Arch Environ Contam Toxicol (2011) 61:401–413
DOI 10.1007/s00244-010-9642-y

Antioxidant Response of Three *Tillandsia* Species Transplanted to Urban, Agricultural, and Industrial Areas

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Received: 27 July 2010 / Accepted: 30 December 2010 / Published online: 30 January 2011

Abstract To evaluate the physiological response of *Tillandsia capillaris* Ruiz & Pav. f. *capillaris, T. recurvata* L., and *T. tricholepis* Baker to different air pollution sources, epiphyte samples were collected from a noncontaminated area in the province of Córdoba (Argentina) and transplanted to a control site as well as three areas categorized according to the presence of agricultural, urban, and industrial (metallurgical and metal-mechanical) emission sources. A foliar damage index (FDI) was calculated with the physiological parameters chlorophyll *a*, chlorophyll *b*, malondialdehyde (MDA), hydroperoxyconjugated dienes, sulfur (S) content, and dry weight-to-fresh weight ratio. In addition, electrical conductivity (E-cond), relative water content (RWC), dehydration kinetics (Kin-H_2O), total phenols (T-phen), soluble proteins (S-prot), and activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase were determined. The parameters E-cond, FDI, SOD, RWC, and Kin-H_2O can serve as suitable indicators of agricultural air pollution for *T. tricholepis* and *T. capillaris*, and CAT, Kin-H_2O, and SOD can do the same for *T. recurvata*. In addition, MDA, T-phen, and S-prot proved to be appropriate indicators of urban pollution for *T. recurvata*. Moreover, FDI, E-cond, and SOD for *T. recurvata* and MDA for *T. tricholepis*, respectively, could be used to detect deleterious effects of industrial air pollution.

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Numerous studies have shown that vascular plants, mosses, and lichens can be used to assess environmental pollution either as bioindicators/biomonitors of air quality or as bioaccumulators of atmospheric pollutants (Bačkor and Loppi 2009; Szczepaniak and Biziuk 2003; Wolterbeek 2002). In general terms, bioindicators are organisms that can be used for the identification and qualitative determination of environmental factors, whereas biomonitors consist of organisms that contain quantitative aspects of the environment (Conti and Cecchetti 2001). Accumulation bioindicators/biomonitors are organisms that accumulate one or more elements and/or compounds from the environment, whereas impact bioindicators/biomonitors are organisms that demonstrate specific or unspecific effects in response to exposure to certain element, compound, or a combination of both (Markert 2007).

Conti and Cecchetti (2001) pointed out that impact bioindicators/biomonitors give information on the effect of pollutants on a living organism that could not be assessed by instrumental measurements. Moreover, instrumental monitoring generally provides accurate physicochemical analysis, but such measurements do not reflect the complex composition of the atmosphere, the possible interactions among pollutants, or its impacts on living organisms (Szczepaniak and Biziuk 2003). In relation to the physiological response, a biomarker is a measurable biological parameter at the suborganismic level (genetic, enzymatic, physiological, or morphological) in which the structural or functional changes indicate environmental influences in general and the action of particular elements, compounds, or mixtures in qualitative and sometimes also quantitative terms (Markert 2007). According to Cuny et al. (2002), an ideal biomarker should be easy to measure and should produce distinctive symptoms that can not be confused with those caused by other environmental stresses.

In this context, the rate of accumulation of S and heavy metals, geographical distributions of trace elements, and physiological changes have been studied in several lichen species in Argentina (Carreras et al. 2005; Pignata et al. 2007). Vascular plants of the *Tillandsia* genus have also been studied as biomonitors in Argentina and other countries (Bermudez et al. 2009; Pignata et al. 2002; Rodriguez et al. 2010; Wannaz et al. 2006, 2008). In Brazil, *T. usneoides* proved to be an efficient bioindicator of atmospheric pollution, which is mainly caused by the presence of heavy metals (Amado Filho et al. 2002; Figueiredo et al. 2007; Segala Alvez et al. 2008; Vianna et al. in press). Moreover, *T. caput-medusae* was used in Costa Rica (Brighigna et al. 1997), and *T. recurvata* in Mexico (Zambrano García et al. 2009), for biomonitoring airborne heavy metals. So far, the biomarkers assessed for *Tillandsia* plants exposed to air pollutants are pigment concentration and degradation, malondialdehide (MDA), water content measured as dry weight-to-fresh weight ratio (DW/FW), hidroperoxy-conjugated dienes, and a foliar damage index (FDI; Pignata et al. 2002; Wannaz and Pignata 2006).

Under stressing conditions, it has been shown that the generation of reactive oxygen species (ROS) increases in aerobic organisms (Cuny et al. 2002). All of the most important air pollutants (O_3, SO_2, and NO_x) can induce production of ROS in plants due to oxidative stress. In addition, they cause direct injury to membranes and DNA, affecting basic components of the cellular metabolism (Melhorn et al. 1990). Plants have complex antioxidant systems that play a main role in the tolerance to oxidative stress (Foyer et al. 1994; Cuny et al. 2002). Mittler et al. (2004) pointed out that superoxide dismutase (SOD), peroxidase (POs), catalase (CAT), α-tocopherol, β-carotenes, glutathione, and ascorbic acid are basic components in the defense mechanisms against ROS. Although many studies have shown that antioxidant systems may be essential as a defense mechanism of plants to atmospheric pollutants (Mittler et al. 2004), they have not been widely used as biomarkers of air pollution or heavy metals in bioindication studies (Cuny et al. 2004; Sun et al. 2010; Weissman et al. 2006). Moreover, to our knowledge, this is the first report on the antioxidant enzymatic response in bromeliads of the *Tillandsia* genus.

In previous biomonitoring surveys carried out in Argentina, we found a high physiological damage in lichens and epiphyte plants in relation to agricultural areas (Bermudez et al. 2009; Pignata et al. 2002; Wannaz et al. 2006). It is important to note that Argentina is one of the major producers of transgenic *Glycine max* L., which is resistant to the glyphosate herbicide. In the province of Córdoba, transgenic soybean represents the most important crop regarding the cultivated area (3.4 million ha), and the production volume (8.4 million tons/y) (Instituto Nacional de Estadística y Censos 2001). This activity has been accompanied by an increase in pesticide application from 39.3 to 124 million kg between 1991 and 1997 (Pengue 2000). In these soybean lands, glyphosate is widely used as are endosulfan, 2-4D, and paraquat.

The aims of this study were as follows: (1) to evaluate the physiological response of *T. capillaris* Ruiz & Pav. f. *capillaris, T. recurvata* L., and *T. tricholepis* Baker transplanted to agricultural, industrial, and urban areas and (2) to find and characterize new biomarkers in *Tillandsia* species exposed to different air pollution sources.

Materials and Methods

Biological Material and Sample Preparation

Plants of *T. capillaris* Ruíz & Pav. f. *capillaris*, *T. recurvata* L., and *T. tricholepis* Baker were collected from trunks of trees in Totoral Department, Córdoba Province (between 30°75′17″ and 30°05′31″ S, and 64°05′41″ and 64°16′46″ W). These areas are considered unpolluted and represent the initial (baseline) conditions of these species previous to transplant.

Twenty-four bags, each containing 300 g *Tillandsia* individuals, were prepared according to Wannaz and Pignata (2006) and transplanted simultaneously to four areas (Fig. 1) with different atmospheric pollution emission sources: (1) traffic (Center of Córdoba city), (2) metallurgical and metal-mechanical industries (south-east of Córdoba city), (3) agricultural activities (Río Primero), and (4) low-pollution control area (Mendiolaza location).

In each sampling area, six bags of each species were hung at 3 m above ground level for periods of 3 months and 6 months (from August 25, 2003, to November 25, 2003 and to February 25, 2004). After each period of exposure, three bags of each species were recovered and preserved at −15°C in the dark until chemical and biochemical analyses were undertaken.

Sampling Areas

Córdoba City (Traffic, Metallurgical, and Metal-Mechanical Industries)

The city of Córdoba is located in the centre of the Republic of Argentina (31°24′S, 64°11′W) at an altitude of approximately 400 m above sea level. The climate is subhumid, with an average annual precipitation of 790 mm, which is concentrated principally in the summer. It has a population of approximately 1.5 million and an irregular topography. Its general structure is funnel-shaped, with an increasing

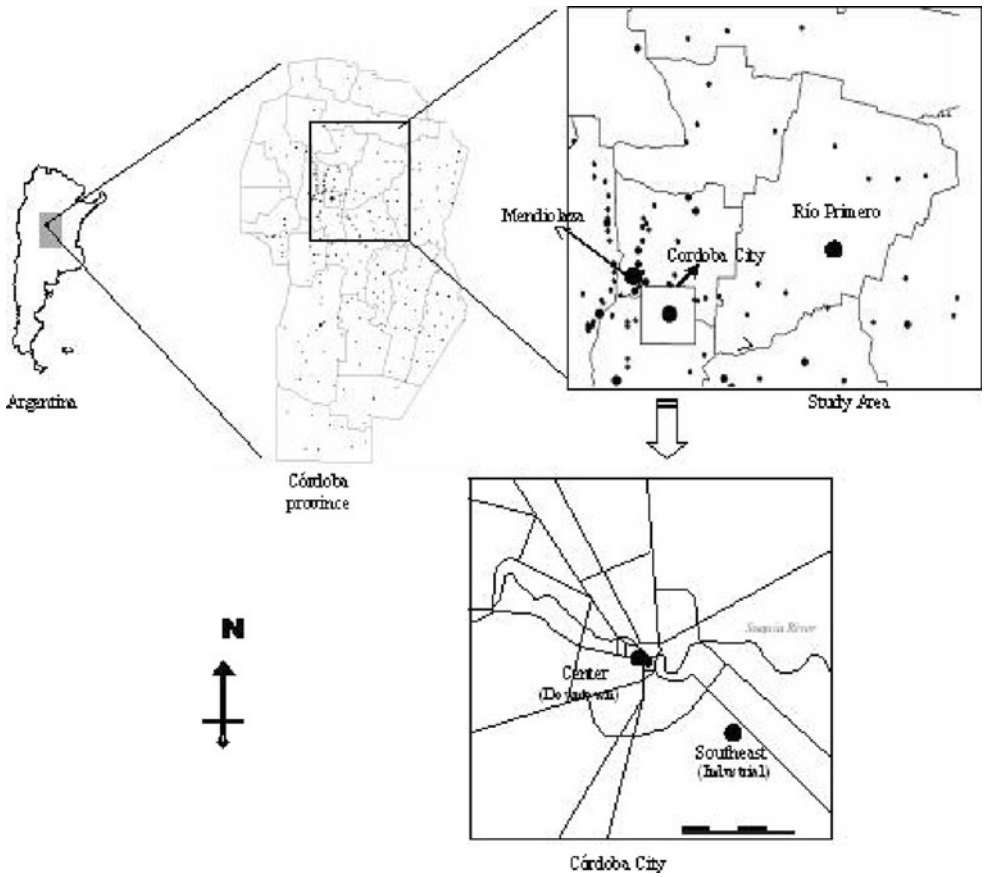

Fig. 1 Location of the four areas in Córdoba Province, Argentina, where the biomonitors were transplanted: (1) Mendiolaza (control), (2) Río Primero (agricultural activities) and Córdoba city (detailed inferior square), (3) center (downtown traffic), and (4) southeast (metallurgical and metal-mechanical industries)

positive slope from the centre toward the surrounding areas. This somewhat concave formation decreases air circulation and causes frequent thermal inversions in both autumn and winter. The main source of air pollution in Córdoba city (downtown) is vehicular emissions (Stein and Toselli 1996; Olcese and Toselli 2002). It also has an important industrial development of mainly metallurgic and metal-mechanical industries located in peripheral areas.

Two areas of Córdoba city were chosen for transplantation (Fig. 1). The southeast is a typically industrial area with metallurgic and metal-mechanic industries. The sampling site was located in the surroundings of an industrial plant in which metallic parts (e.g., power transformers, tanks, radiators, covers, etc.) are made. The sampling site in the centre was located in a densely populated area in which most of the public buildings, government offices, and shops are situated and through which almost all of the local buses run (downtown). We chose these areas because of the high grade of damage they produced in transplanted *Tillandsia* species as described by Wannaz and Pignata (2006), who also found high metal concentrations in atmospheric bulk deposition in downtown and SE Córdoba city. In addition, Carreras et al. (2006) described considerably high levels of total suspended particles occurring in the city center, particularly enriched in the traffic-related elements copper, zinc (Zn), and lead. Furthermore, Bermudez et al. (2009) previously assessed the accumulation of iron, manganese, and Zn in *Tillandsia* species transplanted to agricultural, industrial, and urban areas.

Río Primero (Agricultural Activities)

The Río Primero District is located in the central region of the Cordoba province. The medium annual temperature is 18°C, with average minimum and maximum temperatures of 10°C and 25°C, respectively. Annual precipitations reach 800 mm (Ramírez Sosa and Alé 1997). Córdoba is experiencing a dramatic agricultural expansion that has triggered a severe native forest loss (Aizen et al. 2009; Zak et al. 2008). Related to this, during the last three decades of the 20th century, the land cover changes in northern Córdoba have been dominated by the replacement of forests by crops, with a deforestation rate of approximately 2.75% $year^{-1}$ in the lowlands (Zak et al. 2008).

Forest and agricultural practices, mainly transgenic soybean, have led to the disappearance of great part of these woody formations, running serious risks of desertization (Ramírez Sosa and Alé 1997). The city of Río Primero has a population of approximately 5000 inhabitants.

Mendiolaza (Control Site)

Annual average precipitations in this zone measures 600 to 700 mm. Mendiolaza is part of the "espinal" phytogeographic region. This small location is sparsely populated and has no industrial or agricultural activity. Therefore, it is considered a "clean" control area.

Physiological Determinations

The procedures followed for the quantification of chlorophyll *a*, chlorophyll *b*, MDA, hydroperoxyconjugated dienes, S content, DW/FW, and FDI, all of which have been previously described by Pignata et al. (2002). FDI data were already presented in Bermudez et al. (2009).

Electrical Conductivity

Permeability of cell membranes in *Tillandsia* was estimated by the leakage of electrolytes and was measured according to Tarhanen et al. (1999). One hundred mg fresh leaves were placed in a humidity chamber for 2 h to standardize the flux of ions. Afterward, 50 mL deionized water were added, and the mixture was left for another 2 h at 20°C. Leachate conductivity was measured with an Oakton WD-35610 conductivity meter. The electrical conductivity (E-cond) was expressed as $\mu S\ g^{-1}\ DW\ ml^{-1}$.

Relative Water Content and Dehydration Kinetics

For the relative water content (RWC) determinations, 500 mg fresh leaves were placed in 50 ml deionized water for 12 h at 8°C ± 2°C in darkness. Then the leaves were shaken and allowed to dry at room temperature for 1 h; then the first weight was taken, which was considered the absolute tissue water capacity (100%). RWC was calculated as noted by Peñuelas et al. (2004):

$$RWC = 100 \times (FW - DW)/(TW - DW),$$

where FW is the fresh weight of each sample, DW is the dry weight, and TW is the turgid weight after hydrating the leaves as previously described.

The dehydration kinetics (Kin-H_2O) were performed as cited by Chettri and Sawidis (1997). *Tillandsia* turgid leaves were placed on aluminum paper at 30°C ± 2°C in the darkness. After 45 min, the first weight was taken, and samples were returned to the oven and reweighed every 15 min for 2 h. Each weight was considered FW_t, where $t = 15.\ 30,\ \ldots,\ 120$ min. To avoid the influence of the oversaturation phenomenon (Kogan and Bayer 1996), every FW_t was divided into the RWC of every sample. Hence, the dehydration kinetics were calculated according to the following equation:

$$\text{Kin-}H_2O = (FW_t/RWC)t^{-1},$$

where $t = 15, 30, 45, \ldots, 120$ min. Finally, Kin-H_2O was expressed as g FW_t min^{-1}.

Total Phenols

Total phenolic compounds (T-phen) were measured by the Folin–Ciocalteau micro-method (Singleton et al. 1999). Absorption at 765 nm was measured in a Beckman DU-7000spectrophotometer. T-phen concentration was determined using pyrogallic acid as a standard and was expressed as mg g^{-1} DW.

Soluble Proteins and Enzyme Activities

One hundred milligrams FW leaves were homogenized in a mortar at 4°C with 1.5 ml potassium phosphate buffer (50 mM [pH 7.4]), 1 mM ethylenediaminetetraacetic acid (EDTA), and 100 mg polyvinylpyrrolodone. The homogenate was centrifuged for 30 min at 14000 rpm at 4°C. The supernatants were used for enzymatic assays and determination of protein content. Protein content was determined using the method of Bradford (1976) with bovine serum albumin as a standard and expressed as mg g^{-1} DW.

SOD activity was assayed by its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Beauchamp and Fridovich 1971). The absorbance was recorded at 560 nm, and one unit of enzyme activity was defined as the amount of enzyme causing 50% inhibition of NBT reduction under the assay conditions. The activity was expressed as SOD units mg^{-1} protein. CAT activity was assayed according to the method of Anderson et al. (1995). Adding H_2O_2 the reaction was started, and the decrease in absorbance at 240 nm was recorded for 1 min. Enzyme activity was calculated by using the extinction coefficient ($\varepsilon = 43.6\ mM^{-1}\ cm^{-1}$), and the activity was expressed as nmol min^{-1} mg^{-1} protein. Ascorbate peroxidase activity (APX) was assayed by monitoring the oxidation rate of H_2O_2-dependant absorbance described by Nakano and Asada (1981). The supernatant was passed through a gel-filtration column of Sephadex G-25 (Sigma, St. Louis, MO, USA) (1.5 × 5.0 cm). By doing this, low-molecular interference compounds were removed. The reaction was started with the addition of H_2O_2. Absorbance decrease for 1 min at 290 nm was measured. This procedure was repeated once the leaf extracts were boiled for 5 min to inactivate the enzyme completely. APX activity was determined as the difference between the A_{290} values ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$)

of the nonboiled and boiled tissue samples. The activity was expressed as nmol min^{-1} mg^{-1} protein.

Statistical Analysis

Statistical analyses were based on the mean value of the determinations performed for the three samples of each species extracted from each sampling area. A one-way analysis of variance (ANOVA) for each parameter was carried out considering the area of transplantation as a factor with four levels (control, urban, agricultural, and industrial). Whenever ANOVA indicated significant effects ($p < 0.05$), a pairwise comparison of means by least significant difference (LSD) test was performed. In addition, principal component analysis (PCA) was used, which is generally considered an effective tool by which to exhibit the main factors affecting environmental data contained in a matrix (Bermudez et al. 2010; Carreras et al. 2009; Pignata et al. 2002; Rodriguez et al. 2007). This statistical technique was applied to the data set to identify associations between air pollution sources and physiological parameters. The input data were the values of the physiological parameters, with the air pollution sources being used as a classification criterion.

Results and Discussion

T. tricholepis Baker

After 3 months of exposure, the plants transplanted to the agricultural area had the highest MDA and FDI values, followed by the urban and industrial areas (Table 1). After 6 months, the highest FDI values were also observed in the agricultural and urban areas, followed by the industrial area, which in this case did not differ significantly from the control. These findings are in agreement with those of Pignata et al. (2002) for *T. capillaris* in a passive biomonitoring study, in which the highest FDI values were related to agricultural activities in the Córdoba province, and with those of Wannaz and Pignata (2006), who found high FDI values in urban and industrial areas.

After 3 months of exposure, the plants transplanted to the agricultural area had the highest SOD values. The increased SOD activity compared with the control could be associated with a protective response against the action of pesticides because it has been reported for some other species (Ahsan et al. 2008; Sergiev et al. 2006; Zhang et al. 2007). After 6 months of exposure, the urban area showed the highest SOD activity, with a value almost 20 times greater than the control. These findings are in agreement with studies using lichens as bioindicators, in which an increase of SOD activity was detected in association with SO_2 and O_3 high levels, which are common urban pollutants (Kong et al. 1999; Deltoro et al. 1999; Weissman et al. 2006).

CAT was shown to be sensitive to urban and industrial pollutants after 3 and 6 months of transplantation, respectively, which is in agreement with other studies assessing CAT response to urban contaminants and heavy metals (Weissman et al. 2006; Monnet et al. 2006). For both periods of exposure, the highest APX activity was found in the control area, with the lowest values being observed in the urban and agricultural areas after 3 and 6 months, respectively. These findings are in disagreement with Li (2003), Sergiev et al. (2006), and Zhang et al. (2007), who found an amplified activity of plant POs to urban and agricultural (agrochemical) pollutants, and could be partially explained by the increased CAT activity in the urban areas.

After 3 months of exposure, the highest Kin-H_2O values were found in the agricultural and industrial areas and, after 6 months, in the agricultural area. In addition, the lowest RWC values were registered in the agricultural area after both periods of transplantation. These findings allow us to point out that *T. tricholepis* was sensitive to agricultural and industrial pollution, but to a lesser extent to the latter, in the two periods under study. Moreover, our results are in agreement with many investigators that have shown a direct relationship between the loss of humidity content in lichens and plants and atmospheric pollution (Mansfield 1998; Carreras et al. 2005; Wannaz and Pignata 2006). However, in agreement with Kaiser (1987), who stated that water stress is associated with RWC values <70%, only plants exposed to the agricultural area were stressed, even in the rainy season. These findings indicate that agricultural areas may trigger ROS production and induce antioxidant responses in *Tillandsia* caused by water stress, which is a key ecological factor in determining the physiological status of atmospheric bromeliads (González-Salvatierra et al. 2010; Graham and Andrade 2004; Haslam et al. 2003).

Although E-cond has been widely used in lichens in relation to air pollution effects (Carreras et al. 2009; Garty et al. 1993; Tarhanen et al. 1999), studies on vascular plants are scarce (Neves et al. 2009; Sergiev et al. 2006). In our study, the E-cond values found in agricultural, industrial, and urban areas were greater than the control, meaning that atmospheric pollutants emitted by these sources increased the permeability and subsequent loss of intracellular solutes (Fields and St Clair 1984). Our findings are in agreement with those of Sergiev et al. (2006), who found that maize plants exposed to glyphosate showed increased leaf E-cond, and with Munzi et al. (2009), who assessed electrolyte leakage associated with nitrogen compounds, for which agricultural practices are a major

Table 1 Comparison of mean values (± SE) and results of ANOVA of physiological parameters on *T. tricholepis* Baker in different areas and exposure periods

Mean ± SE					ANOVA
	Urban	Agricultural	Industrial	Control	
FDI (basal 0.633 ± 0.116)					
3 mo	1.843 ± 0.072^{b}	5.790 ± 0.442^{a}	1.413 ± 0.078^{c}	0.671 ± 0.036^{d}	***
6 mo	1.203 ± 0.048^{a}	1.076 ± 0.147^{a}	0.876 ± 0.012^{b}	0.827 ± 0.107^{b}	**
MDA (basal 0.103 ± 0.022 μmol g^{-1} DW)					
3 mo	0.109 ± 0.012^{b}	0.376 ± 0.125^{a}	0.089 ± 0.005^{b}	0.118 ± 0.007^{b}	**
6 mo	0.157 ± 0.011	0.129 ± 0.016	0.147 ± 0.015	0.156 ± 0.010	NS
E-cond (basal 9.520 ± 1.664 μS g^{-1} DW ml^{-1})					
3 mo	8.619 ± 0.628^{a}	9.220 ± 0.933^{a}	8.782 ± 0.984^{a}	2.050 ± 0.293^{b}	***
6 mo	3.370 ± 0.525^{c}	7.861 ± 1.790^{a}	6.032 ± 0.225^{a,b}	4.930 ± 1.028^{b,c}	**
RWC (basal 83.96% ± 4.53%)					
3 mo	87.98 ± 5.01^{a}	51.79 ± 6.89^{c}	73.71 ± 8.33^{b}	86.53 ± 4.29^{a}	***
6 mo	75.20 ± 7.30^{a,b}	69.11 ± 1.60^{b}	78.98 ± 5.12^{a}	84.08 ± 4.32^{a}	*
Kin-H_2O (basal 0.039 ± 0.005 g FW_t min^{-1})					
3 mo	0.036 ± 0.005^{b}	0.087 ± 0.020^{a}	0.050 ± 0.009^{a}	0.034 ± 0.006^{b}	***
6 mo	0.050 ± 0.007^{b}	0.058 ± 0.004^{a}	0.045 ± 0.005^{c}	0.040 ± 0.004^{d}	***
T-phen (basal 434.8 ± 21.5 mg g^{-1} DW)					
3 mo	425.3 ± 23.7	479.2 ± 13.2	498.1 ± 26.4	422.8 ± 63.6	NS
6 mo	447.9 ± 84.1	481.6 ± 61.7	316.0 ± 52.0	375.0 ± 64.4	NS
S-prot (basal 5.515 ± 0.259 mg g^{-1} DW)					
3 mo	4.892 ± 0.156^{b}	5.339 ± 0.124^{a}	4.048 ± 0.160^{c}	5.452 ± 0.107^{a}	***
6 mo	11.80 ± 0.68^{a}	11.23 ± 0.22^{a}	9.745 ± 0.176^{b}	8.797 ± 0.341^{c}	***
CAT (basal 85.39 ± 10.96 nmol min^{-1} mg^{-1} protein)					
3 mo	124.3 ± 20.7^{a}	72.79 ± 13.02^{a,b}	58.57 ± 1.52^{b}	69.14 ± 11.52^{b}	*
6 mo	39.05 ± 2.30^{b}	56.01 ± 5.79^{b}	100.2 ± 15.2^{a}	47.80 ± 2.03^{b}	*
SOD (basal 162.2 ± 13.9 U mg^{-1} protein)					
3 mo	72.81 ± 29.08^{b}	488.9 ± 3.9^{a}	120.3 ± 22.6^{b}	80.93 ± 11.04^{b}	***
6 mo	47.46 ± 8.09^{a}	17.62 ± 1.88^{b,c}	24.56 ± 5.75^{b}	2.692 ± 0.294^{c}	**
APX (basal 2.310 ± 0.042 nmol min^{-1} mg^{-1} protein)					
3 mo	1.961 ± 0.275^{c}	4.029 ± 0.245^{b}	4.048 ± 0.024^{b}	5.871 ± 0.193^{a}	***
6 mo	2.267 ± 0.159^{b}	1.804 ± 0.135^{c}	2.839 ± 0.074^{a}	2.849 ± 0.070^{a}	***

NS not significant

Basal values (before transplantation) are given in *parentheses*

Values on each horizontal line followed by the same letter do not differ significantly ($p = 0.05$)

* Significant at $p < 0.05$

** Significant at $p < 0.01$

*** Significant at $p < 0.001$

source. Moreover, our findings are in accordance with Carreras et al. (2005, 2009), who reported high E-cond values in the lichen *Usnea amblyoclada* transplanted to urban areas. In relation to this, Neves et al. (2009) found a significant increase in electrolyte leakage after *Eugenia uniflora* plants were exposed to acid rain, to which urban atmospheric pollutants are the major precursors (NO_x and SO_2).

S-prot content was greater in agricultural and control areas after 3 months of exposure, whereas after 6 months, the highest concentrations were registered in the agricultural and urban areas. Related to this, Singh et al. (2004) found an increase in soluble protein concentration in tomato plants as an adaptive response to metal stress. Moreover, the high protein concentrations in the agricultural area might show the effect of nitrogen availability (ammonium nitrate, urea, etc.) as pointed out by Pérez-Soba et al. (1994).

Regarding the temporal trends in *T. tricholepis* physiology, FDI, E-cond, CAT, SOD, and APX showed a

decrease from the first (3 months) to the second (6 months) period of transplantation in most areas of exposure. These findings could be partially explained by acclimatization to the air pollution sources and/or by better weather conditions occurring at the end of the 6-month period, such as more rainfall amounts (concentrated in the meridional summer) and greater mean and minimum temperatures than during the 3-month transplantation period, as previously described by Bermudez et al. (2009).

T. recurvata L.

After 3 months of exposure, the plants transplanted to the industrial area showed the highest FDI values, followed by the urban and agricultural areas (Table 2), whereas after 6 months, high FDI values were found in the urban and agricultural areas. The fact that control FDI values were the lowest may indicate that physiological damage caused by different air pollution emission sources was evident in *T. recurvata.*

After 3 and 6 months, the highest MDA concentrations were found in the urban area, which is in agreement with Cañas et al. (1997) using *Ligustrum lucidum*, and with Deniz and Duzneli (2007), who found that urban SO_2 atmospheric levels were positively associated with MDA content in vascular plants. The E-cond values found in the agricultural, industrial, and urban areas were greater than the control after 3 months of exposure. After 6 months, the highest electrolyte leakage was observed in the plants transplanted to the urban and industrial areas. These findings are in agreement with Neves et al. (2009) and Tarhanen et al. (1999), who found high E-cond values in vascular plants and lichens, respectively, exposed to heavy metals and acid rain. Moreover, Rodriguez et al. (2007) assessed that the damage to cell membranes originated by urban air pollutants in two lichen species was even worse in the presence of heavy metals, which are commonly liberated by metallurgical and metal-mechanical industries.

Regarding the hydric balance, the lowest RWC was found in the agricultural area (Table 2), indicating that the irreversible dehydration [RWC $< 30\%$, (Kaiser 1987)] was associated with the agricultural activities. Another related parameter, Kin-H_2O, showed a faster dehydration in samples transplanted to the agricultural area, followed by the industrial (after 3 months) and urban areas (after 6 months of exposure). These findings indicate that the water balance for *T. recurvata* is more sensitive to agricultural pollution than that for *T. tricholepis*, especially in the dry season (after 3 months of transplantation).

Contrary to the results of *T. tricholepis*, the concentration of S-prot was lower than the control in the agricultural areas after 3 and 6 months of exposure. These findings are in agreement with Monnet et al. (2006) and Wang et al. (2009a), who reported a decrease in soluble protein content due to metallic pollution and/or herbicide application. Although the T-phen concentration after 3 months of exposure was lower than the control in the agricultural and industrial areas, after 6 months the highest T-phen concentrations were found in the agricultural and urban areas. These findings are in agreement with Loponen et al. (1998) and Pasqualini et al. (2003), who found an increase in foliar phenol concentration associated with the presence of pollutants, such as SO_2. After 3 months of exposure, the highest SOD and CAT activities were found in the agricultural area, whereas after the 6-month period no significant differences were observed among the transplantation areas for CAT. These findings could be explained by a protective response against the action of pesticides as has been reported for other species (Ahsan et al. 2008; Sergiev et al. 2006; Zhang et al. 2007). In relation to APX response, the lowest and highest activities were found in the industrial areas after 3 and 6 months of exposure, respectively. In addition, the APX activities in *T. recurvata* plants transplanted to the urban areas were lower than the control in both periods of exposure. These findings might indicate a particular sensitivity of APX to urban and industrial pollutants, which is in agreement with Li (2003) and Zhang et al. (2007). Regarding temporal changes in the parameters measured, FDI, E-cond, RWC, and Kin-H_2O indicated an improvement in the physiological status from the first (3 months) to the second (6 months) period of transplantation in most areas of exposure. However, the antioxidant response and the MDA concentration remained constant or even increased with the time of exposure. These findings might indicate a lesser acclimatization to the air pollution sources than *T. tricholepis*, especially in urban and industrial areas.

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After 3 months of exposure, the highest FDI values were found in the agricultural area, followed by the urban and industrial area (Table 3). These findings are in agreement with Pignata et al. (2002) and Wannaz and Pignata (2006) for the same *Tillandsia* species. After 6 months of transplantation, the highest FDI values were detected in the epiphytes exposed to the agricultural and urban areas, but the concentration of MDA did not show significant differences among the areas. After 3 months of exposure, the control E-cond values were the lowest, whereas after 6 months, the highest E-cond values were reported in the industrial area. These findings are in agreement with those of Carreras et al. (2005, 2009), Munzi et al. (2009), Neves et al. (2009), Sergiev et al. (2006) and Tarhanen et al. (1999), who found increased electrolyte leakage in plants

Table 2 Comparison of mean values (± SE) and results of ANOVA of physiological parameters on *T. recurvata* L. among different areas and exposure periods

Mean ± SE					ANOVA
	Urban	Agricultural	Industrial	Control	
FDI (basal 1.030 ± 0.139)					
3 mo	2.139 ± 0.171^{b}	1.718 ± 0.165^{c}	2.495 ± 0.149^{a}	0.595 ± 0.058^{d}	***
6 mo	1.434 ± 0.131^{a}	1.345 ± 0.158^{a,b}	1.155 ± 0.050^{b,c}	0.986 ± 0.092^{c}	**
MDA (basal 0.091 ± 0.018 μmol g^{-1} DW)					
3 mo	0.105 ± 0.006^{a}	0.081 ± 0.011^{b}	0.063 ± 0.016^{b}	0.084 ± 0.014^{a,b}	*
6 mo	0.124 ± 0.017^{a}	0.099 ± 0.003^{b}	0.102 ± 0.006^{b}	0.097 ± 0.002^{b}	*
E-cond (basal 6.971 ± 1.742 μS g^{-1} DW ml^{-1})					
3 mo	11.41 ± 1.39^{a}	11.82 ± 1.07^{a}	12.73 ± 0.80^{a}	0.513 ± 0.047^{b}	***
6 mo	8.551 ± 0.367^{a}	4.474 ± 1.100^{c}	8.867 ± 0.609^{a}	5.932 ± 0.516^{b}	***
RWC (basal 71.70% ± 5.91%)					
3 mo	69.30 ± 10.47^{b}	26.19 ± 2.04^{c}	56.55 ± 11.34^{b}	97.22 ± 0.41^{a}	***
6 mo	74.83 ± 5.35^{b}	58.35 ± 4.87^{c}	92.89 ± 6.61^{a}	99.18 ± 0.16^{a}	***
Kin-H_2O (basal 0.052 ± 0.007 g FW_t min^{-1})					
3 mo	0.050 ± 0.013^{c}	0.259 ± 0.052^{a}	0.071 ± 0.025^{b}	0.026 ± 0.007^{d}	***
6 mo	0.049 ± 0.006^{b}	0.073 ± 0.009^{a}	0.030 ± 0.005^{c}	0.030 ± 0.002^{c}	***
T-phen (basal 412.5 ± 38.4 mg g^{-1} DW)					
3 mo	397.6 ± 10.6^{a}	348.1 ± 15.3^{b}	349.7 ± 18.4^{b}	422.1 ± 20.1^{a}	**
6 mo	339.3 ± 30.0^{b}	333.4 ± 43.8^{a,b}	286.0 ± 12.1^{b,c}	257.5 ± 1.8^{c}	*
S-prot (basal 8.547 ± 1.112 mg g^{-1} DW)					
3 mo	7.233 ± 0.244^{a}	5.910 ± 0.312^{b}	7.320 ± 0.129^{a}	7.352 ± 0.669^{a}	**
6 mo	9.387 ± 0.134^{a}	6.337 ± 0.056^{d}	8.238 ± 0.097^{b}	7.214 ± 0.293^{c}	***
CAT (basal 32.08 ± 2.65 nmol min^{-1} mg^{-1} protein)					
3 mo	64.80 ± 10.69^{b}	366.5 ± 47.2^{a}	26.31 ± 5.06^{b}	39.23 ± 5.60^{b}	***
6 mo	48.65 ± 4.05	40.28 ± 2.12	47.99 ± 3.08	50.64 ± 4.05	NS
SOD (basal 91.18 ± 0.77 U mg^{-1} protein)					
3 mo	147.1 ± 12.9^{a}	148.1 ± 14.2^{a}	124.6 ± 3.1^{ab}	106.7 ± 5.4^{b}	*
6 mo	332.7 ± 51.9^{b}	312.3 ± 15.6^{b}	818.0 ± 98.3^{a}	212.6 ± 26.5^{b}	***
APX (basal 2.634 ± 0.132 nmol min^{-1} mg^{-1} protein)					
3 mo	1.289 ± 0.116^{b}	0.092 ± 0.153^{b}	0.309 ± 0.060^{c}	3.438 ± 0.273^{a}	***
6 mo	0.581 ± 0.055^{c}	2.379 ± 0.055^{b}	3.447 ± 0.195^{a}	2.180 ± 0.128^{b}	***

NS not significant

Basal values (before transplantation) are given between *parentheses*

Values on each horizontal line followed by the same letter do not differ significantly ($p = 0.05$)

* Significant at $p < 0.05$

** Significant at $p < 0.01$

*** Significant at $p < 0.001$

and lichens exposed to urban, industrial and agricultural air pollutants.

After 3 months of exposure, the lowest RWC values were found in the agricultural and urban areas, followed by the industrial area, with water stress (Kaiser 1987) being observed in the three air pollution sources under study. Related to this, the highest Kin-H_2O values were observed in the epiphytes transplanted to the agricultural area in both periods of exposure. These findings indicate that agricultural areas negatively influence the water content and its loss, harmfully affecting a key resource in atmospheric bromeliads (González-Salvatierra et al. 2010; Graham and Andrade 2004; Haslam et al. 2003). T-phen did not show a distinctive response because the highest concentrations were found in the urban and control areas after 3 months of exposure, and no differences were registered after the 6-month period. Regarding the antioxidant response, the lowest and highest CAT activities were found

Table 3 Comparison of mean values (± SE) and results of ANOVA of physiological parameters *T. capillaris* Ruiz & Pav. f. *capillaries* among different areas and exposure periods

Mean ± SE					ANOVA
	Urban	Agricultural	Industrial	Control	
FDI (basal 1.100 ± 0.034)					
3 mo	1.275 ± 0.077^{b}	2.120 ± 0.134^{a}	1.390 ± 0.183^{b}	0.994 ± 0.065^{c}	***
6 mo	1.951 ± 0.280^{a}	2.061 ± 0.250^{a}	1.605 ± 0.123^{a,b}	1.414 ± 0.201^{b}	*
MDA (basal 0.103 ± 0.005 μmol g^{-1} DW)					
3 mo	0.138 ± 0.012	0.145 ± 0.021	0.145 ± 0.021	0.149 ± 0.006	NS
6 mo	0.124 ± 0.015	0.118 ± 0.007	0.141 ± 0.006	0.140 ± 0.025	NS
E-cond (basal 8.232 ± 0.722 μS g^{-1} DW ml^{-1})					
3 mo	7.747 ± 1.781^{a}	8.817 ± 0.981^{a}	8.303 ± 1.024^{a}	0.170 ± 0.020^{b}	***
6 mo	5.097 ± 0.230^{b}	5.679 ± 0.613^{b}	7.541 ± 0.488^{a}	4.660 ± 0.926^{b}	**
RWC (basal 38.35% ± 6.08%)					
3 mo	44.32 ± 5.24^{c}	36.75 ± 3.62^{c}	61.97 ± 11.35^{b}	78.58 ± 9.75^{a}	***
6 mo	81.42 ± 5.89^{a}	65.04 ± 7.56^{b}	82.36 ± 2.75^{a}	79.61 ± 11.38^{a}	*
Kin-H_2O (basal 0.134 ± 0.028 005 g FW_t min^{-1})					
3 mo	0.116 ± 0.028^{b}	0.139 ± 0.028^{a}	0.063 ± 0.015^{c}	0.042 ± 0.008^{d}	***
6 mo	0.041 ± 0.005^{b}	0.060 ± 0.010^{a}	0.041 ± 0.004^{b}	0.043 ± 0.009^{b}	***
T-phen (basal 409.1 ± 10.9 mg g^{-1} DW)					
3 mo	514.2 ± 45.5^{a}	393.6 ± 15.7^{b}	382.8 ± 41.0^{b}	503.9 ± 27.59^{a}	**
6 mo	480.9 ± 70.2	429.2 ± 80.3	480.2 ± 33.3	453.2 ± 35.3	NS
S-prot (basal 9.376 ± 0.554 mg g^{-1} DW)					
3 mo	6.805 ± 0.180^{d}	12.79 ± 0.15^{a}	8.797 ± 0.207^{c}	11.23 ± 0.38^{b}	***
6 mo	10.04 ± 0.21^{b}	9.628 ± 0.249^{b,c}	11.43 ± 0.42^{a}	9.191 ± 0.491^{c}	***
CAT (basal 98.01 ± 6.29 nmol min^{-1} mg^{-1} protein)					
3 mo	83.53 ± 4.91^{a}	34.67 ± 3.36^{b}	63.42 ± 12.68^{a}	80.87 ± 4.62^{a}	*
6 mo	54.47 ± 7.55^{b}	232.0 ± 36.4^{a}	85.03 ± 7.09^{b}	82.03 ± 2.83^{b}	***
SOD (basal 183.4 ± 1.4 U mg^{-1} protein)					
3 mo	128.3 ± 8.4^{a,b}	163.8 ± 3.4^{a}	155.0 ± 17.7^{a}	102.6 ± 8.8^{b}	*
6 mo	1235 ± 97^{b}	1847 ± 98^{a}	850.4 ± 120.2^{c}	1099 ± 98^{b,c}	***
APX (basal 2.082 ± 0.114 nmol min^{-1} mg^{-1} protein)					
3 mo	9.826 ± 0.190^{a}	0.331 ± 0.070^{c}	1.994 ± 0.122^{b}	1.798 ± 0.096^{b}	***
6 mo	1.747 ± 0.065^{b}	1.557 ± 0.047^{b,c}	1.366 ± 0.038^{c}	2.035 ± 0.097^{a}	***

NS not significant

Basal values (before transplantation) are given between *parentheses*

Values on each horizontal line followed by the same letter do not differ significantly ($p = 0.05$)

* Significant at $p < 0.05$

** Significant at $p < 0.01$

*** Significant at $p < 0.001$

in the agricultural areas after 3 and 6 months of transplantation, respectively, which indicate a sensitive behavior of CAT to this source. Likewise, the highest SOD activities were found in the agricultural areas after 3 and 6 months of exposure. These findings are in agreement with Ahsan et al. (2008), Sergiev et al. (2006) and Zhang et al. (2007), who found increased CAT and SOD activities in rice, maize, and cucumber plants exposed to herbicides and fungicides. In contrast, APX activity was significantly greater than the control in the urban area, but lower in the agricultural area, after 3 months of exposure. In the next period, the *T. capillaris* plants transplanted to the control area showed the highest APX values, possibly indicating a specific sensitivity of APX to agricultural, urban, and industrial pollutants, which is in agreement with *T. tricholepis* findings.

Regarding the temporal trends in *T. capillaris* physiology, an increment in FDI values, as well as in CAT, SOD and APX activities, was found from the first (3 months) to the second (6 months) period of transplantation in most of

the areas. Seasonal changes in the antioxidant response have been already described by many investigators in *Ficus*, *Populus*, and *Rhododendron* species (Baycu et al. 2006; Li 2003; Wang et al. 2009b). In relation to this, the hydric balance showed greater RWC values and a lower rate of water loss (Kin-H_2O) after 6 months of exposure, probably accounted for by greater total amounts of precipitation during the meridional summer. However, it is worth noting that even in the *T. capillaris* plants transplanted to the control area, temporal changes in FDI and antioxidant enzymes were observed, which could be associated with weather conditions and acclimatizing processes (Bermudez et al. 2009). In spite of these temporal trends, the differences among areas were used to evaluate the physiological response of *Tillandsia* species to air pollution sources.

PCA

PCA was performed on the physiological data matrix of each species, with the sampling areas being used as a classification criterion. The inertia of the principal axes was expressed as the percentage of explained variance.

For *T. tricholepis* (Fig. 2), PCA showed that the damage in the biological membranes (E-cond, Kin-H_2O, and MDA), SOD, FDI, and the T-phen and RWC (inversely) were associated with agricultural activities. For *T. recurvata* (Fig. 3), PCA showed that FDI, E-cond, and SOD were related to the industrial area, whereas CAT, Kin-H_2O, SOD, and RWC (inversely) were associated with the agricultural area. In addition, S-prot, T-phen, and MDA were related to the urban area. For *T. capillaris* (Fig. 4), FDI, CAT, SOD, E-Cond, Kin-H_2O, and RWC (inversely)

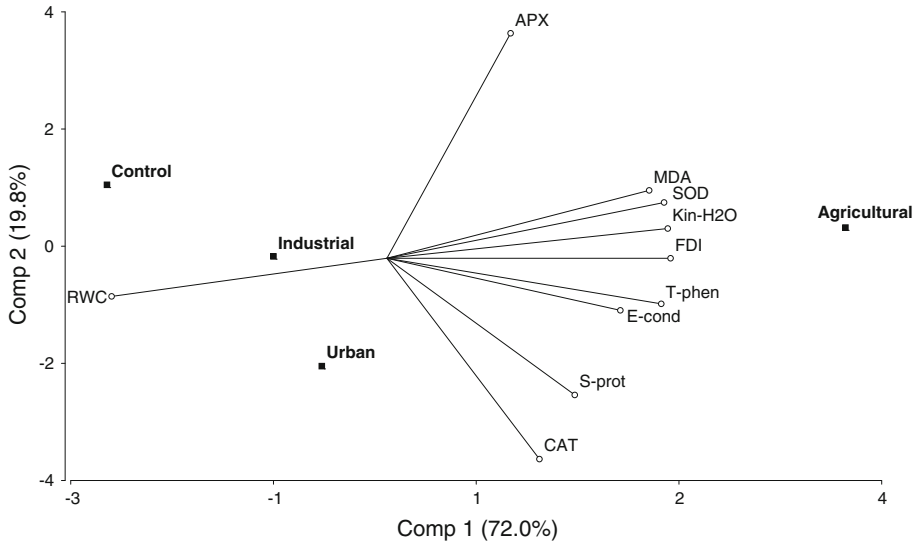

Fig. 2 Variable scores based on first two main components of PCA showing the clustering of different physiological variables and air pollution emission sources as classification criterion for *T. tricholepis* Baker. Percentages of total explained variance are given between *brackets*

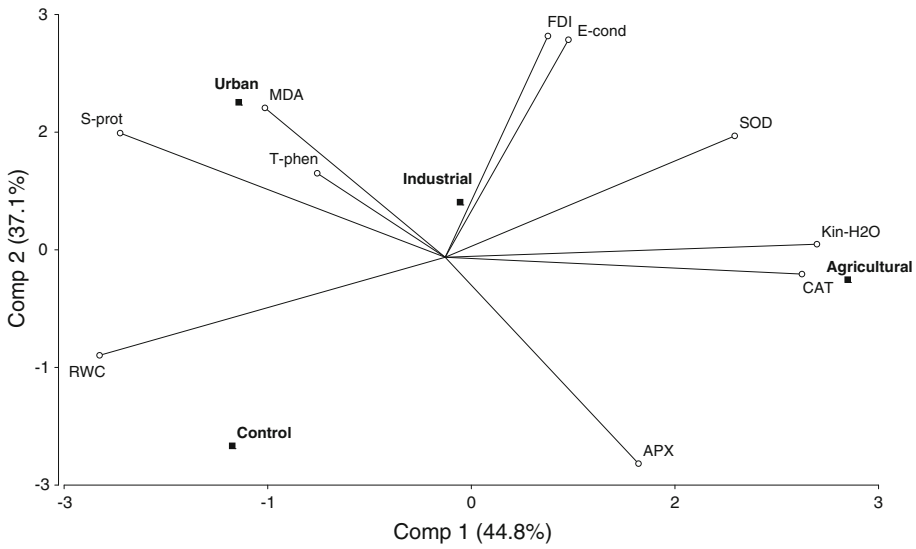

Fig. 3 Variable scores based on first two main components of PCA showing the clustering of different physiological variables and air pollution emission sources as classification criterion for *T. recurvata* L. Percentages of total explained variance are given between *brackets*

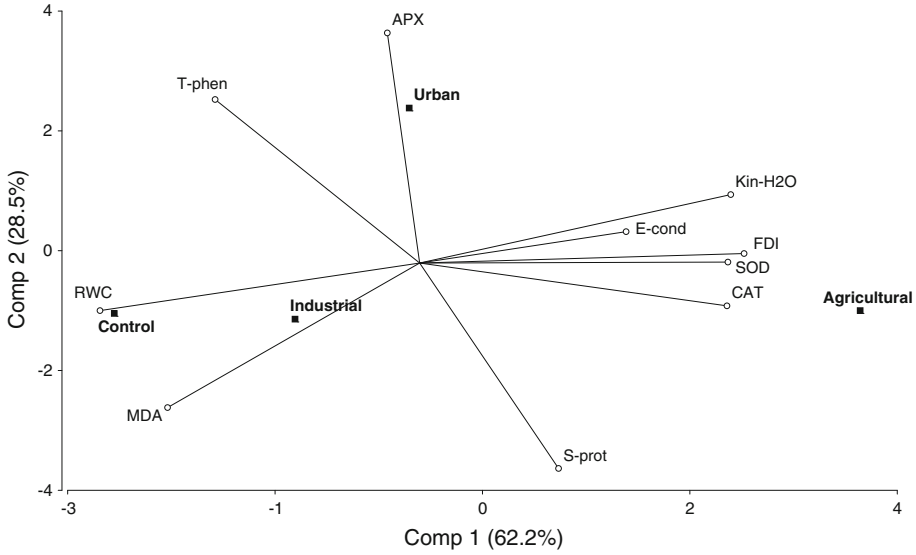

Fig. 4 Variable scores based on first two main components of PCA showing the clustering of different physiological variables and air pollution emission sources as classification criterion for *T. capillaris* Ruiz & Pav. f. *capillaris*. Percentages of total explained variance are given between *brackets*

were associated with the agricultural area. Moreover, APX activity was related to the urban area and MDA concentration to the industrial area.

Conclusion

In *Tillandsia* species, biomarkers that have already been used to study the effects of pollutants include lipid peroxidation products, such as MDA; photosynthetic pigments and degradation derivatives; and FDI. However, there is a growing trend toward the use of earlier and more sensitive biomarkers that could provide information before irreversible damage is caused. As yet, antioxidant enzymes have received little attention as a way of monitoring air pollution with lichens or epiphytic plants.

In the present study, the antioxidant response and physiological damage to three *Tillandsia* species transplanted to urban, industrial, and agricultural areas was assessed. Although the changes were species dependent, the majority of the parameters measured were affected by agricultural sources. In relation to this, *T. tricholepis* and *T. capillaris* were more sensitive to agricultural activity, whereas *T. recurvata* was more sensitive to urban and industrial air pollution sources.

Considering that air pollution is comprised of a complex mixture containing metals, organic compounds, and secondary photochemical compounds, it is difficult to ascribe the physiological disturbances observed to a particular agent. Regardless, the parameters E-cond, FDI, SOD, RWC, and Kin-H_2O behaved as suitable indicators of agricultural air pollution for *T. tricholepis* and *T. capillaris* and CAT, Kin-H_2O and SOD for *T. recurvata*. In addition, MDA, T-phen, and S-prot proved to be appropriate indicators of urban pollution for *T. recurvata*. Moreover, FDI, E-cond, and SOD for *T. recurvata* and MDA for *T. tricholepis*, respectively, could be used to detect deleterious effects of industrial air pollution.

Temporal changes in the physiological parameters were found in *T. tricholepis*, *T. recurvata*, and *T. capillaris* between the 3- and 6-month periods of exposure, indicating an acclimatization response on one hand and/or the positive effects of better whether conditions on the other. Although *T. capillaris* seemed to be the most sensitive species along this survey, further studies still need to be carried out to determine the extent to which the temporal physiological response in *Tillandsia* species is explained by the seasonal conditions or the length of the transplantation period.

Finally, our study may give further support to the hypothesis that agricultural activities represent a source of air pollution having a great impact on the native flora. Therefore, the use of the before-mentioned physiological parameters may play an important role as indicators of environmental disruptions.

Acknowledgments This work was partially supported by the Agencia Nacional de Promoción Científica y Tecnológica (FONCyT), the Secretaría de Ciencia y Técnica de la Universidad Nacional de Córdoba (SECYT-UNC), CONICET and the Instituto de Fitopatología y Fisiología Vegetal (IFFIVE-INTA). We are thankful to IFFIVE and especially to Dr. Mariana Melchiore.

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