Structural Analysis of *Tillandsia usneoides* L. Exposed to Air Pollutants in São Paulo City–Brazil

Edenise Segala Alves · Bárbara Baêsso Moura · Marisa Domingos

Abstract Bioindicators plants are important for the evaluation of air quality and *Tillandsia usneoides* L., an atmospheric epiphyte bromeliad, has been used for this purpose. The present study aims at evaluate the structural pattern of the leaf of this species when exposed to urban air pollutants, and determining whether the leaves present structural parameters that could be used as indicators of such pollutants. Samples of *T. usneoides* were exposed in São Paulo, the biggest city of Brazil, for 8, 16 and 24 weeks, and compared with others kept in a rural area. The urban pollution of São Paulo affected the structure of the leaves of *T. usneoides* causing alterations, especially in the scales, density of stomata and epidermis thickness. Qualitative alterations in the mesophyll were not observed in plants exposed at the polluted sites. These structural characteristics of *T. usneoides* seem to account for its high tolerance to heavy metal accumulation. The percentage of anomalous scales may potentially be used as an alternative bioindicator parameter.

Keywords Bioindication · Bromeliads · CAM · Leaf anatomy · Scales · Urban air pollution

E. S. Alves (✉) · B. B. Moura · M. Domingos
Instituto de Botânica,
Caixa Postal 3005, 01061-970 São Paulo, São Paulo, Brazil
e-mail: ealves@ibot.sp.gov.br

1 Introduction

The main air pollutants emitted by motor vehicles in urbanized areas are particles of different sizes and composition, gases such as nitrogen, sulfur and carbon oxides, volatile organic compounds (VOCs), as well as photochemical oxidants such as ozone, peroxyacetyl nitrate (PAN), aldehydes, cetones, peroxides (Sawyer et al. 2000). In those areas, the concentration of primary pollutants in the air generally diminishes during the night, since the traffic of vehicles, the main pollutant source, slows down. Regarding secondary pollutants, especially ozone, the highest levels are found between 10 A.M. and 4 P.M (CETESB 2004).

According to Klumpp et al. 2001, the use of bioindicator plants is suitable to detect the effects of atmospheric pollutants on living organisms, by showing characteristic symptoms, although it does not replace the physico-chemical measures that indicate their concentrations in the environment. Therefore, bioindicator plants are complements to those methods.

There are many plant species known as biomonitors, due to their high capacity of accumulating heavy metals and hydrocarbons (Arndt and Schweizer 1991; VDI 1999; Falla et al. 2000). Among the tropical species, representative epiphytes from the Bromeliaceae family are under evaluation. From those, the *Tillandsia* genus has been receiving special attention. This genus is found all over Latin America, living independently from the soil, on trees or on inert

substrates, absorbing nutrients by scales present on the leaf surface (Brighigna et al. 1997). Some studies already indicated the potential of *T. usneoides* for biomonitoring heavy metals (Calasans and Malm 1997; Amado Filho et al. 2002; Figueiredo et al. 2007). This species inclusively was proposed by Arndt and Schweizer (1991) as a universal accumulative bioindicator, since it is an air epiphyte that depends exclusively on the atmosphere for its survival, being part of the atmospheric bromeliads (Brighigna et al. 1997).

According to Benzing et al. (1976), root development in this plant is sporadic or virtually non-existent, occurring only at the germination stage. For these reasons, this species is especially suitable for air pollution monitoring, since the symptoms presented are clearly independent from soil conditions, making it easier to establish patterns for biomonitoring studies.

Furthermore, *Tillandsia usneoides* is a plant with CAM metabolism (crassulacean acid metabolism) according to Loeschen et al. (1993), which consists of fixation of CO_2 during the night, when the plant opens its stomata. Thus, the plant has its stomata closed during the day and gas exchange does not occur when the concentration of atmospheric pollutants is high, due to the intense traffic of vehicles. Moreover, Amado Filho et al. (2002), exposing the species to atmospheric Hg, verified that this element was present in relatively lower amounts in the epidermal cell layer than in scales or in stem surfaces and no Hg was detected in the inner parts of the plant, including the mesophyll parenchyma, the sclerenchymatous fibres or in the vascular system.

Considering *Tillandsia usneoides* potential for biomonitoring, the present study aims at evaluating the structural pattern of this species, with emphasis on the leaf surface, when exposed to urban air pollutants, and test the hypothesis that the leaves present anatomical parameters that could be used as alternative indicators of such pollutants.

2 Materials and Methods

2.1 Plant Material and Exposure sites

Tillandsia usneoides L. (Bromeliaceae) was collected in a public park in Paranapiacaba, state of São Paulo–Brazil. That region is predominantly occupied by por-

tions of the Atlantic Forest and is not affected by urban air pollutants. Samples were exposed in the city of São Paulo that is situated in a lower region of the South American Atlantic Plateau, occupying an area of approximately 500 km^2, with an altitude of 715–900 m above sea level. According to the Köppen system, its climate is Cwa type with annual rainfall around 1,300 mm (Setzer 1966). Three polluted sites in São Paulo City were chosen. Two of them situated in avenues exposed to air pollutants produced by heavy traffic of cars, buses and trucks, specially SO_2, NOx, CO, PM10: the first site was at Bandeirantes Avenue, near the "Congonhas" air quality monitoring station of CETESB (the governmental agency for air quality control); the second site was at Doutor Arnaldo Avenue, near the "Cerqueira César" monitoring station, next to the Public Health School of São Paulo University. The last site chosen was Ibirapuera Park, near a monitoring station with the same name, where high concentrations of O_3 are generally registered.

Tillandsia plants were also placed in a rural area with low influence of traffic in Caucaia do Alto, a city 50 km far from São Paulo. According to the Köppen system, the climate of Caucaia do Alto is Cfb type, temperate, without dry season (average temperatures of the hottest month below 22°C and of the coldest month below 18°C) and with an annual rainfall of approximately 1,500 mm (Setzer 1966). Although the air quality data for the rural site is not available, CETESB considers the air as clean and non-polluted. Guimarães et al. (2003) confirmed the satisfactory air quality at this rural site by means of instantaneous colorimetric techniques in test tubes coupled to an aspiration pump (Dräger-Lubeck, Germany). They detected mean values of ozone around 40 $\mu m\ m^{-3}$ and 0 ppm of CO.

Each sample for exposure was composed of 5 g of entangled green mass of plants packed in a polyethylene net bag (1 cm mesh). Each plant inserted in this sample was about 20 cm length. The samples were hanged in a gyrator apparatus (six bags per apparatus and three apparatus for each site), which turned according to the wind direction, so that a homogenous exposure to air contaminants was guaranteed. The exposure time was from January to July of 1999. Every 8 weeks, one apparatus with six composed samples was collected. Average levels as well as maximum and minimum values of air pollutants during

these periods in the urban sites, hereafter referred to as Congonhas, Cerqueira César and Ibirapuera, are shown in Fig. 1. SO$_2$, NO$_x$ and PM10 were in higher concentrations at Congonhas and Cerqueira César and O$_3$ at the Ibirapuera Park. Higher levels of air pollutants from vehicular emissions were observed in March to July and of O$_3$ in January to March/1999.

2.2 Microscopy Methods

The samples were immediately fixed in FAA$_{70}$ (formalin and acetic acid: alcohol 70%) remaining in this solution for 48 h; afterwards, six samples of each site in each period of exposure, were stored in alcohol 70%. One fragment of the apical third of the leaves (0.5 cm from each sample), which grew during the exposure, were embedded in polyethylene glycol 2000 according to the technique described by Richter (1981). Cross-sections of each fragment with 10–15 μm were stained with safranin-astra blue 9:1 and permanently mounted on microscope slides with cover slips using Permount®.

Epidermis thickness was measured using a microscope equipped with a camera for image capturing and a semi-automatic measuring system (Olympus Model: BX41-BF-III) along with image analysis software: Image-Pro Express 4.0.1-Media Cybernetics.

Additionally, temporary slides were prepared with the epidermis of the apical third of leaves of all the samples. Fragments of the leaves in longitudinal section were immersed in a 1:1 mixture of acetic acid and hydrogen peroxide diluted in distilled water at 80%, remaining in a stove at 60°C for 24 h, later to be filtered and washed, then stained with 1% safranin and 1% astra blue and mounted on glycerin. Scale and stomata densities were determined in this material. Densities were established by projecting the image of the leaf epidermis on a 0.5 mm-side square. Forty-five different fields were evaluated in each material.

The typical scale was determined after analysis of a great amount of scales taken from the material before its exposure in São Paulo (time zero), both the reference material and that exposed in the city, based on bibliography. Afterwards, the rate of anomalous scales, defined according to the arrangement and number of cells in the external ring, was estimated in each sample, by counting the number of anomalous scales among 100 scales. The results were expressed as percentage.

2.3 Statistical Analysis

In order to determine the differences between quantitative variables, nonparametric analysis of variance (ANOVA; Kruskal–Wallis test) was applied to estimation of stomata and scale densities. The parametric ANOVA test (F test) was used to identify differences in the thickness of the epidermis cells and of percentage of anomalous scales. Once the level of significance ($P<0.05$) of the analysis was reached, Student–Newman–Keuls test of multiple comparisons was used in order to identify differences between treatments. During each period of exposure, we took into account variations in time for the same site as well as variations in the sites.

Fig. 1 Minimum, average and maximum concentration levels of air pollutants during the different periods of exposure, (1999). Data from CETESB stations. 0–8 weeks: January to March/1999; 8–16 weeks: March to May/1999; 16–24 weeks: May to July /1999. **a** PM10 (μg m^{-3}, average for 24 h), **b** NO$_2$ (μg m^{-3} maximum of 1 h), **c** SO$_2$ (μg m^{-3}, average for 24 h), **d** O$_3$ (μg m^{-3}, maximum of 1 h). Ibirapuera (*white bar*), Cerqueira César (*grey bar*) and Congonhas (*dark grey bar*)
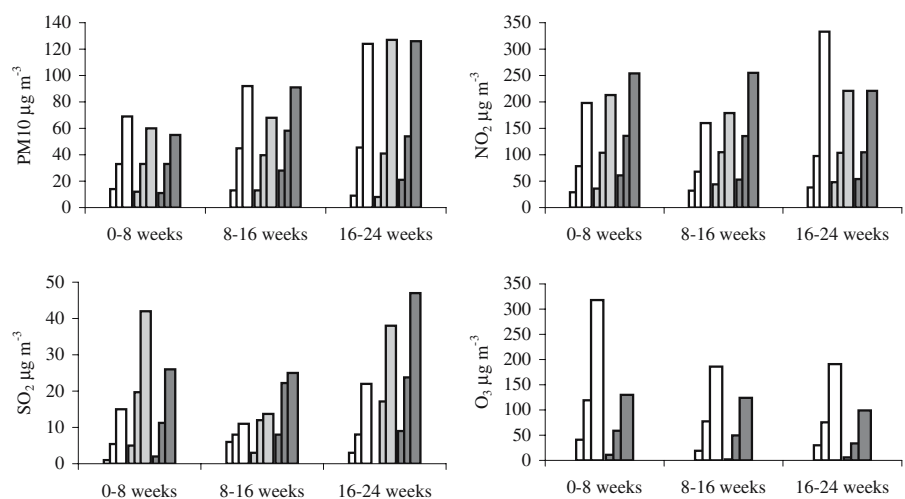
3 Results

Leaves of *Tillandsia usneoides* are needle-shaped and with an approximately semicircular cross section. The epidermis is uniseriate and composed, in transversal section, by cells of reduced dimensions and slightly thick homogeneous walls (Fig. 2a). In surface view epidermal cells have rectangular shape and straight anticlinal walls (Fig. 2b). It presents anomocytic stomata in a level lower than the other epidermis cells (Fig. 2b, c). The leaves are composed mainly of round thin walled parenchyma cells that store water. These cells differ from the chlorophyll parenchyma in that they present a larger size and absence of chloroplast. The mesophyll is compact, showing little intercellular air space.

The leaf is covered by scales composed of a stalk of live cells that are deeply inserted in the other epidermis cells, the two last ones (foot cells) being in direct contact with the mesophyll, which demonstrates its importance in the absorption of water (Fig. 2d, e). At the tip of the stalk, we find the shield composed of a central disc of four cells, all triangularly shaped, united by its sides and surrounded by two series of cells (ring cells), consisting of 8 and 16 cells respectively, concentrically displayed. Around this last series of cells, there is the shield formed by dead cells radially arranged (Fig. 2e).

The plants studied also presented anomalous scales with the last disc composed of cells in numbers other than 16 and a less concentric arrangement (Fig. 3a, b). The plants exposed at Ibirapuera presented twice as
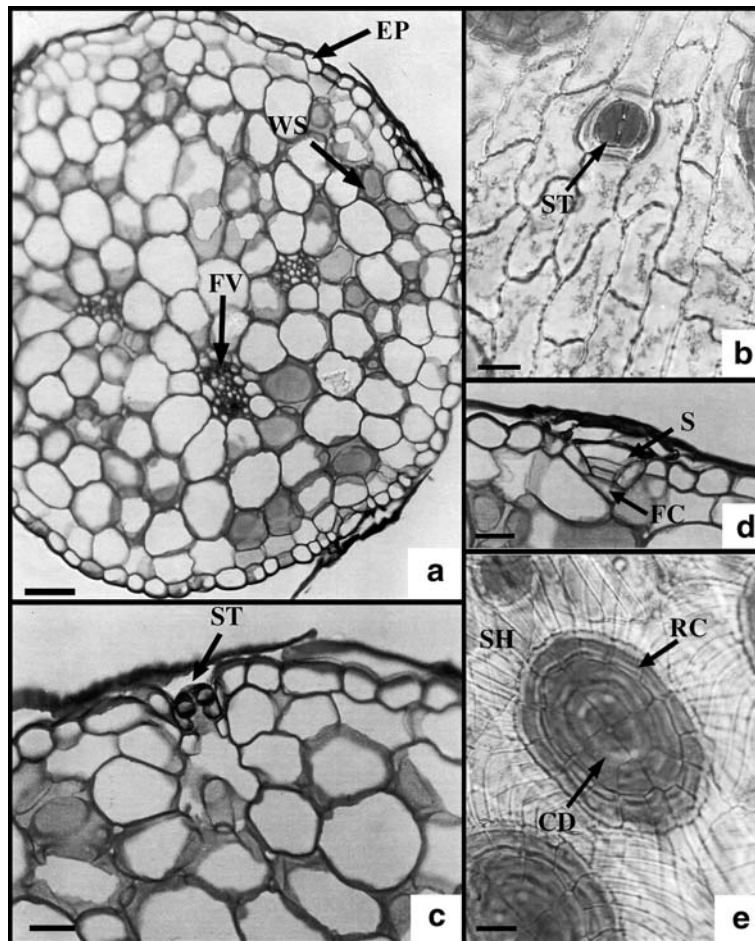

Fig. 2 a–e Apical portion of the leaf of *Tillandsia usneoides*. **a–c** Cross sections showing: fiber-vascular tissue (*FV*), water-storage cell (*WS*), epidermis (*EP*), stomata (*ST*). **b, e** General appearance of the surface of the leaf. **b** Stomata. **e** Scale showing central disc cells (*CD*), ring cells (*RC*) and shield cells (*SH*). **d** Scale in longitudinal section showing stalk (*S*) and foot cells (*FC*). Bar=50 μm in **a** and bars=20 μm in **b–e**

Fig. 3 Frontal view of the scales of *Tillandsia usneoides* showing anomalies in the ring cells (arrows). **a** Plant collected in Congonhas after 8 weeks of exposure. **b** Plant collected in the same place after 24 weeks of exposure. Bars=20 μm
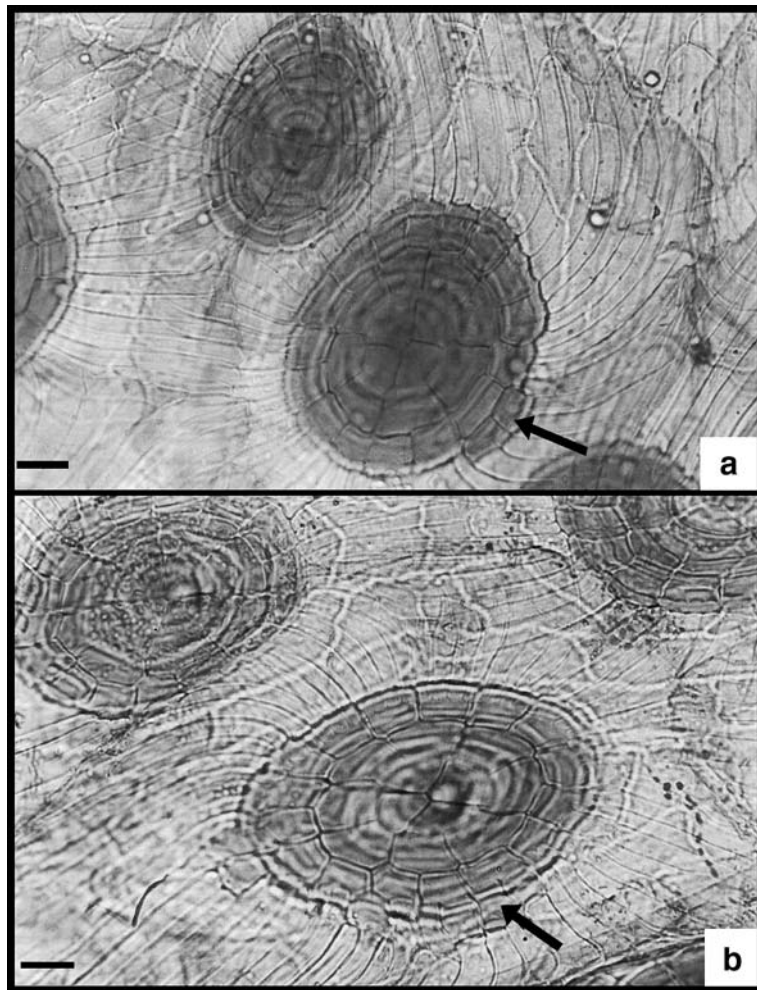
many anomalous scales when compared to those kept at the rural area (Caucaia) after all periods of exposure in situ (Fig. 4a). Significantly higher percentages of anomalous scales were also observed in plants kept at Cerqueira César and Congonhas.

Concerning other quantitative parameters measured in the epidermis, we could observe a significant decrease in the stomata density and an increase in the epidermis thickness after eight weeks in the plants exposed at the polluted sites of São Paulo, when compared to the reference site (Fig. 4c, d). No other clear trends were observed for those parameters. On the other hand, the density of scales observed in plants from Ibirapuera and from Cerqueira César was only significantly higher after 24 weeks of exposure (Fig. 4b).

The density of scales was the only quantitative parameter that decreased in leaves of *T. usneoides*

kept in Caucaia during the exposure. Other parameters did not change among exposure times. The densities of scales and of stomata and epidermis thickness were mainly altered after 24 weeks in plants from Ibirapuera and after 16 weeks in plants from Cerqueira César. No variations among exposure times were observed in plants maintained at Congonhas (Fig. 4a–d). Qualitative alterations in the mesophyll were not observed in plants exposed at different sites or different periods when compared to those kept at the rural area.

4 Discussion

Evident structural changes were observed in the scales of *T. usneoides* exposed in São Paulo, mainly in the plants exposed at the site highly affected by O_3
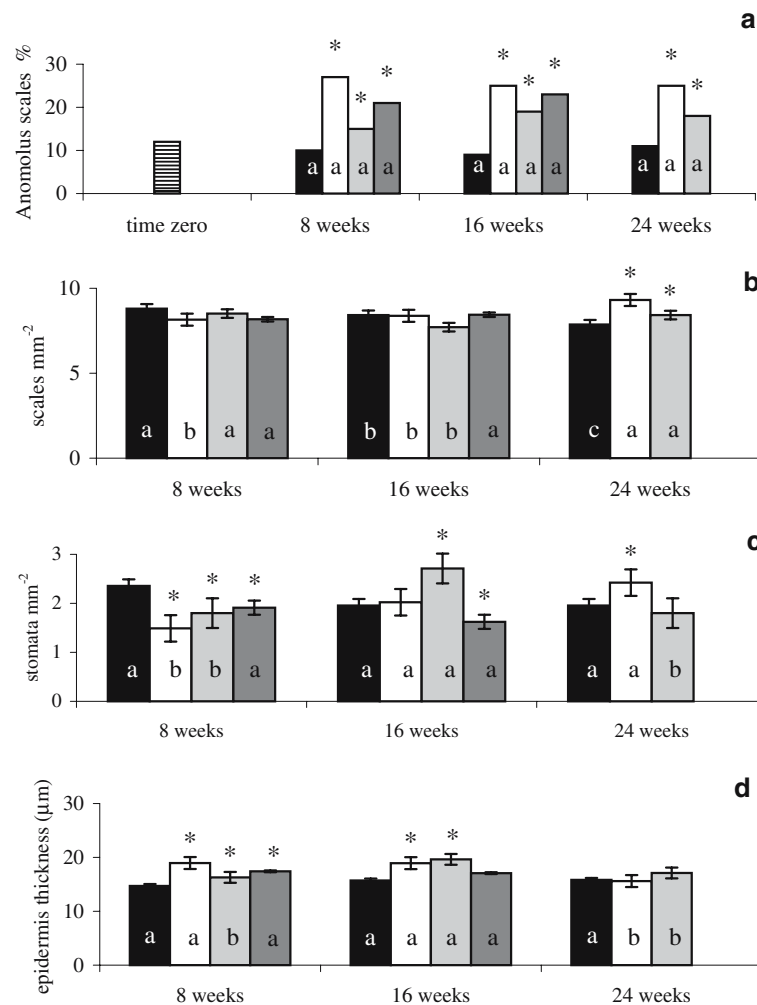
Fig. 4 Average values and standard errors (*bars*) of percentage of anomalous scales (**a**), scale density (**b**), stomatal density (**c**) and epidermis thickness (**d**) in leaves of *Tillandsia usneoides* exposed for 8, 16 and 24 weeks at a rural area. Time zero: plants collect in Paranapiacaba (*striped bar*) Caucaia (*black bar*) and three urban sites of São Paulo city -Brazil: Ibirapuera (*white bar*), Cerqueira César (*grey bar*) and Congonhas (*dark grey bar*). The averages obtained at the polluted sites marked with *asterisks*, in each exposure time, differ significantly at the $P<0.05$ from those obtained at the rural site. *Distinct letters* indicate significant variations at the $P<0.05$ among the exposure times on structural parameters in the same site

(Ibirapuera). There were changes in the central disc, resulting in a pattern different from the usual (4+8+ 16 cells), which may be attributed to oxidative effect on cellular level during the leaf development. Strehl and Arndt (1989) observed damage to the central cells of the scales could also be observed in the *T. aeranthus* e *T. recuvata* fumigated with HF and SO_2. It's believed that these observed changes are also a result of the air pollution action on the process of cell division of those cells that form the disc.

Since the preparation of the material and the visualization of the scales in *T. usneoides* is a simple process, we suggest that the percentage of anomalous

scales could be used as an alternative bioindicator parameter to the known accumulator potential of chemical elements from air pollution (Strehl and Arndt 1989; Benzing et al. 1992; Calasans and Malm 1997; Figueiredo et al. 2001; Amado Filho et al. 2002).

The variations in the number of cells in the external ring of cells of the scales were noticed after eight weeks of exposure (January to March), which shows that this interval of time is enough for observing structural changes in the epidermis cells. Likewise Brighigna et al. (2002), using tropical bromeliads (*Tillandsia* spp.) for monitoring atmospheric pollution in the town of Florence-Italy,

analyzed the species two months after the beginning of plant exposure to urban air pollution. Figueiredo et al. (2007) found increasing concentrations of some trace element in *T. usneoides* exposed during 8 weeks in São Paulo City. However, in a more stressing condition, the exposure time could be reduced. Calasans and Malm (1997) and Amado Filho et al. (2002) reported that *T. usneoides* can accumulate appreciable Hg amounts, especially in the scales, when exposed during 15 days in an electrolysis room of a chlore-alkali industry.

There are studies that relate the increase in scale density to a higher resistance to the entrance of gaseous pollutants through stomata (Sharma 1989). Others mention changes in stomata density in plants exposed to air pollutants. For example, Sharma (1989) and Matyssek et al. (1993) observed an initial decrease in stomata density in plants and Pääkköen et al. (1995; 1997) and Evans et al. (1996) registered an increase of stomata density in plants exposed to air pollutants. In C3 plants, which open their stomata during the day, the reduction of stomata density can diminish the entrance of pollutants, whereas its increase, almost always followed by a reduction of stomata size, represents a way to maximize the its closure efficiency, an important feature for plants under hydric stress and/or air contaminants (Larcher 2000).

Due to CAM metabolism, the increase in stomata density in *T. usneoides*, as observed at the polluted sites of São Paulo, does not seem to affect the entrance of pollutants in the plant, since the stomata open during the night, when the concentration of urban air pollutants is generally lower (CETESB 2004). Additionally, they are protected by scales.

The absence of qualitative changes in the mesophyll of *T. usneoides* exposed to urban pollutants in São Paulo may be explained by the high compactation of the mesophyll that characterizes this plant species, as pointed by Billings (1904) and Scatena and Segecin (2005) and has generally been associated with a lower plant sensitivity to air pollutants (Ferdinand et al. 2000, Gravano et al. 2003).

5 Conclusions

On the basis of the results obtained, we conclude that the urban pollution of São Paulo City affected the structure of the leaves of *T. usneoides*, causing alterations especially in the scales that are in direct contact with the air and that play an important role in the regulation of water and gas entrance in the plant.

The percentage of anomalous scales may potentially be used as an alternative bioindicator parameter.

References

Amado Filho, G. M., Andrade, L. R., Farina, M., & Malm, O. (2002). Hg localization in *Tillandsia usneoides* L. (Bromeliaceae), an atmospheric biomonitor. *Atmospheric Environment*, *36*, 881–887.

Arndt, U., & Schweizer, B. (1991). The use of bioindicators for environmental monitoring in tropical and subtropical countries. In H. Ellenberg, U. Arndt, R. Bretthauer, B. Ruthsatz, & L. Steubing (Eds.) *Biological monitoring signals from the environment* (pp. 199–259). Berlin: Vieweg.

Benzing, D. H., Arditti, J., Nyman, L. P., & Temple, P. J. (1992). Effects of ozone and sulfur dioxide on four epiphytic bromeliads. *Environmental Experimental Botany*, *32*, 25–32.

Benzing, D. H., Henderson, K., Kessel, B., & Sulak, S. (1976). The absorptive capacities of bromeliad thichomes. *American Journal of Botany*, *63*, 330–348.

Billings, F. H. (1904). A study of *Tillandsia usneoides*. *Botanical Gazette*, *38*, 99–121.

Brighigna, L., Papini, A., Mosti, S., Cornia, A., Bocchini, P., & Galletti, G. (2002). The use of tropical bromeliads (*Tillandsia* spp.) for monitoring atmospheric pollution in the town of Florence, Italy. *Revista de Biologia Tropical*, *50*, 577–584.

Brighigna, L., Ravanelli, M., Minelli, A., & Ercoli, L. (1997). The use of an epiphyte (*Tillandsia capuy-medusae morren*) as bioindicator of air pollution in Costa Rica. *The Science of the Total Environment*, *198*, 175–180.

Calasans, C. F., & Malm, O. (1997). Elemental mercury contamination survey in a chlor-alkali plant by the use of transplanted Spanish moss. *Tillandsia usneoides* (L). *The Science of the Total Environment*, *208*, 165–177.

CETESB Companhia de Tecnologia de Saneamento Ambiental (1999). *Relatório da qualidade do ar no estado de São Paulo*. São Paulo: Série Relatório.

CETESB Companhia de Tecnologia de Saneamento Ambiental (2004). *Relatório da qualidade do ar no estado de São Paulo*. São Paulo: Série Relatório.

Evans, L. S., Adamski II, J. H., & Renfro, J. R. (1996). Relationships between cellular injury, visible injury of leaves, and ozone exposure levels for several dicotyledonous plant species at Great Smoky Mountains National Park. *Environmental and Experimental Botany*, *36*, 229–237.

Falla, J., Laval Gilly, P., Henryon, M., Morlot, D., & Ferard, J. F. (2000). Biological air quality monitoring: a review. *Environmental Monitoring and Assessment*, *64*, 627–644.

Figueiredo, A. M. G., Nogueira, C. A., Saiki, M., & Domingos, M. (2007). Assessment of atmospheric metallic pollution in São Paulo city, Brazil, employing *Tillandsia usneoides* L. as biomonitor. *Environmental Pollution*, *145*, 279–292.

Figueiredo, A. M. G., Saiki, M., Ticianelli, R. B., Domingos, M., Alves, E. S., & Market, B. (2001). Determination of elements in *Tillandsia usneoides* by neutron activation analysis for environmental biomonitoring. *Journal of Radioanalytical and Nuclear Chemistry*, *249*, 391–395.

Ferdinand, J. A., Fredericksen, T. S., Kouterick, K. B., & Skelly, J. M. (2000). Leaf morphology and ozone sensitivity of two open pollinated genotypes of black cherry (*Prunus serotina*) seedlings. *Environmental Pollution*, *108*, 297–302.

Gravano, E., Giulietti, V., Desotgiu, R., Bussotti, F., Grossoni, P., Gerosa, G., et al. (2003). Foliar response of an *Ailanthus altissima* clone in two sites with different levels of ozone-pollution. *Environmental Pollution*, *121*, 137–146.

Klumpp, A., Ansel, W., Klumpp, G., & Fomin, A. (2001). Um novo conceito de monitoramento e comunicação ambiental: a rede européia para a avaliação da qualidade do ar usando plantas bioindicadoras (EuroBionet). *Revista Brasileira de Botânica*, *24*, 511–518.

Larcher, W. (2000). *Ecofisiologia vegetal*. São Carlos: Rima Artes e Textos.

Loeschen, V. S., Martin, C. E., Smith, M., & Eder, S. (1993). Leaf anatomy and CO_2 recycling during crassulacean acid metabolism in twelve epiphytic species of *Tillandsia* (Bromeliaceae). *International Journal of Plant Sciences*, *154*, 100–106.

Matyssek, R., Günthardt-Georg, M. S., Landolt, W., & Keller, T. (1993). Whole-plant growth and leaf formation in ozonated hybrid poplar (*Populus* x *euramericana*). *Environmental Pollution*, *81*, 207–212.

Pääkköen, E., Holopainen, T., & Kärenlampi, L. (1995). Ageing-related anatomical and ultrastructural changes in leaves of Birch (*Betula pendula* Roth.) clones as affected by low ozone exposure. *Annals of Botany*, *75*, 285–294.

Pääkköen, E., Holopainen, T., & Kärenlampi, L. (1997). Differences in growth, leaf senescence and injury, and stomatal density in birch (*Betula pendula* Roth.) in relation to ambient levels of ozone in Finland. *Environmental Pollution*, *96*, 117–127.

Richter, H. G. (1981). Anatomia des sekundarem xylems und der Rinde der Lauraceae. (Hamburg: Vereins Hamburg 5, Paul Parey).

Guimarães, E. T., Domingos, M., Alves, E. S., Caldini, N., Lobo, D. J. A., Lichtenfels, A. J. F. C., et al. (2003). Detection of the genotoxicity of air pollutants in and round the city of São Paulo (Brazil) with the *Tradescantia*-micronucleus (Trad-MCN) assay. *Environmental and Experimental Botany*, *44*, 1–8.

Sawyer, F. R., Harley, R. A., Cadle, S. H., Norbeck, J. M., Slott, R., & Bravo, H. A. (2000). Mobile sources critical review: 1998 NARSTO assessment. *Atmospheric Environment*, *34*, 2161–2181.

Scatena, V. L., & Segecin, S. (2005). Anatomia foliar de *Tillandsia* L. (Bromeliaceae) dos Campos Gerais, Paraná, Brasil. *Revista Brasileira de Botânica*, *28*, 635–649.

Setzer, J. (1966). *Atlas climático e ecológico do Estado de São Paulo*. São Paulo: Comissão Interestadual da Bacia do Paraná, Uruguai/Centrais Elétricas de São Paulo.

Sharma, K. G. (1989). Cuticular and morphological dynamics in *Salix nigra* L. and *Quercus alba* L. in relation to air pollution.. In J.B. Bucher & I. Bucher-Wallin (Eds.), *Air pollution and Forest Decline*. (pp 527–529). Birmensdorf: Proceedings of the 14th International meeting for specialists in air pollution effects on forest ecosystems, IUFRO).

Strehl, T., & Arndt, U. (1989). Alterações apresentadas por *Tillandsia aeranthos* e *Tillandsia recurvata* (Bromeliaceae) expostas ao HF e SO_2. *Iheringia Série Botânica*, *39*, 3–17.

VDI-Verein Deutscher Ingenieure. (1999). *Biological measuring techniques for the determination and evaluation of effects of air pollutants on plants. Fundamentals and aims. VDI 3975/1. VDI/DIN vol 1a*. Berlin: Handbtch Reinhaltung der Luft, Beuth.