestrial plants. These microclimatic differences within the canopy provide a structured habitat that may be exploited by different species (Hietz and Briones, 2001) possessing the ability to establish and develop in an environment where the water a major limiting factor.

There are a number of stresses brought about by, or in association with, extreme water loss (Farrant, 2000; Walters et al., 2002). Two of the associated stresses in photosynthetically active tissues are light and temperature. According to Lebkuecher and Eickmeyer (1993) these physiological "state changes" may take place over a wide range of plant temperatures depending upon time of year, and temperature may interact significantly with light to predispose the plants to damage. Photoinhibitory damage and chlorophyll loss could be extremely harmful if they impair the plant's ability to recover photosynthetic competence rapidly when rewetted. Then minimizing photoinhibitory damage and chlorophyll bleaching may be essential for the survival of this species this kind of habitat.

Under water-limiting conditions, excitation energy harnessed by chlorophyll cannot be dissipated via photosynthesis and can lead to the formation of oxygen free radicals, which, if left unquenched, can cause considerable subcellular damage (Smirnoff, 1993). According to Hietz and Briones (2001), photoinhibition is stronger in plants under water stress and photoprotective mechanisms should therefore be very important.

Many of these mechanisms are present in plants that have the ability to desiccation tolerant. Desiccation tolerance may depend on different physiological and biochemical strategies carried out by the plants in order to survive and to regain normal metabolic processes upon rehydration (Bewley and Krochko, 1982).

Acquisition of desiccation tolerance is a complex process that is probably due to an elaborate set of specific adaptations (Djilianov et al., 2010). It has been postulated that different species may use a mixture of different protective metabolites to allow complete desiccation tolerance (Kranner et al., 2002; Kranner and Birtic, 2005). Such as the reduction in the percentage of the components in their photosynthetic apparatus (Hietz, 2010); the accumulation of carotenoids (Bukhov et al., 2001), phenols (Sgherri et al., 2004), flavonoids (Rozema et al., 2002; Tattini et al., 2004); and carbohydrates (Zˇivkovic´ et al., 2005).

According to Hietz (2010), there are many species of ferns that show tolerance traits to desiccation. From the moment there is limitation on the availability of water, the fronds of ferns that show this effect, curl and get a lifeless aspect (Lambers et al., 2008). This happens due to the change in the form of the walls of the cells that promote the folding of the cells, reducing the mechanical stress during the desiccation (Thompson and Platt, 1997) and also provides additional protection against possible damages caused by the high incidence of light (Hietz, 2010).

The light conditions epiphytes are subjected to range from almost full sun on exposed branches to deep shade on the stem base, and the gradients in relative humidity, evaporative demand and amount of water-storing substrate accumulating on branches will produce stronger gradients of drought in different canopy positions that on the ground, where at least the water-holding capacity of the soil is more homogeneous (Hietz and Briones, 2001).

Many studies have reported a preference for areas of epiphytes within the canopy and few attempts have been made to relate the distribution of epiphytes to their physiological traits. Comparing the physiology of epiphytes from different microhabitats provides a more detailed picture of the advantages of physiological adaptations and helps to explain the structure of the epiphyte community. According to Scott (2000) the presence of desiccation

tolerance capacity would promote a competitive advantage compared to others that don't have this feature in the occupation of certain ecological niches.

The aim of this study was to compare the degree and mechanisms of desiccation tolerance of four epiphytic ferns that commonly occur on the same phorophyte, by quantifying and comparing changes in leaf water content, chlorophyll fluorescence, total phenols and soluble carbohydrates. We tested the hypothesis that species with characteristics of desiccation tolerance have physiological adaptations more pronounced during periods of desiccation and rehydration than those that do not express this behavior.

MATERIAL AND METHODS

Plant Material

The four species of ferns used in this study pertain to the Polypodiaceae and were selected by co-occurring and by demonstrating, through the previous visual analysis, potential characteristics of tolerance or desiccation avoidance. The species were considered tolerant when they curled their fronds in periods of low relative humidity, as previously describe by Hietz (2010), and resistant to that one that remained with its form unchanged.

Pleopeltis pleopeltifolia (Raddi) Alston, *Polypodium hirsutissimum* (Raddi) de la Sota and *Polypodium polypodioides* var. *minimum* (Bory) Kuhlm & Kuhn, presented characteristics of tolerance to desiccation, while *Microgramma squamulosa* (Kaulf.) de la Sota has been considered drought avoider (see article one).

In spite of the co-occurrences, not always the four species overlap in the same host tree, such as: *Ligustrum lucidum* W.T. Aiton. (Oleaceae Family), *Melia azedarach* L. (Meliaceae Family) and *Jacaranda mimosaefolia* D. Don (Bignoniaceae Family). *Polypodium polypodioides* occur preferably over the shaft and rarely over the bulkier basal ramifications

of the host tree, mostly covering a vast area. On the other hand the other three species prefer more apical ramifications and differ from this according to the habit. *Polypodium hirsutissimum* and *Pl. pleopeltifolia* are species that are characterized by having a short stem-reptile while *Microgramma* has long-stem reptile, allowing to this one a more vast occupation in the phorophyte than the others. Amongst the studied species, *P. hirsutissimum* differed by its bigger leave and by the presence of a great quantity of trichomes, while *P. polypodioides* var. *minimum* was the species with the smallest foliar area (Table 1).

The species were identified by specialist and the *vouchers* were entrusted to the Herbal ICN of the Department of Botany / Institute of Biological Sciences of the Universidade Federal do Rio Grande do Sul (UFRGS), under the registry numbers: ICN 182858 (*P. polypodioides* var. *minimum*); ICN 182859 (*P. hirsutissimum*); ICN 182860 (*Pl. pleopeltifolia*) and ICN 182861 (*M. squamulosa*).

Collect of Material

The collects were realized in two different occasions during the month of November/2010 in Porto Alegre, RS, Brazil (30º03'23"S - 51º13'15"O). In the first collect fronds were used to desiccation process and in the second collect they are used for the rehydration process. Only mature and well hydrated fronds of the four species cited above were collected, totalizing 180 fronds by species, excepting *P. polypodioides* for which the number of fronds needed to be doubled due to its little foliar area. Right after being taken from the host tree, the fronds were packed in plastic bags and taken to the Seeds Technology Laboratory of the Fundação Estadual de Pesquisa Agropecuária (FEPAGRO).

Table 1. General characteristics of the sampled species. Observations and measurements we made during a dehydration period. ab = apical branches; bb = basal branches, Bo = bole. Values of leaf area are based on 10 individuals from each species.

Species	Location on host	Leaf curling	Trichomes	Leaf area (cm^2)
P. polypodioides var. *minimum*	Bo, bb	whole frond	-	2.64
P. hirsutissimum	bb, ab	Partial	+	28.97
Pl. pleopeltifolia	bb, ab	whole frond	-	22.77
M. squamulosa	Bo, bb, ab	None	-	6.67

Sample proceeding

The relative content of water (RCW), the content of total phenols and soluble sugar, as well the index of fluorescence were evaluated in different gaps of time during the phases of desiccation and rehydration of the fronds in three conditions of humidity. These values were expressed in a relative way, considering 100% the highest measured value.

Desiccation and rehydration process

In both processes the fronds were transferred from the plastic bags to paper envelopes to facilitate the separation between the gaps of time and the different relative humidity. The fronds exposed to the process of desiccation were submitted to three different conditions of relative humidity (RH): 0% (silica in desiccator placed in a dry chamber), 60-70% (ambient temperature) and 100% (germinator with controlled temperature and humidity). Different gaps of time were used for each of humidity level: 0, 12, 24, 36 and 48 hours for the desiccation, with the measurements related to time zero to those realized right after the collect. The fronds used for the rehydration were previously exposed to 0% RH/48h in the desiccator with the finality of resembling the physiological conditions of the fronds to those that passed by the process of desiccation. After this period, the fronds were immersed in deionized water in the following gaps of time: 0, 2, 4, 6, 8 e 24 hours. In each of these gaps of time, the same measurements were realized in the desiccation, with the related to time zero were realized right after the fronds have been taken from the dissector.

Only the central region of the fronds was evaluated, except by *P. polypodioides* for which option was to evaluate the entire front due its reduced size, avoiding the excessive collection of leaves.

Relative Water Content (RWC)

At each of the time intervals (t) during the dehydration and rehydration processes, the fresh weight (FW) of frond sections was measured, followed by their immersion in deionized water for 24 hours in order to determine the turgid weight (TW). They were then oven-dried (70°C/24h) for evaluation of the dry weight (DW). The RWC was calculated according to the equation (Barrs, 1968): RWC (%) = {[(FW or FW_t) – DW] / (TW – DW)} x 100.

Total soluble sugar

The total soluble sugar was determined using the phenol-sulfuric method adapted (Dubois *et al*., 1956). The material was oven-dried (60°C/48h) and extraction was made in 80% ethanol at 75°C from 10mg of dry plant tissue. The absorbance of the solution was read in spectrophotometer (SpectraMax Plus384 Absorbance Microplate Reader) in the wavelength of 620 nm. Glicose was used as standard sugar for calibration curve. The content of soluble sugar was calculated using the following linear equation based on the equation of the calibration curve: $y = 0{,}241x - 0{,}029$, where y is the absorbance and x is the content of soluble sugars in $mg.g.DW^{-1}$.

Total phenols

The total phenolic content was determined using the Folin-Ciocalteau method (Bärlocher and Graça, 2005) with tannic acid as standard. The material was oven-dried (60°C/48h), grinded and about 100mg of tissue were used to make the measurements in both desiccation and rehydration process. The extraction occurred in 5 ml of acetone for 1 hour at 4°C. The absorbance of the solution was read in spectrophotometer (SpectraMax Plus384 Absorbance Microplate Reader) in the wavelength of 760 nm. The content of phenols was calculated using the following linear equation based on the equation of the calibration curve

(r^2 = 0,985): $y = 1{,}766x - 0{,}007$, where y is the absorbance and x is the content of phenol in $\mu g.g^{-1}$.

Chlorophyll fluorescence

The determination of chlorophyll *a* fluorescence was carried out using a portable fluorometer (Opti-Science/OS-30p) on intact leaves. The data were collected in four fronds/species of each different gaps of time during desiccation process and rehydration. During fluorescence measurements the temperature was maintained constant (~25°C). F_0, the minimum fluorescence of dark-adapted leaves, was measured in fronds dark-adapted by covering the part to be measured with a leaf clip for at least 20 min, while maximal fluorescence yield (F_m) was determined after exposure to a 0.8 s saturating flash of light. Maximal photochemical PSII yield (Y_0) was calculated as $Y_0\,(F_v/F_m) = (F_m - F_0)/F_m$, where F_m is the maximal fluorescence of dark-adapted leaves during a saturation pulse (Maxwell and Johnson, 2000; Hietz and Briones, 2001). Maximal PSII photochemical efficiency (FV/FM) was estimated as the ratio of variable (FV) to maximal fluorescence yield (Schreiber et al., 1994).

Statistical analysis

Data collected during the desiccation process were submitted to three-way ANOVA, for comparison of the four species at different periods of time (five levels) and relative humidity conditions (three levels). In process of rehydration was used two-way ANOVA, for comparison between the four species and six different periods of time.

Fischer's test ($P \leq 0.05$) was used to mean separation procedure in the process of desiccation and rehydration. Statistical analyses were performed using Sigma Plot statistical program, version 11.0 (SPSS Inc.).

RESULTS

Patterns of reduction in relative water content (RWC) during the desiccation period varied among species and relative humidities to which fronds were exposed (Fig. 1). At time zero, most species had a RWC around 70-80%, except for *Pl. pleopeltifolia* that differ statistically from the other species. *Microgramma squamulosa* underwent the lowest dehydration at all RH, and its RWC did not drop much below 50%. *Polypodium polypodioides* followed the same pattern of dehydration as *M. squamulosa* at a RH of 100%, didn't differ statistically from each other in the gaps of time 0h, 12h and 24h. However its dehydration was much faster and more pronounced at lower RH, particularly at 0%, where it reached a minimum RWC of about 20%. *Polypodium hirsutissimum* exhibited a very fast initial dehydration, displayed similar patterns at all three values of RH, and reached minimum values of RWC between 30 and 40%. Finally, *Pl. pleopeltifolia* typically had the lowest RWC at all times and RH, attaining, together with *P. polypodioides*, minimum values of around 20% at the lowest RH didn't differ statistically after 48 hours of desiccation. *Polypodium polypodioides* and *P. hirsutissimum* did not differ statistically from each other throughout the desiccation process in 60-70%RH had intermediate values of RWC% when compared to other species.

The increase in RWC during rehydration was more pronounced in the fronds of *P. polypodioides*, reaching values around 80% after 8 hours. *Microgramma squamulosa* didn't differ of *P. polypodioides* and neither *P. hirsutissimum* after 24 hours of desiccation. *Pleopeltis pleopeltifolia* fronds had higher values in final rehydration than those at the beginning of the desiccation process, 70% and 40-60% respectively. The variations in fluorescence parameters, phenols contents and total sugars, were found to be dependent on RWC (Figs. 2 to 4).

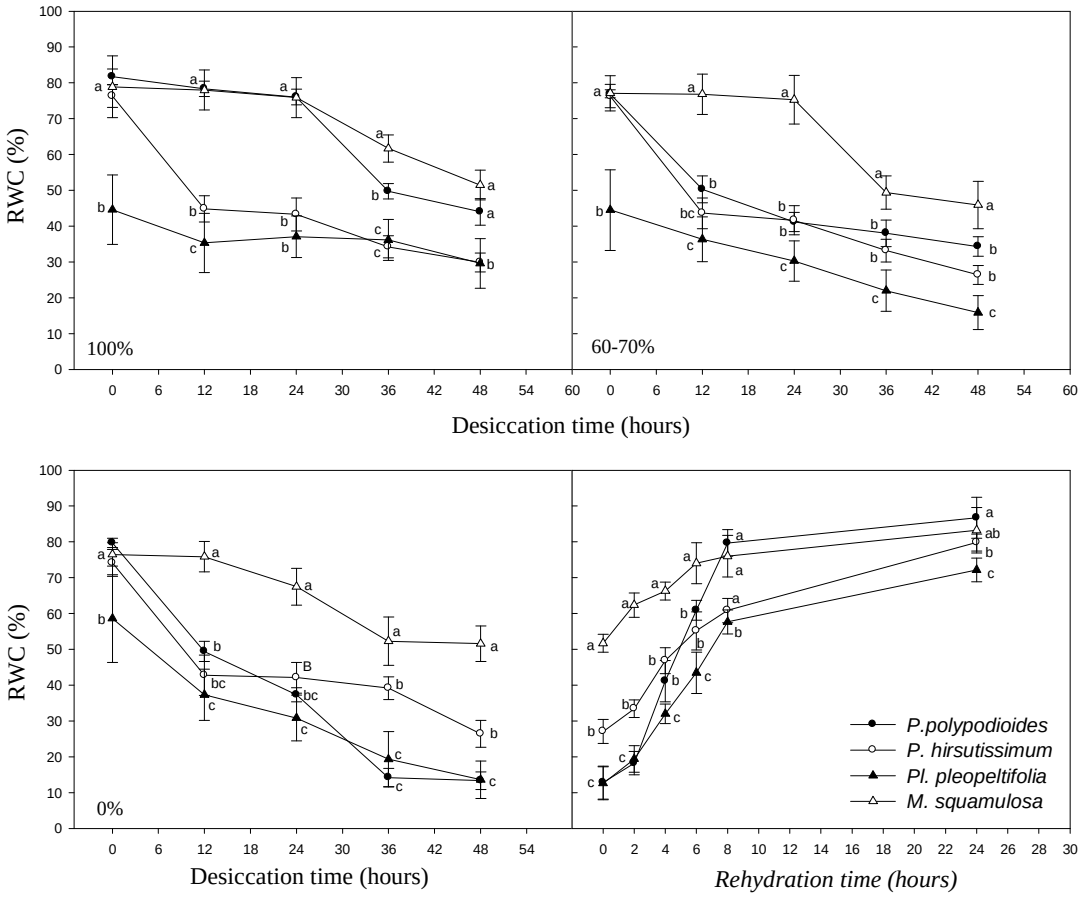

Fig. 1. Relative water content (RWC) in relation to desiccation time (hours) in three different conditions of relative humidity (0%, 60-70% e 100% RH) and in relation to *rehydration time (hours)* in fronds of the four species studied. Each point represents the average ± SE (standard error) of ten fronds. Different letters indicate significant differences between means within the same gaps of time at $P<0.05$ (ANOVA; Fischer LSD test).

There is a statistically significant interaction between species, different relative humidity and gaps of time (P<0.05) in relation to fluorescence, phenols and total sugars during desiccation and rehydration process (Table 2).

The F_v/F_m ratio doesn't seems to be affected in *M. squamulosa*, keeping values around 0.8 during desiccation and rehydration process, unlike *P. polypodioides* showed lower values in all situations (Fig. 2). The four species did not differ during desiccation at relative humidities of 60-70% and 100%. *Microgramma squamulosa* and *P. hirsutissimum* fronds did not differ in any of the intervals of time when exposed to 0%RH. *Polypodium polypodioides* and *Pl. pleopeltifolia* were the two species most affected by the decrease of RWC in relative humidity of 0% reaching values close to 0.2 and 0.4, no difference to the interval of 24 hours of desiccation and 60-70% RWC reaching values close to 0.3 and 0.4, respectively.

The Fv/Fm ratio show that the chlorophyll was ressintetizada in studied species, achivied final values in rehydration closed to initial values in desiccation process (Fig. 2). Despite the initial difference in hydration of the fronds *M. squamulosa* and *P. hirsutissimum*, 50% and 30% respectively, no statistical differences between them in any time interval. *Polypodium polypodioides* and *Pl. pleopeltilfolia* also did not differ after 4 hours of rehydration when presented approximately 30% and 40% RWC and not the other species at the end of the process (24h: p=0.539).

The total phenols content increased for the four species studied during the desiccation process, no matter if the relative humidity conditions to which the fronds were submitted (Fig. 3). *Polypodium polypodioides* and *P. hirsutissimum* showed similar patterns during early desiccation, with values below 300 µmol.g^{-1}DW after reaching some 50% RWC on their

Table 2. Summary statistics for general linear models performed on fronds trait data of *P. polypodioides* var. *minimum*, *P. hirsutissimum*, *Pl. pleopeltifolia* and *M. squamulosa* during desiccation and rehydration process. Bold values denote significant differences ($P < 0.05$). Where Sp = species, RH = relative humidity (0%, 60-70% and 100%) and GT = gaps of time (0, 12, 24, 36 and 48 hours).

Desiccation process

		F_v/F_m		Phenols		Total soluble sugars	
Variables	DF	F	*P*	F	*P*	F	*P*
Sp	3	462.5	**<0.001**	991.9	**<0.001**	393.5	**<0.001**
RH	2	117.2	**<0.001**	537.2	**<0.001**	283.9	**<0.001**
GT	4	449.9	**<0.001**	2330.7	**<0.001**	425.2	**<0.001**
Sp X RH	6	43.5	**<0.001**	54.8	**<0.001**	8.9	**<0.001**
Sp X GT	12	58.5	**<0.001**	61.8	**<0.001**	10.8	**<0.001**
RH X GT	8	15.9	**<0.001**	61.5	**<0.001**	29.9	**<0.001**
Sp X RH X GT	24	7.4	**<0.001**	11.3	**<0.001**	2.4	**0.001**

Rehydration Process

		F_v/F_m		Phenols		Total soluble sugars	
Variables	DF	F	*P*	F	*P*	F	*P*
Sp	3	354.5	**<0.001**	269.6	**<0.001**	161.9	**<0.001**
GT	5	238.4	**<0.001**	802.5	**<0.001**	291.4	**<0.001**
Sp X GT	15	47.8	**<0.001**	20.5	**<0.001**	4.6	**0.006**

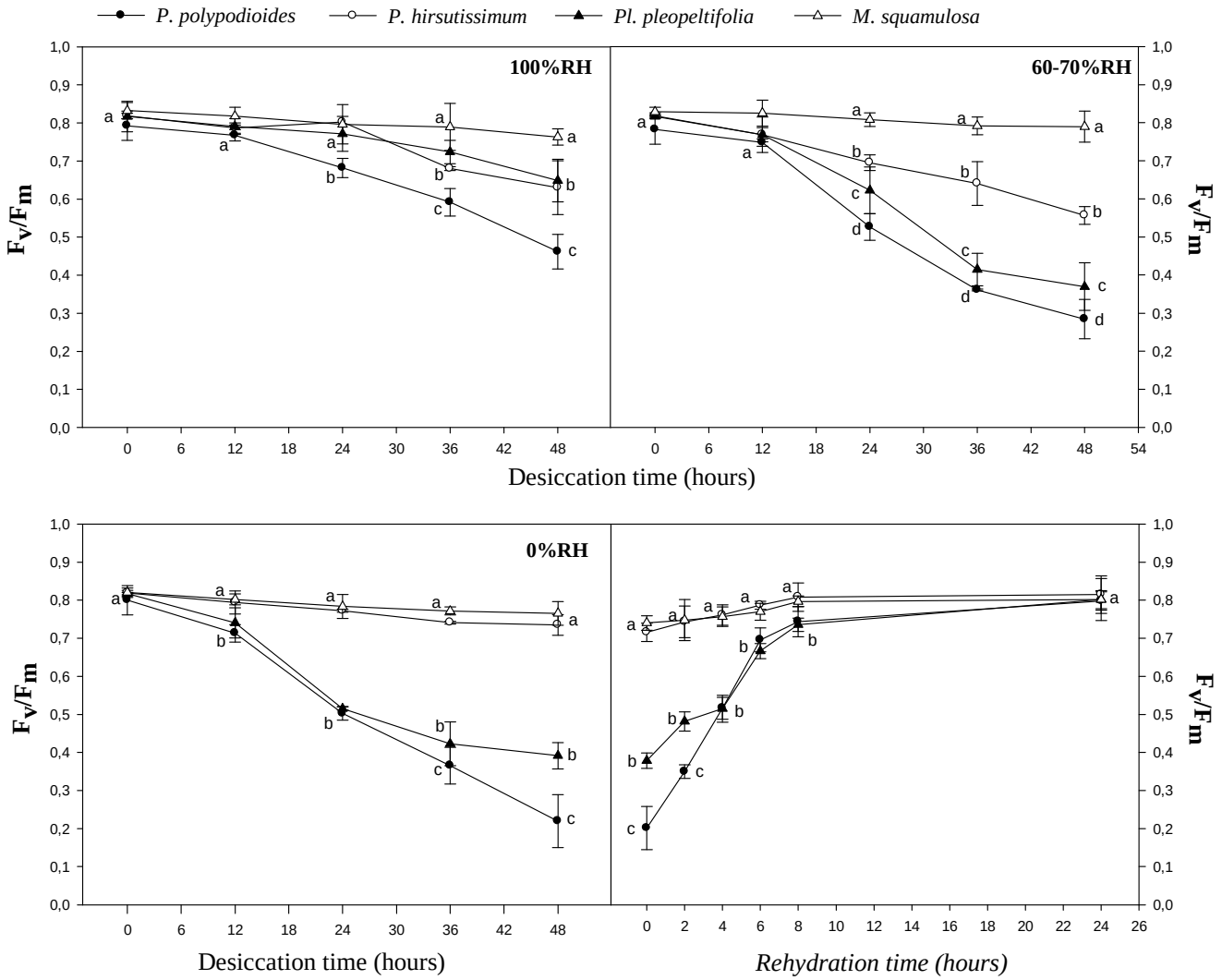

Fig. 2. Maximum quantum efficiency of PSII (F_v/F_m) during desiccation (0%, 60-70%, 100%RH) and rehydration process in relation to time (hours) in fronds of *P. polypodioides* var. *minimum*, *P. hirsutissimum*, *Pl. pleopeltifolia* and *M. squamulosa*. Each point represents the mean ± standard error of ten fronds. Different letters indicate significant differences between species means within the same gaps of time at $P<0.05$ (ANOVA; Fischer LSD test).

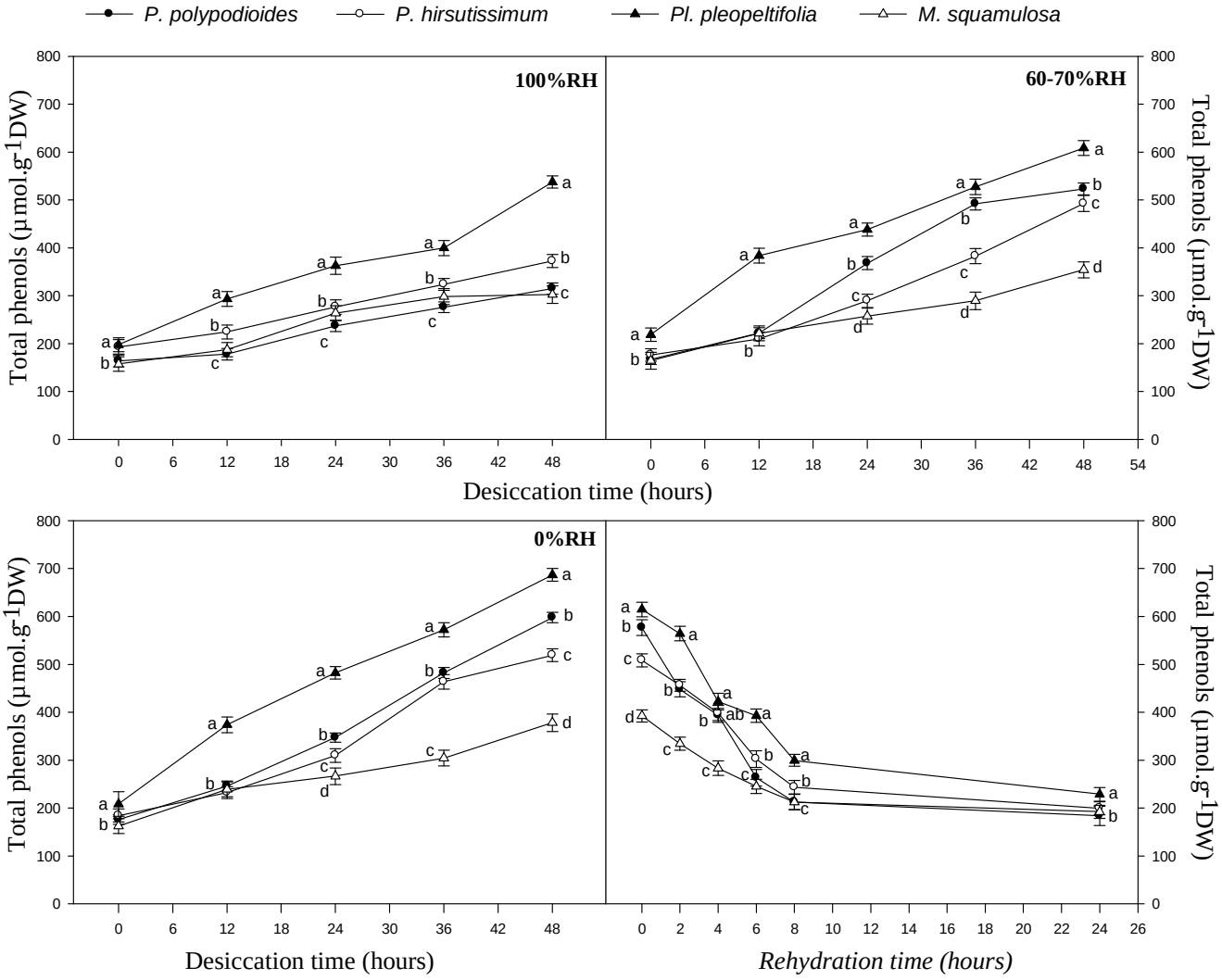

Fig. 3. Total phenols during desiccation (0%, 60-70%, 100%RH) and rehydration process in relation to time (hours) in fronds of *P. polypodioides* var. *minimum*, *P. hirsutissimum*, *Pl. pleopeltifolia* and *M. squamulosa*. Each point represents the mean ± standard error of ten fronds. Different letters indicate significant differences between species means within the same gaps of time at $P<0.05$ (ANOVA; Fischer LSD test).

fronds. *Polypodium polypodioides*, *P. hirsutissimum* and *M. squamulosa* no differ statistically in 0% UR. The accumulation of phenols in *Pl. pleopeltifolia* occurred more markedly in the three RH conditions compared the other species, reaching values above 600 µmol.g^{-1}DW the relative humidity of 0 e 60-70%. Already in *M. squamulosa* the accumulation of phenols was more pronounced up to about 75% RWC, only the largest relative humidity which their fronds were exposed, then the accumulation occurred more slowly, not exceeding 400 µmol.g^{-1}DW.

During rehydration the total content of phenols reduced for the four species, reaching around 200 µmol.g^{-1}DW the end of the process. The most pronounced decrease was observed in *P. polypodioides*, with reduced 313 µmol.g^{-1}DW six hours after rehydration and less marked in *M. squamulosa*, with reduced 147 µmol.g^{-1}DW after the same period.

As the total content of phenols, total soluble sugar for the four species increased during the dehydration process and reduced in rehydration (Fig. 4). *Microgramma squamulosa* showed the highest values and stood out from other species by having a higher accumulation of soluble sugars started soon after dehydration. This species showed a tendency to reduction in the concentration of the end of the process, showing higher values in the range 36h than at 48h, when RWC was greater than 50%. This pattern also differed from the tolerant species that showed a sharper drop of the RWC of their fronds in early desiccation and increased the accumulation of soluble sugar only after the RWC of their fronds were below 50%.

Polypodium polypodioides, *P. hirsutissimum* and *Pl. pleopeltifolia* did not differ at the beginning of dehydration in three relative humidity conditions. *Pleopeltis pleopeltifolia* had the lowest concentrations of soluble sugars in relative humidity of 0%. No significant difference was observed between species after 48 hours of dehydration. In 0% RH *M. squamulosa* didn't differ *P. hirsutissimum* nor *P. polypodioides* of *Pl. pleopeltifolia*

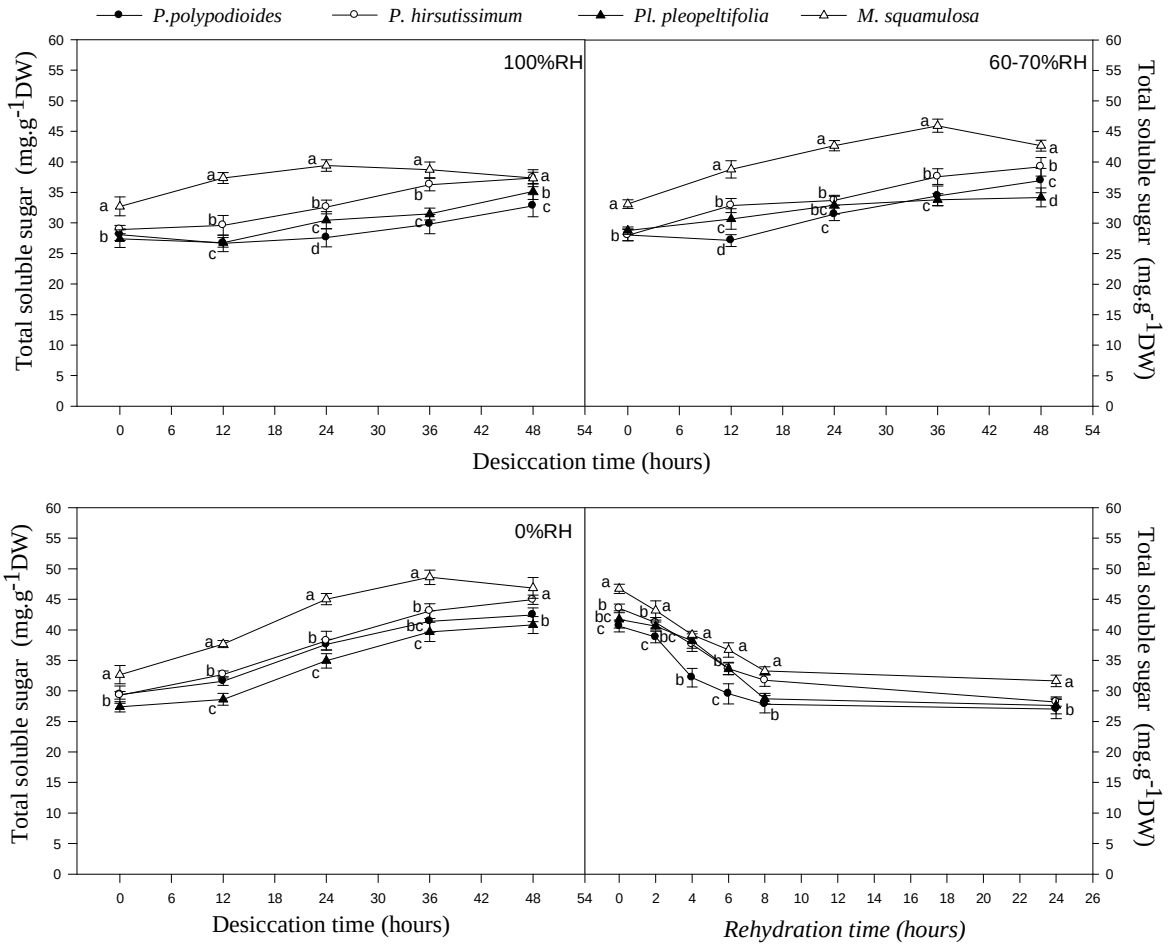

Fig. 4. Total soluble sugar concentration during desiccation (0%, 60-70%, 100%RH) and rehydration process in relation to time (hours) in fronds of *P. polypodioides* var. *minimum*, *P. hirsutissimum*, *Pl. pleopeltifolia* and *M. squamulosa*. Each point represents the mean ± standard error of ten fronds. Different letters indicate significant differences between species means within the same gaps of time at $P<0.05$ (ANOVA; Fischer LSD test).

and in 100% RH only *P. hirsutissimum* and *M. squamulosa* didn't differ.

Microgramma squamulosa showed a higher concentration of soluble sugars in both the process of desiccation and rehydration. However, unlike the other species that apparently had not yet stabilized this accumulation after 36 hours of desiccation, this species showed the beginning of a decline in its fronds when they reached about 60% RWC. This pattern also differed from the tolerant species, which showed a sharper drop of the RWC of their fronds in early desiccation and increased the accumulation of soluble sugar only after the RWC of their fronds were below 50%.

As soon begin rehydration process, there was a reduction in the concentration of soluble sugars for the four species, stabilizing the values after 8 hours. The initial concentration of total soluble sugar in frond used in the rehydration was similar to the fronds had been exposed to the conditions of 0% RH during the desiccation process. However, the species that presented the lowest values of soluble sugars during this process at all time intervals was *P. polypodioides*.

DISCUSSION

The epiphytic ferns studied exhibit different adaptations, reflecting the diversity of epiphytic habitats on single tree. From the stem base to the outer branches, increasing wind speed, radiation, temperature oscillations and vapour pressure deficit contribute to increase evaporation and transpiration (Parker, 1995). Additionally, more dead organic matter and bryophytes which store water and nutrients for rooting epiphytes are found on inner than on outer branches (Hofstede et al., 1993), thus were reinforcing the effects of microclimate. Due to all these factors, drought stress for epiphytes will be more severe in the upper canopy and on small branches (Hietz and Briones, 1998).

Although there are shared features among the four species examined, there are also significant differences in the ecological strategies and mechanisms of protection induced against mechanical and oxidative stress.

We consider that *P. polypodioides* var. *minimum*, *P. hirsutissimum* and *Pl. pleopeltifolia* are desiccation-tolerant species because they are often desiccated and curled in their natural habitat, beyond reduce levels of chlorophyll during desiccation and resynthesize after rehydration as observed in article one.

Polypodium polypodioides var. *minimum* was the species with the smallest leaf area of the four species studied, followed by *M. squamulosa*, *Pl pleopeltifolia* and *P. hirsutissimum.* According Kappen and Valladares (2007) the leaf small size is typical of the recognized frequently the shape of the poikilohydrous (lack of control of water relations).

Individuals of *P. polypodioides* var. *minimum* usually cover a large area of the host tree and as soon as there is increased moisture in the air, its leaves unfold to take advantage and capture light energy before they are shaded by other species of fronds with same behavior. Features such as the slope of the frond, the thickness of the blade, the smallest leaf area (added in the form of pinnate fronds) and shorter length of the frond can be understood as strategies to reduce the deleterious effects of light and water deficit according to Boeger et al. (2007).

The smaller leaf size in this species becomes an advantage over the other, it provides the ability to quickly reduce the RWC of their fronds by transpiration when exposed to lower RH. Associated with this feature, the absence of tissues that store water in their leaves or rhizomes, restrict their presence to wetter areas as the base and thicker branches phorophytes, as also observed by Hietz and Briones (2001) in *Trichomanes bucinatum*. Thus, *P. polypodioides* is able to take advantage of any increase in RH that occurs as a quick rain or dew.

Water uptake in poikilohydrous vascular plants can be very complex because of interactions between different organs (Kappen and Valladares, 2007). Stuart (1968) found individuals of the fern *Polypodium polypodioides* was not able to rehydrate by substrate moistening if the air was dry, and the leaves reached only 50% of their maximal water content within 2–3 days, even in a water vapor-saturated atmosphere. Similar results found for *P. polypodioides* var. *minimum* in this study, which decreased the RWC of their fronds even when they were exposed to high relative humidity conditions as 100%.

Matthes-Sears et al. (1993) found that fronds of *Polypodium virginianum*, which are highly desiccation-tolerant, were not able to absorb Deuterium-labeled water from air. Thus, the capacity of the leaves to take up water vapor varies significantly among species (Kappen and Valladares, 2007). In contrast, rapid water uptake fronds can be observed in *P. polypodioides* var. *minimum* during the rehydration and Stuart (1968) for to same species. Kappen and Valladares (2007) suggest that in pteridophytes the water uptake through leaves is an important mechanism for reestablishing water relations of the whole plant and for resuming xylem function. Furthermore, the cuticle of poikilohydrous vascular plants may also enhance water uptake by leaves although generally to be considered an efficient protection against water loss.

Although *Pl. pleopeltifolia* and *P. hirsutissimum* also present characteristics of desiccation tolerance differ from *P. polypodioides* var. *minimum* occupy a more apical branches and by having a short stem-reptile, which may occur more isolated. However, the highest location of these species leads to greater amount of sunlight, increased susceptibility to the winds and increased evapotranspiration in the driest days. Thus, strategies become necessary for these plants are able to establish and develop well in these places.

Polypodium hirsutissimum differed by its bigger foliar and by the presence a thick rhizome possibly able to assist in water storage and of a great quantity of trichomes.

According to Grammatikopoulos and Manetas (1994) trichomes in the leaf blade have been shown to contribute to water uptake and the second Kappen and Valladares (2007) they absorb water more easily than the leaf epidermis. In *Polypodium puberulum* the dense hair-like trichomes on the adaxial surface retain a water film and thus increase the period during which liquid water may be taken up (Hietz and Briones, 1998), as observe in the lower surface of the curled and folded leaflets of *Ceterach officinarum*, densely covered with trichomes (Oppenheimer and Halevy, 1962).

However, it may be that the presence of trichomes retarded water uptake because of the air trapped, as observed in leaves of several species of *Ceterach* and *Cheilanthes* (Oppenheimer and Halevy, 1962; Gaff, 1977; Gebauer, 1986) and the fronds of *P. hirsutissimum* during the drying process in this study. The reduction in RWC of the fronds of *P. hirsutissimum* was similar at three different relative humidity conditions probably due to a barrier against the ingress of water caused by the layer of air that is trapped between the leaf epidermis and trichomes. On rehydration, when the fronds were submerged in liquid water, the presence of hairs was added to the absence of air layer, resulted in rapid absorption and damage cell membranes (see article one). This same effect is observed in *R. myconi* (and of other *Ramonda* species), where spraying of the detached hairy leaves resulted in less water uptake than immersion in water or spraying hairless leaves (Kappen and Valladares, 2007).

Pleopletis pleopeltifolia besides presenting leaf area intermediate the two species of *Polypodium*, lacks trichomes, features added to the fact that occur in more apical branches in the host tree which makes it more susceptible to environmental variations. The ecological adaptation by this species, also tolerant to desiccation, seems to be the ability to completely expand their fronds with RWC around 50%, as observed in fronds collected at the beginning of this study. The lowest percentage of RWC in *Pl. pleopeltifolia* possibly represents a great advantage, because it enhances the area for light incidence and the realization of

photosynthesis, while the others require higher RWC for the complete expansion of its fronds (personal observation).

Microgramma squamulosa fronds become more exposed to environmental changes due to long-stem reptile that allowing this one to a more broad occupation, from base to apical regions of the host tree. Unlike the other three species of this study, *M. squamulosa* have thick leaves and thick rhizomes that appear to serve as a water reservoir, does not curl their fronds in low RH (Hietz, 2010; see article one), characterized as drought avoider.

The three species tolerant to desiccation of this study showed reductions in the fluorescence during desiccation in different proportions. *Polypodium polypodioides* var. *minimum* showed the greater reduction F_v/F_m in the three relative humidity conditions which their fronds were exposed, followed by *Pl. pleopeltifolia* and *P. hirsutissimum*. The lower reduction in *P. hirsutissimum*, especially at 0% RH, was probably due to the presence of dense hair on their fronds, which promoted an additional protection to the photosynthetic pigments (see article one). It may be that low F_v/F_m reflects a constitutive photo/desiccation protective strategy that is highly expressed in desiccation tolerant plants (Csintalan et al., 1999), thus avoiding the photoinhibition (Öquist et al., 1992).

These reductions in the fluorescence caused a significant increase in the amounts of thermal energy dissipation in desiccation-tolerant species (data not shown). According to Angelopoulos et al. (1996) the induction and increase this thermal energy dissipation is associated with a down-regulation of photosynthesis and it has been considered as a photoprotective process. In some cases light-dependent inactivation of PSII reaction centres is associated with a decline in both F_m and F_v/ F_m and with an increase of initial fluorescence yield, F_o (Demmig-Adams and Adams, 1992; Long and Humphries, 1994).

Values of F_v/F_m found in M. squamulosa were around 0.8, showing that there was no photoinhibition on their fronds in any of the processes of desiccation and rehydration, as suggested by Björkman and Demming (1987) with ranged between 0.8 and 0.83.

Although *P. polypodioides* and *Pl. pleopeltifolia* presented the lowest F_v/F_m in the early process of rehydration, after eight hours had values close to the start of desiccation. Similar behavior was observed by Hietz and Briones (2001) in two other species of same genus *Polypodium plebeium* and *Pleopeltis mexicana*. These findings are consistent with previous research, such as Deltoro et al. (1998b) and Csintalan et al. (1999), suggesting that highly desiccation-tolerant bryophytes maintain rapidly reversing, regulatory photoprotective mechanisms in the desiccated state, which may be adaptive in environments with variations in the water supply.

Another protective mechanism is constituted by curling of leaves when it begins the process of desiccation Korte and Porembski (2012). *Polypodium polypodioides* var. *minimum*, *P. hirsutissimum* and *Pl. pleopeltifolia*, independent of the speed of response to desiccation, also have the ability to curl their fronds during desiccation causing shading of the inner surfaces from light and protect the photosynthetic apparatus of the high incidence of light. As observed in leaves of *Craterostigma wilmsii* and *Myrothamnus flabellifolius* by Farrant (2000).

Sherwin and Farrant (1998) proposed that desiccation-tolerant plants use leaf folding and antioxidants pigments accumulation as a means of minimizing light chlorophyll interaction and thus photo-oxidative damage. A wide range of other compounds such as flavonoids, sugars, polyols, proline and polyamines also have antioxidant properties (Smirnoff, 1993), and particularly phenols (Rice-Evans et al., 1996).

According to Kranner et al. (2002) desiccation, as other biotic and abiotic stresses, ultimately cause loss of control mechanisms that maintain low ROS concentrations.

Therefore, effective antioxidant machinery is essential trait of desiccation tolerant organisms, because the increase in ROS will lead to deteriorative processes such as ageing and eventually death (Beckmann and Ames, 1998), as is also important in the reverse process on rehydration (Smirnoff, 1993; Sherwin and Farrant, 1998; Farrant, 2000).

The role of phenols together with flavonoids is act as 'sun screen' pigments, shading the desiccated photosynthetic apparatus, and this will help avoiding O_2 formation. This appears to be particularly important in resurrection plants as suggested by Farrant et al. (2003). Antioxidative defense systems may be involved in different ways in preventing damage. These antioxidants were also maintained high in the inactive, completely dehydrated state. Hence, they may confer protection upon the onset of rehydration, when photosynthesis is still inactive and excitation pressure can be high (Farrant et al., 1999).

The increase observed in the phenol concentration in the four species of this study added to that of flavonoids (see article one) gave an important antioxidant protection during desiccation. As observed by Farrant et al. (1999) in leaves of the resurrection *Ramonda serbica* that possess different photoprotective mechanisms allowing them to withstand profound dehydration during drought periods, maintaining the integrity of metabolism.

Desiccation-tolerant plants also have the ability of a membrane mechanical stabilization by means of compatible solutes (Vander Willigen et al., 2003; Djilianov et al., 2011), like soluble sugars that are involved in signaling processes and plant stress tolerance (Solfanelli et al., 2006). According to Khurana et al. (2008) soluble sugars promote vitrification as water is removed from cells while the glass phase prevents collapse of cells by maintaining the macromolecular structures. Recently also has been recognized that sugar signalling is involved in scavenging of reactive oxygen species (ROS) (Bolouri-Moghaddam et al., 2010), perhaps in coordination with phenolic compounds (Van den Ende and Valluru, 2009).

According to Allison et al. (1999) in water deficit conditions sucrose interacts with polar head groups of membrane phospholipids, thus preserving the membrane native structure and preventing leakage due to phase transition. Furthermore, soluble sugar interacts with proteins to form hydrogen bonds with specific polar groups on the protein surface, not allowing the formation of hydrogen bonds within the proteins themselves that would irreversibly change their tertiary structure.

Polypodium polypodioides var. *minimum*, *P. hirsutissimum* and *Pl. pleopeltifolia* increased the concentration of soluble sugars when their fronds were exposed to desiccation and decreased after rehydration. This same pattern of accumulation soluble carbohydrate was observed by Muller et al. (1997) in *Ramonda nathaliae* and *R. myconi*, indicating that soluble carbohydrate accumulation is connected to desiccation tolerance in Gesneriaceae. In a study of several species of resurrection, Ghasempour et al. (1998) found a wide variation that can occur in the accumulation of sugars, as already mentioned *Ramonda myconi* and *Tripogon jacquemontii* that presented, respectively, 47.9 / 7.5 mg sugar / g DW in leaves hydrated and 52.8 / 35.9 mg sugar / g DW in dried leaves. In the three species tolerant to desiccation of this study, the difference between the accumulation of sugars from the beginning to the end of the dehydration process increased with decreased relative humidity to which the fronds were exposed, revealing the importance of these solutes in the protection of cell integrity in response to changes of humidity that occur in their habitat.

Microgramma squamulosa showed the same patterns of accumulation of soluble sugars and reducing that species tolerant of this study. However, these levels were higher in all conditions which their fronds were exposed. These patterns may possibly have taken place in order to protect the cellular integrity of the species, since it is considered drought avoider and showed a slight reduction in chlorophyll content during the drying process, in rehydrated quickly recovered as demonstrated in the article one.

In summary, our results showed that during desiccation the fronds of four species study were able to recover when exposed to conditions of extreme desiccation with the damage of the photosynthetic apparatus limited to a repairable level and the physiological integrity maintained in the dry state enabling full recovery after rehydration.

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CONSIDERAÇÕES FINAIS

Apesar das pteridófitas serem mais comuns em ambientes úmidos e sombreados, muitas espécies também são encontradas em habitats propensos à seca, ou em ecossistemas áridos ou em locais com suprimento de água descontínuo, como galhos ou rochas onde a capacidade de estocagem é muito baixa (Hietz 2010). As espécies de hábito epifítico apresentam uma grande desvantagem quanto aos recursos hídricos quando comparadas as espécies terrestres, pois não têm acesso a água disponível no solo. Entretanto, muitas pteridófitas epifíticas adquiriram ao longo da evolução a capacidade de tolerar a dessecação (Zotz & Hietz 2001), característica que as coloca em vantagem na ocupação deste habitat em relação às outras espécies. Todavia, a tolerância à dessecação pode depender de diferentes estratégias fisiológicas e bioquímicas realizadas pelas plantas para sobreviver e recuperar os processos metabólicos normais após a re-hidratação (Bewley & Krochko 1982).

Nesse sentido, os resultados deste trabalho indicam que aquelas pteridófitas epifíticas que apresentaram um evidente enrolamento foliar durante períodos de baixa disponibilidade hídrica, também demonstraram respostas fisiológicas protetoras mais proeminentes do que a espécie sem este comportamento. Além disso, essas plantas possuem a capacidade de recuperar o seu metabolismo após re-hidratação, confirmando-as como espécies tolerantes à dessecação.

Nas pteridófitas epifíticas tolerantes estudadas foi possível a detecção de diferentes graus de tolerância. *Polypodium polypodioides* var. *minimum* foi a espécie mais tolerante, seguida de *Pleopletis pleopeltifolia* e *Polypodium hirsutissimum*. Enquanto *Microgramma squamulosa* demonstrou evitar à dessecação, com padrões de respostas fisiológicas aos processos de dessecação e re-hidratação, não tão proeminentes quanto às espécies tolerantes e,

também, pela capacidade de estocagem de água em seus tecidos, favorecendo-a em períodos de baixa disponibilidade hídrica.

A área foliar influenciou a velocidade de resposta das espécies as variações na disponibilidade hídrica do ambiente. *Polypodium polypodioides* var. *minimum* que possui a menor área foliar apresentou resposta mais rápida, enquanto a outra espécie de mesmo gênero, *Polypodium hirsutissimum*, de área maior e uma grande quantidade de tricomas, respondeu mais lentamente.

Apesar de ter ocorrido redução nos teores de clorofila durante a desidratação, não houve à ausência deste pigmento em nenhuma das espécies, classificando-as como homoclorofilas (plantas que mantêm a clorofila durante a dessecação). Todavia, estas reduções ocorreram em diferentes escalas. *Polypodium polypodioides* var. *minimum* foi a espécie com maior redução nos teores de clorofila, seguida de *Polypodium hirsutissimum* e *Pleopeltis pleopeltifolia* com padrões intermediários e *M. squamulosa* com os maiores teores.

O aumento na concentração de flavonóides durante a dessecação revelou sua ação protetora ao aparato fotossintético nas quatro espécies. *Microgramma squamulosa*, ao contrário das espécies tolerantes, não reduziu o teor de flavonóides quando suas frondes foram expostas a re-hidratação, podendo este pigmento também apresentar ação protetora como anti-fúngico, conforme Grayer & Harborne (1994).

As mudanças fisiológicas ocorridas nas espécies tolerantes à dessecação foram fundamentais para a preservação da estrutura das membranas celulares tanto durante o processo de dessecação quanto na re-hidratação. As três espécies tolerantes estudadas, quando expostas a diferentes condições de disponibilidade hídrica, demonstraram índices de injúria mais baixos às membranas do que *M. squamulosa*, espécie que evita à dessecação.

A redução nos teores de clorofila foi acompanhada pela diminuição da fluorescência nas espécies tolerantes à dessecação quando expostas às situações de estresse hídrico.

Polypodium polypodioides var. *minimum*, foi a espécie com maior redução dos teores de clorofila e com os menores índices de fluorescência entre as espécies estudadas, situação oposta a *M. squamulosa* que não apresentou alterações significativas.

Assim como já foi revelado o papel protetor dos flavonóides ao aparato fotossintético, os teores totais de fenóis reafirmam esta ação protetora aumentando sua concentração durante o processo de dessecação e reduzindo na re-hidratação, quando esta proteção já é assumida pela ação de outros pigmentos, como os carotenóides.

As membranas celulares das espécies tolerantes à dessecação sofreram menos injúrias durante períodos de estresse hídrico, em decorrência de uma maior síntese de açúcares solúveis totais em comparação com espécies sensíveis. Entretanto, ficou evidenciado que em *M. squamulosa*, espécie que evita à dessecação, que os açúcares solúveis totais também têm importante papel protetor das estruturas celulares.

A partir destas informações, podemos inferir que estas quatro espécies apresentam diferentes estratégias ecofisiológicas tanto para facilitar a co-ocorrência delas em um mesmo forófito, otimizando a captura de luz em períodos mais favoráveis para cada uma, quanto em relação às eficientes respostas metabólicas frente às variações hídricas as quais estão diariamente expostas.

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