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Morphometric differences of *Microgramma squamulosa* (Kaulf.) de la Sota (Polypodiaceae) leaves in environments with distinct atmospheric air quality

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ABSTRACT

Plants growing in environments with different atmospheric conditions may present changes in the morphometric parameters of their leaves. *Microgramma squamulosa* (Kaulf.) de la Sota is a neotropical epiphytic fern found in impacted environments. The aims of this study were to quantitatively compare structural characteristics of leaves in areas with different air quality conditions, and to identify morphometric parameters that are potential indicators of the effects of pollution on these plants. Fertile and sterile leaves growing on isolated trees were collected from an urban (Estância Velha) and a rural (Novo Hamburgo) environment, in Rio Grande do Sul, Brazil. For each leaf type, macroscopic and microscopic analyses were performed on 192 samples collected in each environment. The sterile and fertile leaves showed significantly greater thickness of the midrib and greater vascular bundle and leaf blade areas in the rural environment, which is characterized by less air pollution. The thickness of the hypodermis and the stomatal density of the fertile leaves were greater in the urban area, which is characterized by more air pollution. Based on the fact that significant changes were found in the parameters of both types of leaves, which could possibly be related to air pollutants, *M. squamulosa* may be a potential bioindicator.

Key words: atmospheric pollution, bioindicator, epiphytism, fern, leaf anatomy.

INTRODUCTION

The leaf is the part of the plant that shows the greatest plasticity in response to environmental variations. Therefore, it is able to change its structure to adapt to a specific environmental condition (Dickison 2000). The stomata is the main route of entry of pollutants into plants (Bobrov 1955). Pollutants may cause metabolic, physiological, and anatomical damage (Rocha et al. 2004). Leaf size and thickness and stomatal density are the major morphological and anatomic features which show more differences among plants growing in environments with different atmospheric conditions (Sharma and Tyree 1973, Eleftheriou 1987, Alves et al. 2001, 2008). These leaf parameters may determine if the plant is tolerant or sensitive to urban pollutants (Pedroso and Alves 2008, Cabrera et al. 2009).

Epiphytes are efficient air pollution indicators because they absorb chemicals directly from the atmosphere (Elias et al. 2006). Ferns constitute a group of plants that deserve special attention in the epiphytic environment, considering that it has

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been estimated that 2,600 fern species around the world are epiphytes (Kress 1986). Polypodiaceae is a pantropical family containing about 1,200 species that represent most epiphytic ferns (Smith et al. 2006). Floristic surveys have often reported this family as one of the richest in terms of epiphytes (Kersten and Silva 2002, Dittrich and Waechter 2005, Schmitt and Windisch 2010). These plants' anatomy and morphology vary widely because of their ability to adapt to very different environmental conditions (Dubuisson et al. 2009). Ferns are highly abundant in areas of high humidity, with some species being drought tolerant (Kessler and Siorak 2007, Kreier et al. 2008, Dubuisson et al. 2009, Peres et al. 2009).

Microgramma squamulosa (Kaulf.) de la Sota (Polypodiaceae) is a neotropical epiphytic fern commonly found in Peru, Bolivia, Brazil, Argentina, Paraguay, and Uruguay (Tryon and Stolze 1993). It is often found in trees of primary and secondary forests, but it also grows on isolated trees in anthropic environments, including public parks in the urban area of cities (Sehnem 1970, Gonçalves and Waechter 2003, Blume et al. 2010, Schmitt and Goetz 2010). This species has a long rhizome from which the petioles of dimorphic fertile and sterile leaves arise (Sehnem 1970). Anatomical studies on *M. squamulosa* have mainly focused on the taxonomic differences and structural features related to the medicinal action of this species (Hirsch and Kaplan 1974, Suffredini et al. 1999, 2008, Jaime et al. 2007). Recently, Rocha et al. (2013) described and compared the anatomical characteristics of the dimorphic leaves of *M. squamulosa* specifically with the purpose of defining those characteristics that contribute to adaptations in the epiphytic environment.

Bioindicators constitute an important parameter that in addition to traditional methodologies provide environmental diagnostics with information about the negative synergistic effects of pollutants on living organisms (Markert 2007, Merlo et al. 2011). In Brazil, among the native species indicative of pollution *Tillandsia usneoides* (L.) L. has been used due to a morphological adaptation to remove substances from the atmosphere through its scales (Figueiredo et al. 2004). *Tradescantia pallida* (Rose) D.R. Hunt var. *purpurea* Boom, an introduced species well adapted to sub-tropical and tropical climates is considered to be an efficient bioindicator of atmospheric pollution due to its high sensitivity to genotoxic agents (Ma et al. 1994, Costa and Droste 2012).

Considering that *M. squamulosa* is a native species commonly found in environments with different levels of anthropic activity, the objectives of the present study were: (i) to quantitatively compare macroscopic and microscopic structural characteristics of fertile and sterile leaves in rural and urban areas with different air quality conditions, and (ii) to identify morphometric parameters that are potential indicators of the effects of pollution on these plant leaves.

MATERIALS AND METHODS

STUDY AREA

Fertile and sterile leaves of *M. squamulosa* growing on isolated trees were collected from two environments (urban and rural) in the State of Rio Grande do Sul (RS), Brazil. The predominant regional climate is classified as Cfa type according to Köppen, being humid-temperate, with rainfall throughout the year (Moreno 1961).

The urban environment is a public park located downtown in the municipality of Estância Velha (29°39’05” S and 51°10’24” W, alt 40 m). This municipality has about 42,000 inhabitants, and 98% of its total area is urban (21.6 km^2). The main economic activity in the region is focused on leather and the footwear industry. The vehicle fleet consists of 13,264 cars, 758 trucks, and 156 buses (IBGE 2012).

The rural environment is located in the municipality of Novo Hamburgo, in an Area of Special Environmental Interest (29°46’51” S and 50°58’31” W, alt. 55 m). The total area of the rural environment comprises 148.3 km^2 and the economic activities in the region are geared towards leisure and tourism, family agriculture and farming (Schütz 2001).

On the year of sample collection, as well as in the three previous years, the atmospheric air of the urban environment where the leaves of *M. squamulosa* were collected showed mean concentrations of total suspended particulates (TSP) with up to 50 µm between 37.17 and 53.99 µg m^{-3}, inhalable particles up to 10 µm in diameter (MP 10) between 29.76 and 32.48 µg m^{-3}, and sulfur dioxide (SO_2) up 13.09 µg m^{-3}, according to the state foundation for environmental protection (FEPAM 2009). High genotoxic potential of the atmospheric air in the same location was detected by biomonitoring using *Tradescantia pallida* (Rose) D.R. Hunt var. *purpurea* Boom. Mean frequencies of up to 8.13 micronuclei were detected in the meiotic tetrads. Conversely, in the rural area where the leaves of *M. squamulosa* were also collected*, T. pallida* var. *purpurea* showed significantly lower frequencies of micronuclei (up to 1.26). Thus, this environment was classified as a white spot (Costa and Droste 2012).

SAMPLING

In June 2009, six isolated host trees (phorophytes) covered by extensive rhizomes of *M. squamulosa* were selected in the rural and urban areas, respectively. Considering each leaf type (sterile and fertile), 192 samples were collected, 32 from each phorophyte. The leaves were collected from the internal area of the phorophyte canopy, where they received sunlight from the East and were exposed to a range of luminosity from 22.99 to 38.73 µmol m^{-2} s^{-1}. Voucher material was deposited in the *Herbarium* Anchieta of the Universidade do Vale do Rio dos Sinos (PACA 108022, 108023), in São Leopoldo, RS, Brazil.

MACROSCOPIC AND MICROSCOPIC ANALYSES

Macroscopic analyses of 120 leaves of each type were performed. These leaves were digitalized using a desktop scanner connected to a computer. The leaves were dehydrated in an oven at 65°C until reaching constant mass. The sclerophylly index was calculated according to Rizzini (1976). The other 72 leaves of each type were used for microscopic analyses. An area of 25 mm^2 in the midline portion of the leaves was selected and fixed in FAA 70 for 48 hours (Johansen 1940) and stored in 70% ethanol (Berlyn et al. 1976) until processing. The permanent slides of cross-sections were obtained after 36 samples of fertile leaves and 36 samples of sterile leaves were embedded in methacrylate (HistoResin™, Leica), as described by Feder and O'Brien (1968), and according to the manufacturer's instructions. The samples were embedded transversally. Samples were sectioned at a thickness of 7 µm using a rotary microtome (Leica RM 2125 RT) with disposable blades (Leica 818). The sections were stained with 0.05% toluidine blue (Sakai 1973) and mounted in synthetic resin (Entellan™, Merck). The semi-permanent slides of paradermal sections were obtained after dissociation (Franklin 1946) of other 36 leaves of each type, which were later stained with 0.05% toluidine blue (Sakai 1973), mounted in 50% glycerin, and luted with clear nail polish (Purvis et al. 1964). Slides were mounted with epidermis samples of the two faces of the leaves to classify the leaves according to the occurrence of stomata.

Sections of permanent and semi-permanent slides were digitalized using a photomicroscope (Olympus CX 41) coupled to a DC 3000 camera (Micrometrics™) and software Micrometrics SE Premium® 2.9. The sections were described according to Van Cotthem (1970), Ogura (1972), White (1974), Sen and Hennipman (1981), and Rocha et al. (2013).

After digitizing the macroscopic and microscopic images, thicknesses (leaf blade, epidermis, hypodermis, midrib, and sclerified layer), areas (leaf blade, vascular bundle, and stomata), and stomatal density were calculated using the Micrometrics SE Premium® 2.9 software, according to the method adapted from Godoi et al. (2010) and Santos et al. (2010). The thickness of the hypodermal tissue was calculated as the mean of the thickness of the adaxial and abaxial hypodermis of each leaf. Stomatal density was analyzed after the software provided a random definition of 1 mm^2 areas for each paradermal section, and one quadrant per section was examined for each leaf.

The statistical software SPSS version 20 was used to compare the quantitative parameters of each leaf type between the rural and urban areas. The Shapiro-Wilk test was applied to confirm normal data distribution. As the hypothesis of normal distribution was rejected, the Mann-Whitney test was used, and the level of significance was set at 5%.

RESULTS

The structure of the fertile and sterile *M. squamulosa* leaves collected in the rural and urban areas showed no qualitative differences. The front view of leaves revealed epidermal cells with sinuous walls in the adaxial and abaxial faces of leaves. Anomocytic stomatal complexes were only observed in the abaxial face (Fig. 1A, B).

The cross-section revealed uniseriate epidermis on both faces, followed by 1-2 layers of hypodermic cells. In the hypodermis, the mesophyll had homogeneous chlorenchyma and small vascular bundles. In the midrib region, the vascular bundle was wrapped in a sclerified layer, whose cells were filled with brownish contents. Inside this layer, we observed the endodermis and the pericycle. The phloem was located externally to the xylem (Fig. 1C, D).

The sterile and fertile leaves showed significantly greater thickness of the midrib and greater vascular bundle and leaf blade areas in the rural environment, which is characterized by less air pollution. Additionally, the sterile leaves presented significantly greater thickness of the epidermis on the adaxial face, as well as greater areas of the stomata in this environment. No significant quantitative variations in terms of thickness of the hypodermis, epidermis on the abaxial face, and sclerified layer, as well as stomatal density and sclerophylly index between both environments with different air quality conditions were found (Table I).

Conversely, the thickness of the hypodermis and the stomatal density of the fertile leaves were greater in the urban area, which is characterized by more air pollution. There were no significant quantitative variations in terms of thickness of the epidermis on the adaxial and abaxial faces, sclerified layer, and leaf blade, as well as the stomatal area between both environments with different air quality conditions (Table II). Both leaf types were classified as sclerophyllous in the urban and rural areas.

DISCUSSION

The structure of the fertile and sterile leaves of *M. squamulosa* showed no qualitative variations between the environments with different air quality conditions. There were only quantitative structural changes between the same type of leaves collected from different environments.

Variations in the conduction system were detected in fertile and sterile leaves, which showed lower thickness of the midrib and smaller area of the vascular bundle in the environment with higher levels of pollution. A minor diameter of the conducting elements related to increased air pollution was found in the needle-like leaves of *Picea abies* (L.) H. Karst. (Masuch et al. 1992) and in the leaves of *Tradescantia* clone 4430 (Alves et al. 2001). Whenever plants are subjected to abiotic stress, smaller diameter vessels increase safety in the transport of sap (Alves et al. 2001).

In the polluted environment, the adaxial face of the epidermis and the parenchyma of the sterile leaves showed significant reduced thickness, contributing to the decrease in total leaf thickness.

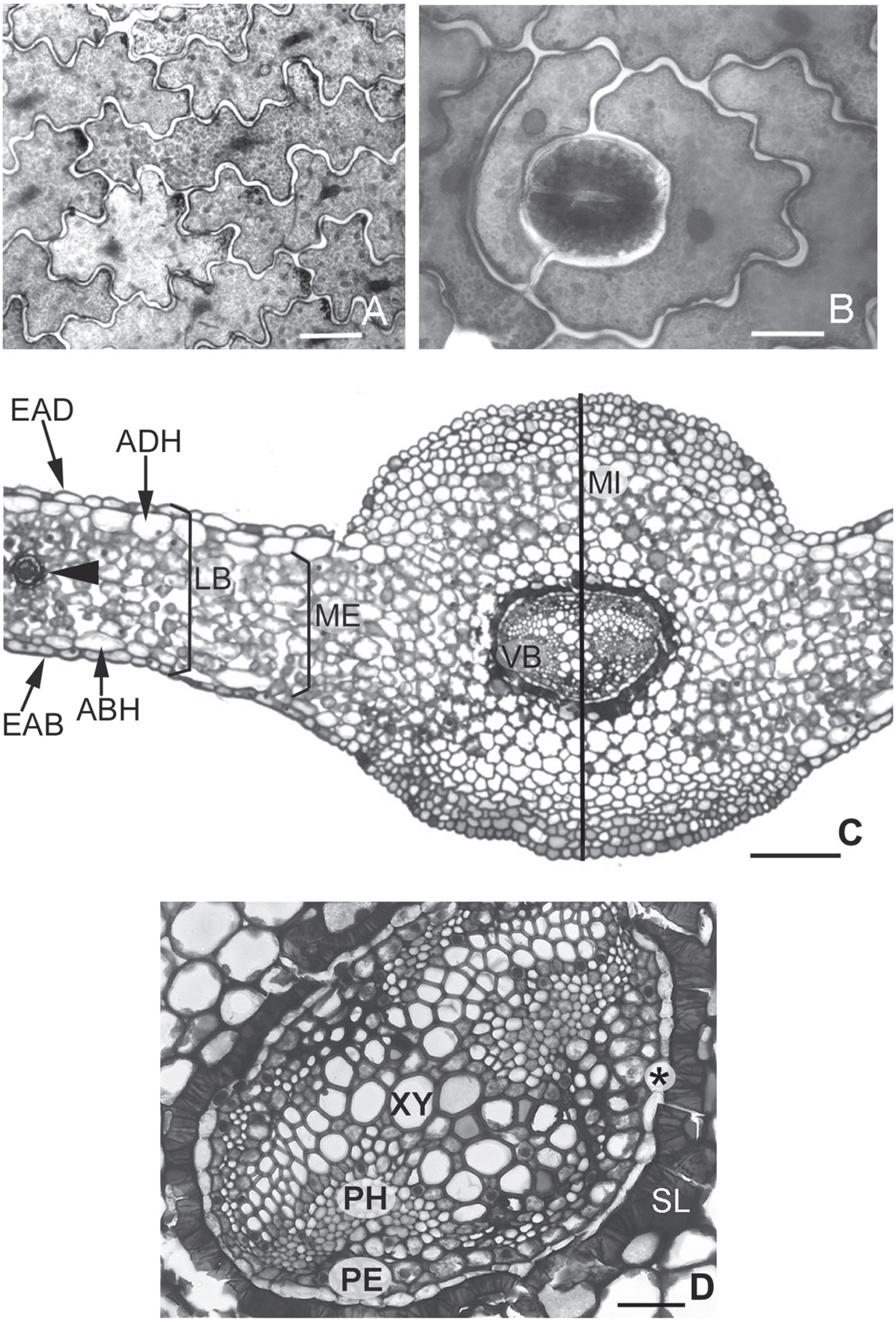

Figure 1 - Leaf sections of *Microgramma squamulosa*. **A.** Epidermis adaxial face. Bar = 20 µm. **B.** Epidermis abaxial face: anomocytic stomatal complex. Bar = 15 µm. **C.** Leaf blade (LB) in cross-section evidencing: epidermis adaxial face (EAD), epidermis abaxial face (EAB), adaxial hypodermis (ADH), abaxial hypodermis (ABH), mesophyll (ME), midrib (MI) and vascular bundle area (VB and arrowhead). Bar = 100 µm. **D.** Midrib: detail of sclerified layer (SL), endodermis (*), pericycle (PE), phloem (PH) and xylem (XY). Bar = 10 µm.

TABLE I
Values (mean, minimum and maximum) of morphometric parameters of *Microgramma squamulosa* sterile leaves in urban and rural environments (N=number of measured leaves per environment; SD=standard deviation; U=Mann Whitney test; P=probability).

	Parameters	N	Urban environment			Rural environment			U	P
			Minimum	Mean ± SD	Maximum	Minimum	Mean ± SD	Maximum		
Thickness (µm)	Epidermis-adaxial face	36	8.98	12.93 ± 3.60	23.09	7.62	18.39 ± 5.15	30.81	253.0	<0.001
	Hypodermis	36	13.34	29.33 ± 9.87	54.40	0.00	27.60 ± 19.90	77.65	2453.5	0.678
	Epidermis-abaxial face	36	2.00	15.09 ± 4.87	25.81	9.00	16.82 ± 3.83	28.00	536.0	0.212
	Sclerified layer	36	16.00	37.38 ± 16.12	81.30	21.83	38.75 ± 9.61	60.67	511.5	0.124
	Parenchyma	36	48.75	146.38 ± 45.59	220.91	18.16	182.45 ± 35.63	235.54	346.0	<0.001
	Leaf blade	36	112.97	233.63 ± 56.91	341.24	199.82	271.42 ± 41.96	369.01	329.0	<0.001
	Midrib	36	214.70	425.94 ± 89.12	612.28	261.73	514.57 ± 104.05	726.52	338.0	<0.001
Area	Vascular bundle (x 10^2 µm^2)	36	6.31	84.86 ± 36.17	168.99	51.68	227.89 ± 310.85	1653.24	332.0	<0.001
	Stoma (x 10^2 µm^2)	36	4.69	8.55 ± 2.12	14.30	4.26	25.62 ± 16.87	60.30	207.0	<0.001
	Leaf blade (cm^2)	120	1.69	10.10 ± 3.84	34.08	5.88	14.80 ± 6.21	32.40	3797.0	<0.001
Stomatal density (stomata mm^{-2})		36	17	25.97 ± 5.24	37	12	27.75 ± 6.68	39	537.5	0.217
Sclerophylly index (g cm^{-2})		120	120	0.004 ± 0.002	0.023	0.002	0.133 ± 0.341	2.012	6682.0	0.351

The thickness of the chlorenchyma in the leaves of *Tillandsia stricta* Sol. ex Sims, an epiphytic bromeliad, decreased in the presence of higher concentrations of volatile organic pollutants commonly found in urban areas (Godoi et al. 2010). Comparing leaves of olive trees in rural and urban areas, Eleftheriou (1987) observed a reduction in the mesophyll of those leaves collected in the urban environment. The same fact was observed by Evans et al. (1996) in herbaceous species of *Rudbeckia laciniata* L., *Rubus canadenses* L. and *Sassafras albidum* (Nutt.) Nees exposed to ozone. *Tradescantia* clone 4430 had thinner leaves when subjected to high concentrations of primary pollutants (Alves et al. 2001). The leaves of *Eugenia uniflora* L. were also thinner in the environment with the highest level of pollution. The decrease in intercellular spaces may be an adaption to hinder the displacement of gaseous pollutants within the leaf, consisting of an adaptive strategy to the environment with large amounts of toxic gases (Alves et al. 2008). The fertile leaves of *M. squamulosa* also showed a trend to parenchymal compression; however, this was not translated into a significant statistically difference between the two environments.

In both environments, the fertile and sterile leaves of *M. squamulosa* were classified as sclerophyllous leaves, suggesting an ability to reduce excessive water loss (Sobrado and Medina 1980). In addition, the hypodermis of the fertile leaves was statistically thicker in the urban environment. Hypodermal tissue in *M. squamulosa* is a typical characteristic of xeromorphic leaves (Rocha et al. 2013). In epiphytes, this tissue is considered the most common structure responsible for water storage (Madison 1977).

There was a significant reduction in leaf area in the fertile and sterile leaves of *M. squamulosa*

TABLE II

Values (mean, minimum and maximum) of morphometric parameters of *Microgramma squamulosa* fertile leaves in urban and rural environments (N=number of measured leaves per environment; SD=standard deviation; U=Mann Whitney test; P=probability).

Parameters		N	Urban environment			Rural environment			U	P
			Minimum	Mean ± SD	Maximum	Minimum	Mean ± SD	Maximum		
Thickness (μm)	Epidermis-adaxial face	36	7.28	14.45 ± 4.49	25.08	7.00	16.98 ± 5.51	28.65	483.5	0.062
	Hypodermis	36	16.30	33.00 ± 11.74	62.44	0.00	26.98 ± 22.10	85.93	1963.0	0.027
	Epidermis-abaxial face	36	9.10	18.25 ± 5.33	33.42	5.00	19.65 ± 18.88	126.12	583.0	0.461
	Sclerified layer	36	18.44	42.47 ± 14.99	72.45	14.00	38.18 ± 16.85	90.03	525.5	0.174
	Leaf blade	36	142.76	252.06 ± 44.75	322.39	157.80	250.41 ± 47.62	336.64	639.0	0.916
	Parenchyma	36	57.08	167.19 ± 39.62	246.22	123.94	236.38 ± 47,68	260.22	526.0	0.169
	Midrib	36	315.25	475.64 ± 71.03	632.41	269.85	534.75 ± 119.23	799.16	412.0	0.013
Area	Vascular bundle (x 10^2 μm^2)	36	18.40	110.70 ± 156.96	1009.99	54.71	144.22 ± 631.71	324.24	288.0	<0.001
	Stoma (x 10^2 μm^2)	36	3.67	23.89 ± 16.89	64.21	2.72	25.01 ± 17.56	60.38	631.0	0.853
	Leaf blade (cm^2)	120	3.08	8.18 ± 2.60	16.64	2.88	10.05 ± 3.96	22.24	5309.5	<0.001
Stomatal density (stomata mm^{-2})		36	17	25.67 ± 5.68	37	12	21.97 ± 3.48	28	433.5	0.011
Sclerophylly index (g cm^{-2})		120	0.001	0.010 ± 0.014	0.048	0.002	0.103 ± 0.262	1.238	6910.0	0.587

collected in the urban environment. In *Liquidambar styraciflua* L., Sharma and Tyree (1973) found a decrease in the leaf length of trees exposed to high levels of primary pollutants, especially particulates. Fares et al. (2006) reported reduced leaf size of *Populus alba* L. indirectly exposed to ozone. Alves et al. (2008) found a significant decrease in the leaf length and width of *E. uniflora* in plants exposed to heavy traffic areas, with great loads of particulate matter and primary pollutants (sulfur dioxide, nitrogen oxides, carbon monoxide) in São Paulo, Brazil. These findings showed that under polluted environments plants invest less in increasing leaf area.

Stomatal density increased significantly in the fertile leaves of plants exposed to higher concentrations of pollutants, which was also demonstrated in tree species (Masuch et al. 1992, Päänkkönen et al. 1997, Alves et al. 2008, Cabrera et al. 2009). Stomatal density increases and stomatal pore surface decreases due to increasing levels of air pollution optimizing the closure efficiency of the stomata (Balasooriya et al. 2009). Such increase was not found in sterile leaves. Stomatal density was not statistically different in both environments, but the stomatal area was approximately three times smaller in the more polluted area. *Tradescantia* clone 4430 showed reduced stomatal size on the abaxial face of the leaf epidermis without an increase in its frequency in highly polluted areas in the city of São Paulo. This may suggest plant adaptation to polluted environments (Alves et al. 2001) since the formation of smaller stomata could minimize the uptake of pollutants and reduce the water loss (Balasooriya et al. 2009). Smaller stomata may have been observed only in sterile leaves because sterile leaves have a longer life span than fertile leaves in ferns with dimorphic leaves (Farrar et al. 2008). Fertile leaves die soon after releasing their spores, whereas sterile leaves, whose main functions include the photosynthesis and vegetative growth of the plant, tend to live longer in the environment (Lee et al. 2009).

The results support the idea that morphometric differences in *M. squamulosa* leaves reflect different air quality conditions. This species may be considered a potential bioindicator, with the advantages of being native and widely distributed in the Neotropics, commonly found in urban and rural environments. Based on the fact that significant changes were found in some parameters of both types of leaves, such as the size of the vascular cylinder and leaf blade and the thickness of the midrib, which are possibly related to the concentration of air pollutants, the findings indicate that the species respond in a measurable manner to differences in the quality of atmospheric air. Controlled studies involving active exposure to pollutants may contribute to increase the knowledge about *M. squamulosa* as a potential bioindicator of atmospheric air pollution.

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RESUMO

Plantas crescendo em ambientes com diferentes condições atmosféricas podem apresentar mudanças nos parâmetros morfométricos de suas folhas. *Microgramma squamulosa* (Kaulf.) de la Sota é uma samambaia epifítica neotropical encontrada em ambientes impactados. Os objetivos deste estudo foram comparar quantitativamente características estruturais de folhas em áreas com diferentes condições de qualidade do ar e identificar parâmetros morfométricos que são indicadores potenciais dos efeitos da poluição sobre estas plantas. Folhas férteis e estéreis crescendo sobre árvores isoladas foram coletadas de ambientes urbano (Estância Velha) e rural (Novo Hamburgo), no Rio Grande do Sul, Brasil. Para cada tipo foliar, as análises macroscópicas e microscópicas foram realizadas em 192 amostras coletadas em cada ambiente. As folhas estéreis e férteis apresentaram nervura central significativamente mais espessa e feixe vascular e áreas da lâmina foliar maiores no ambiente rural, que é caracterizado por menor poluição do ar. A espessura da hipoderme e a densidade estomática das folhas férteis foram maiores na área urbana, que é caracterizada por uma poluição maior do ar. Baseado no fato de que foram encontradas mudanças significativas nesses parâmetros em ambos os tipos de folhas, que são possivelmente relacionadas com poluentes do ar, *M. squamulosa* pode ser uma potencial bioindicadora.

Palavras-chave: poluição atmosférica, bioindicador, epifitismo, samambaia, anatomia foliar.

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