

Physiological responses of root-less epiphytic plants to acid rain

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Abstract Selected physiological responses of *Tillandsia albida* (Bromeliaceae) and two lichens (*Hypogymnia physodes* and *Xanthoria parietina*) exposed to simulated acid rain (AR) over 3 months were studied. Pigments were depressed in all species being affected the most in *Tillandsia*. Amounts of hydrogen peroxide and superoxide were elevated and soluble proteins decreased only in AR-exposed *Hypogymnia*. Free amino acids were slightly affected among species and only glutamate sharply decreased in AR-exposed *Xanthoria*. Slight increase in soluble phenols but decrease in flavonoids in almost all species suggests that the latter are not essential for tolerance to AR. Almost all phenolic acids in *Tillandsia* leaves decreased in response to AR and activities of selected enzymes (phenylalanine ammonia-lyase, polyphenol oxidase, ascorbate- and guaiacol-peroxidase) were enhanced by AR. In lichens, considerable increase in metabolites (physodalic acid, atranorin and parietin) in response to AR was found but amount of ergosterol was unchanged. Macronutrients (K, Ca, Mg) decreased more pronouncedly in comparison with micronutrients in all species. *Xanthoria* showed higher tolerance in comparison with *Hypogymnia*, suggesting that could be useful for long-term biomonitoring.

Keywords Antioxidative enzymes · Epiphytic plants · Phenolic metabolism · Proline · Reactive oxygen species

Introduction

Increasing industrial activities lead to elevated release of by-products which may negatively affect environment. Production of carbon, nitrogen and sulphur oxides is one of the most adverse consequences of such activities resulting to potential injuries of all aerobic organisms. Species of Bromeliaceae family represent considerable part of epiphytic vegetation in tropical America (Monteiro et al. 2009) and the genus *Tillandsia* includes epiphytic plants with extraordinary capacity to obtain water and nutrients from the atmosphere where pollutants are present (Pignata et al. 2002). Lichens are symbiotic entities formed by the algal photobiont (usually green alga or cyanobacteria) and fungal mycobiont. This unique symbiotic nature of lichens is responsible for the production of more than 800 specific metabolites produced by mycobiont hyphae (Hauck and Huneck 2007). A wide range of functions has been suggested for these metabolites including filtration of UV light, regulation of heavy metal uptake or antifeedant effect (Edwards et al. 2003a, b).

In most countries, pH of acid rain (AR) ranges from 4.4 to 2.7 and values above 3.5 did not act as primary stress but below 2.0 it may induce visible injuries (Gabara et al. 2003 and the references therein). Acid rain, in addition to other environmental stresses, stimulates increase in reactive oxygen species (ROS) production as judged from enhancement of antioxidative enzyme activities (Gabara et al. 2003; Wyrwicka and Skłodowska 2006) but little is known about non-enzymatic antioxidants such as phenolic metabolites (Suomela et al. 1998) or proline. These data are

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even absent in epiphytic plants and lichens. Membrane permeability and K^+ leakage in AR-treated lichens were not considerably affected in lichen *Bryoria* in two-year study (Tarhanen et al. 1999).

With respect to growth adaptations of epiphytic plants, they are potentially sensitive to atmospheric pollution. Several studies have presented use of epiphytic plants such as *Tillandsia* species in biomonitoring of atmospheric quality (Pignata et al. 2002 and the references therein) but deeper data about basic metabolic parameters and physiological responses to AR are not available. Lichens are also suitable objects for biomonitoring since adverse effects of industrial emissions on lichens are frequently ascribed to elevated SO_2 level (Tarhanen et al. 1999). Lichens which we used are moderately tolerant to SO_2 and heavy metals (Hauck and Huneck 2007) but *Hypogymnia* occurs mainly on the periphery of Košice city, while *Xanthoria parietina* is common lichen even in the city centre (Bačkor et al. 2003).

Root-less epiphytic plants are therefore good indicator of air pollution since they gain water and nutrients directly from rain/air. However, physiological data related to effects of AR on such species are almost absent. It was therefore the main aim of the present research to study quantitative changes of selected physiological parameters in three epiphytic plants, *Tillandsia albida* Mez and Purpus (Bromeliaceae) and two lichens (*Hypogymnia physodes* (L.) Nyl. and *Xanthoria parietina* (L.) Th. Fr.), exposed to simulated acid rain (pH 3.0) over 3 months. Selected basic physiological parameters (chlorophylls, soluble proteins, free amino acids, mineral nutrients) and specific metabolites/parameters (lichen's metabolites, phenolic acids and enzymatic activities in *Tillandsia*, soluble phenols and flavonoids in all species) were assessed in order to compare resulting responses. Many of these parameters are reported here for the first time. Selected biochemical data are compared with vascular plant *Matricaria chamomilla* L. (chamomile), our standard experimental object.

Materials and methods

Plant material and simulated acid rain

Plants of *Tillandsia albida* were kindly provided by Dr. Klára Repčáková (Botanical Garden of P. J. Šafárik University in Košice); they were ca. 5 years old ones placed on branches of *Vitis vinifera*. Lichens *Xanthoria parietina* and *Hypogymnia physodes* were collected on the locality "Harmanec-jaskyňa" (central Slovakia) and grew on branches of *Larix decidua* (Fig. 1). We note absence of differences in mineral nutrients composition of lichens thalli growing on different trees and the same was found for

Tillandsia (data not shown). Five individual branches for lichens and *Tillandsia* were used as control and five as acid rain-treated (AR). Cultivation was realised in the Laboratory of Plant Stress Physiology (Department of Botany, Faculty of Science, P. J. Šafárik University in Košice) during May–August 2009 (day/night temperature 25–30/18–23°C). Branches were placed on the window where they received natural full sun light (ca. 7:00–11:00 a.m.) with average daily irradiation $\sim 650 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR. We have decided to culture plants in these semi-controlled conditions since artificial light with strict photoperiod in cultivation room is strongly different in comparison with conditions where plants previously grew. Control and AR-treated samples were watered using distilled water with pH 5.5 and 3.0, respectively. These values have been selected based on the literature survey and pH was corrected using 1 M H_2SO_4 (Tarhanen et al. 1999). Note that pH 5.5 is considered as natural rain. Since these plants are root-less, they were irrigated daily (4 L from each pH to 15 branches for either control or AR-treated samples) and solutions were applied in two doses within 1 h in order to maintain plants and branches wet for at least 3 h each day. Solutions were applied using hand-made sprayer and water from branches flowed into the washbasin. No artificial mineral nutrients were applied in order to prevent side effects on the metabolic parameters and composition of mineral nutrients. After 3 months, plants of *Tillandsia* were powdered in liquid N_2 and aliquots were taken for assay of fresh mass-requiring parameters mentioned below; the residue was dried at 70°C in order to determine tissue water content allowing recalculation of parameters measured in fresh samples. Free amino acids, phenolic acids and mineral nutrients were measured in dried samples. Lichens were carefully removed from branches using forceps and since they are poikilohydric, a known amount of biomass was immersed in several drops of HEPES buffer in closed plastic tubes 24 h before measurements (Bačkor et al. 2009a). Spectrophotometry has been carried out with Uvi Light XTD 2 (Secomam, ALES Cedex, France).

Assay of pigments and ROS

Chlorophylls and total carotenoids were extracted from *Tillandsia* using pure methanol (Kováčik and Bačkor 2007a) and from lichens using DMSO (Bačkor et al. 2009a). Measurement and calculation was done according to equations for specific extraction solvent proposed by Wellburn (1994). We also verified efficiency of pigment extraction from *Tillandsia* using different solvents (80% acetone, methanol and DMSO) and non-significant differences were found (data not shown).

Hydrogen peroxide and superoxide were measured in 50 mM potassium phosphate buffer homogenates



Fig. 1 Representative photo of epiphytic plants after 3 months of exposure to simulated acid rain (note absence of visible damage). **a** *Tillandsia albida*, **b** *Xanthoria parietina*, **c** *Hypogymnia physodes*

containing 5 mM polyvinyl polypyrrolidone (pH 7.0). H_2O_2 was measured using $TiCl_4$ (Jana and Choudhuri 1981): to 0.50 ml of supernatant, 0.25 ml of 0.1% titanium chloride (Sigma–Aldrich, Germany) in 20% H_2SO_4 (v/v) was added and the mixture was centrifuged at 15,000 g for 20 min at 4°C. Absorbance was measured at 410 nm. The amount of H_2O_2 was calculated from standardized curve with known H_2O_2 concentrations. Superoxide was estimated according to Elstner and Heupel (1976) by monitoring formation of nitrite from hydroxylamine at 530 nm. Reaction mixture contained 0.27 ml of potassium phosphate buffer, 0.03 ml of 10 mM hydroxylamine, 0.3 ml of supernatant, 0.3 ml of 17 mM sulphanylamine, 0.3 ml of 7 mM α -naphthylamine and 0.3 ml of diethyl ether. The amount of nitrite was calculated from standardized curve with known $NaNO_2$ concentrations.

Quantification of phenolic and lichen's metabolites

Total soluble phenols and flavonoids were extracted with 80% methanol from fresh material. Phenols were quantified using Folin-Ciocalteu method (Singleton and Rossi 1965) with gallic acid as standard according to slightly modified protocol (Kováčik and Bačkor 2007b). Flavonoids were estimated using $AlCl_3$ method (Ordoñez et al. 2006) with quercetin as standard: to 0.5 ml of methanol supernatants, 1 ml of 2% $AlCl_3$ in methanol (w/v) was added. Samples were mixed and absorbance was measured 1 h later at 420 nm.

The selected cinnamic and benzoic acid derivatives in the *Tillandsia* plants were measured in 80% methanol extracts. Extraction and re-purification by solid phase extraction procedure at computer controlled robot Aspec XL, Gilson (USA), HPLC conditions and detection by a mass selective HP MSD quadrupole detector (G1946A, Hewlett-Packard, Palo Alto, USA) was done as described earlier (Kováčik et al. 2007; Kováčik et al. 2009c).

Lichen's metabolites were extracted from dry material using pure acetone. Measurement and calculation using gradient HPLC system with UV-detection were done as described in detail previously (Pöykkö et al. 2005). Ergosterol has been extracted by 99% ethanol and analysed by HPLC as described earlier (Bačkor et al. 2009a).

Measurement of soluble proteins and free amino acids

Proteins were quantified in 50 mM potassium phosphate buffer homogenates (pH 7.0) according to Bradford (1976) using 20 μ l of supernatants and bovine serum albumin as standard (595 nm). For calculation of PAL activity, proteins were quantified in respective homogenates (see below).

Free amino acids were extracted with 80% aqueous ethanol and analyses were performed on an HP 1100 liquid chromatograph (Hewlett Packard, Waldbronn, Germany) with fluorometric detector FLD HP 1100 and using pre-column derivatization with *o*-phthalaldehyde and 9-fluorenylmethyl chloroformate as described earlier (Kováčik et al. 2009a).

Assay of enzymatic activities in *Tillandsia*

Polyphenol oxidase (PPO, EC 1.10.3.2), ascorbate peroxidase (APX, EC 1.11.1.11) and guaiacol peroxidase (GPX, EC 1.11.1.7) activities were assayed in supernatants prepared using 50 mM potassium phosphate buffer containing 5 mM polyvinyl polypyrrolidone (1 g FW/5 ml of buffer) and assayed as the oxidation of catechol, ascorbate and guaiacol at 420, 290 and 470 nm, respectively (Kováčik and Bačkor 2007a; Kováčik et al. 2009c).

Activity of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) was determined as the production of *trans*-cinnamic acid from phenylalanine using the HPLC method with homogenates prepared using sodium borate buffer

(pH 8.7) with UV detection at 275 nm (dos Santos et al., 2004; Kováčik et al. 2007). For all enzymes, randomly selected supernatants were boiled to destroy enzymes and to check that the observed reactions were enzymatic.

Quantification of mineral nutrients

Samples for quantification of mineral nutrients were prepared as described elsewhere (Kováčik et al. 2009c, d). Briefly, dry material was kept overnight in HNO₃ and H₂O₂ mixture (10 ml + 10 ml, Suprapur, Merck) at laboratory temperature and next day evaporated to dryness at 90°C in a water bath (4–5 h). Dry residue was dissolved in 5% HNO₃. Measurements were carried out using an atomic absorption spectrometer AA30 (Varian Ltd., Mulgrave, Australia) and an air-acetylene flame.

Statistical analyses

Data were evaluated using either ANOVA followed by a Tukey's test (MINITAB Release 11, Minitab Inc., State College, Pennsylvania) or Student's *t*-test. Number of replications (*n*) in tables/figures denotes number of samples = number of individual branches (then *n* = 5 as described at the beginning of Materials and Methods section). Two independent repetitions of whole experiment were performed in order to check reproducibility.

Results

Effect of AR on pigments and ROS

Content of chlorophyll *a* decreased in all AR-treated plants, being expressed the most in *Tillandsia* (−90%) while content of chlorophyll *b* showed both negative and positive

changes in AR-exposed plants (Table 1). Accumulation of carotenoids decreased in *Tillandsia* and *Hypogymnia* in response to acid rain. Chlorophyll *a* pheophytinization (ratio of absorbance 435/415) increased in AR-treated *Tillandsia* and *Xanthoria* (expressed as decrease of chlorophyll *a* integrity in this value, Table 1).

Within reactive oxygen species (ROS), accumulation of hydrogen peroxide and superoxide was significantly elevated in AR-exposed *Hypogymnia* only (+133 and +47%, respectively).

Quantitative changes of proteins and free amino acids

Content of soluble proteins was the highest in *Tillandsia* and AR caused significant decrease only in *Hypogymnia* (−21%, Table 2).

Among 17 detected free amino acids, accumulation of glutamate and alanine was exceptionally high in *Xanthoria* control samples contributing to the highest sum of free amino acid in this taxon (Table 2). Both these amino acids significantly decreased in AR-treated samples (−45 and −64% for glutamate and alanine, respectively). *Hypogymnia* showed increase in the sum of amino acids after AR treatment and mainly serine (2.2-fold) and arginine (2.2-fold) contributed to this increase. *Tillandsia* showed the lowest sum of free amino acids and AR had no effect on this sum. Notwithstanding this, methionine strongly increased (Table 2). Accumulation of proline, a stress-induced amino acid, has only slightly increased in *Xanthoria* but decreased in *Hypogymnia* (Table 2).

Effect of AR on phenolic metabolites and lichen's specific metabolites

Total soluble phenols were found to be the most accumulated in *Hypogymnia* control samples, followed by

Table 1 Content of pigments (mg g^{−1} DW), chlorophyll *a* pheophytinization (A435/A415) and amount of reactive oxygen species (ROS)—hydrogen peroxide (μmol g^{−1} DW) and superoxide radical (μg g^{−1} DW) in a studied species exposed to simulated acid rain over 3 months

	<i>Tillandsia</i>		<i>Hypogymnia</i>		<i>Xanthoria</i>	
	Control	Acid rain	Control	Acid rain	Control	Acid rain
<i>Pigments</i>						
Chlorophyll <i>a</i>	1.55 ± 0.09 c	0.14 ± 0.03 f	0.74 ± 0.05 d	0.44 ± 0.008 e	2.87 ± 0.28 a	2.23 ± 0.19 b
Chlorophyll <i>b</i>	0.52 ± 0.029 b	0.035 ± 0.002 e	0.24 ± 0.027 d	0.37 ± 0.031 c	0.75 ± 0.030 a	0.61 ± 0.028 b
Carotenoids	0.39 ± 0.031 b	0.17 ± 0.028 cd	0.25 ± 0.019 c	0.13 ± 0.016 d	1.06 ± 0.14 a	1.13 ± 0.08 a
A435/A415	1.22 ± 0.006 b	1.02 ± 0.019 c	0.57 ± 0.015 d	0.59 ± 0.023 d	1.38 ± 0.004 a	1.09 ± 0.011 c
<i>ROS</i>						
Hydrogen peroxide	46.3 ± 4.34 a	45.5 ± 3.08 a	2.29 ± 0.16 c	5.34 ± 0.55 b	1.02 ± 0.16 d	1.45 ± 0.21 d
Superoxide	23.0 ± 2.12 a	23.4 ± 3.22 a	4.01 ± 0.28 c	5.91 ± 0.14 b	1.82 ± 0.28 d	2.43 ± 0.19 d

Data are means ± SDs (*n* = 5). Values within horizontal lines, followed by the same letter(s), are not significantly different according to Tukey's test (*P* < 0.05). *nd* not detected

Table 2 Accumulation of free amino acids ($\mu\text{mol g}^{-1}$ DW) and soluble proteins (mg g^{-1} DW) in a studied species exposed to simulated acid rain over 3 months

	<i>Tillandsia</i>		<i>Hypogymnia</i>		<i>Xanthoria</i>	
	Control	Acid rain	Control	Acid rain	Control	Acid rain
Aspartic acid	1.13 b	0.76 bc	0.91 bc	0.94 bc	1.56 a	0.67 c
Glutamic acid	0.54 d	0.37 d	4.13 c	4.36 c	12.24 a	6.79 b
Serine	1.19 b	1.64 b	1.18 b	2.61 a	2.52 a	2.59 a
Histidine	0.14 b	0.13 b	0.084 b	0.12 b	0.79 a	0.87 a
Glycine	0.42 d	0.71 c	0.67 c	0.92 bc	1.24 ab	1.68 a
Threonine	0.58 b	0.69 b	0.60 b	0.86 b	1.69 a	1.57 a
Arginine	0.13 d	0.14 d	1.23 c	2.74 a	1.21 c	1.67 b
Alanine	0.81 d	0.93 d	3.22 b	2.60 b	4.62 a	1.66 c
Tyrosine	0.45 b	0.48 b	0.053 c	0.051 c	0.75 a	0.68 a
Cysteine	0.51 bc	0.78 a	0.26 d	0.48 c	0.49 c	0.65 ab
Valine	0.53 cd	0.65 bc	0.25 d	0.39 d	1.76 a	0.91 b
Methionine	0.026 c	0.50 a	0.13 b	0.14 b	0.17 b	0.14 b
Phenylalanine	0.29 d	0.41 c	0.038 e	0.068 e	0.85 a	0.67 b
Isoleucine	0.28 c	0.35 c	0.14 d	0.16 d	0.98 a	0.68 b
Leucine	0.40 bc	0.50 b	0.13 d	0.22 cd	0.88 a	0.99 a
Lysine	0.18 d	0.28 c	0.097 e	0.24 cd	1.70 b	2.46 a
Proline	0.12 c	0.18 c	0.35 a	0.28 b	0.17 c	0.28 b
<i>Sum</i>	7.76 e	9.55 e	13.49 d	17.12 c	33.67 a	24.98 b
Proteins	5.08 a	4.60 ab	4.21 b	3.34 c	2.75 cd	2.24 d

Data are means ($n = 5$); for the lucidity of table, SDs are not shown. Values within horizontal lines, followed by the same letter(s), are not significantly different according to Tukey's ($P < 0.05$)

Tillandsia. *Xanthoria* showed the lowest accumulation of total soluble phenols (Fig. 2). Acid rain stimulated increase in soluble phenols in *Tillandsia* (+27%) and *Hypogymnia* (+20%) but had no effect in *Xanthoria*. In contrast, flavonoids have decreased in *Hypogymnia* (−42%) and *Xanthoria* (−24%).

Among 12 derivatives of benzoic and cinnamic acid (so-called phenolic acids) detected in *Tillandsia* leaves, only vanillin increased in response to AR. Accumulation of chlorogenic acid showed the most pronounced decrease (Table 3).

Lichen's specific aromatic metabolites showed increase in response to AR (parietin, atranorin, physodalic acid). Ergosterol, principal sterol of mycobiont (fungal) plasma membranes, showed tendency to increase but differences were not strong enough to be significant (Table 3).

Enzymatic activities in *Tillandsia*

Activities of two common phenolic metabolism-related enzymes were enhanced in AR-exposed samples: PAL +48% and PPO +89%. Two common peroxidases also showed increased activities in response to AR (APX +34% and GPX +146%, Fig. 3).

Effect of AR on mineral nutrients

At the level of mineral nutrients, accumulation of potassium and magnesium decreased in AR-treated samples

from each species (−34/−43/−26% for K^+ and −14/−83/−37% for Mg^{2+} in *Tillandsia/Hypogymnia/Xanthoria*, Table 4). Content of calcium decreased in the lichens (−38 and −50% in *Hypogymnia* and *Xanthoria*, respectively) and Zn in *Tillandsia*. Amounts of Na, Fe and Cu were not affected by AR in any of species (Table 4).

Discussion

Negative effects of environmental stress are usually obvious at the level of chlorophyll which was also depleted in the present study (Table 1). This was the most pronounced in *Tillandsia* samples as the accumulation of chlorophyll *a* decreased ca. 10-times in response to AR. Since no direct damage of leaves were visible and chlorophyll *a* pheophytinization increased only slightly we assume that its biosynthesis was affected. This may also be judged from decrease of chlorophyll *b* and its concentration was extremely low in AR samples. Chlorophyll *a/b* ratio was ca. 3.0 in control samples, similarly to value found in vascular plants such chamomile measured by the same method in methanol extracts (Kováčik and Bačkor 2007a). In the literature, there are similar results presented on this ratio in the genus *Tillandsia*, e.g. 1.1 in *T. fasciculata* (Koh and Davies Jr 2001) or 3.0 in *T. usneoides* (Haslam et al. 2003) and other Bromeliaceae such as *Aechmea* and *Guzmania* have these ratios also ca. 3.0 (Croonenborghs et al. 2009). Sum of chlorophyll was found to be 1–2 mg g^{-1} DW in

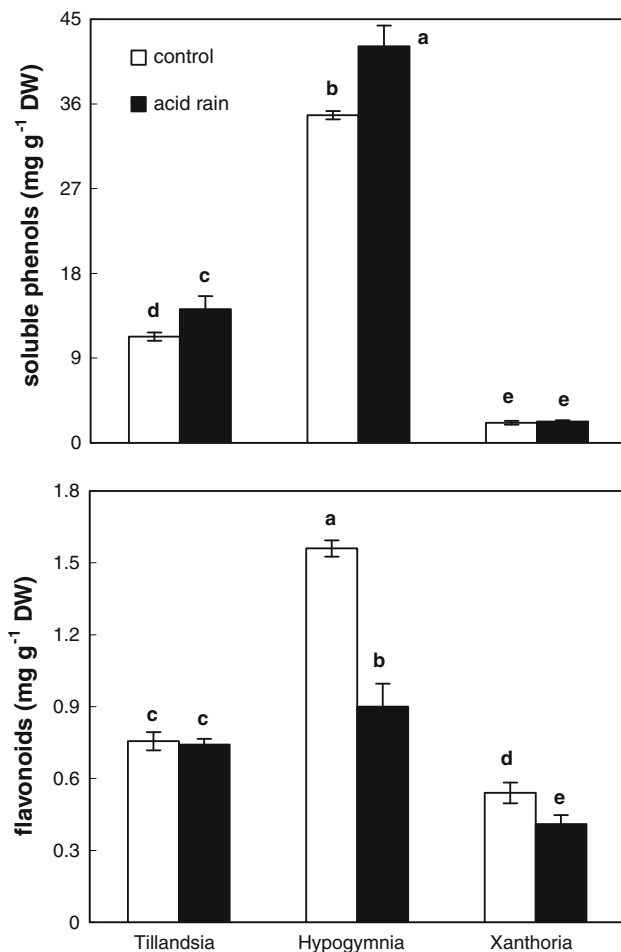


Fig. 2 Amounts of total soluble phenols and flavonoids in epiphytic plants after 3 months of exposure to simulated acid rain. Data are means \pm SDs ($n = 5$). Values within each graph followed by the same letter are not significantly different according to Tukey's test ($P < 0.05$)

different *Tillandsia* species (Koh and Davies Jr 2001; Haslam et al. 2003) including our present data (Table 1). *Xanthoria* showed similar decrease of both chlorophyll *a* and *b*, indicating effect of AR on biosynthesis. We note that this decrease was very small in relation to duration of treatment and other kinds of stresses applied on lichens, such as excess of copper, caused more dramatic decrease (Bačkor et al. 2009a). This is good evidence that this species is well adapted to polluted environment and its populations really occur on the urban localities where more sensitive lichens are absent (Bačkor et al. 2003). In contrast, decrease of chlorophyll *a* but increase in chlorophyll *b* which we observed in AR-treated *Hypogymnia* samples confirms conversion of chlorophyll *a* to *b* which is mediated by the oxidation of methyl group on ring II to the aldehyde (Bačkor and Váczi 2002). However, decrease in chlorophylls alone in response to AR treatments was the most visible (in comparison with other parameters measured in

Table 3 Accumulation of selected phenolic acids in *Tillandsia* (ng g⁻¹ DW), parietin in *Xanthoria* (mg g⁻¹ DW), atranorin and physodalic acid in *Hypogymnia* (mg g⁻¹ DW) and ergosterol in lichens (mg g⁻¹ DW) after 3 months of simulated acid rain

	Control	Acid rain
<i>Tillandsia</i>		
Protocatechuic a.	758.2 \pm 50.8	544.9 \pm 39.4***
Protocatechuic ald.	834.9 \pm 37.8	899.8 \pm 56.6 ^{ns}
<i>p</i> -OH-benzoic a.	960.9 \pm 145.1	617.6 \pm 100.6**
<i>p</i> -OH-benzoic ald.	2508 \pm 362	2101 \pm 117.7 ^{ns}
Vanillic a.	6017 \pm 229.1	6014 \pm 214.2 ^{ns}
Vanillin	4289 \pm 291	5443 \pm 282.7**
Syringic a.	549.2 \pm 28.0	387.7 \pm 15.2***
Salicylic a.	252.7 \pm 22.3	186.4 \pm 18.8**
Chlorogenic a.	17 474 \pm 390.3	5285 \pm 273.8***
Caffeic a.	2332 \pm 223.9	1799 \pm 150.2**
<i>p</i> -coumaric a.	1450 \pm 127.4	984.1 \pm 21.1***
Ferulic a.	1318 \pm 52.4	1148 \pm 107.8*
<i>Hypogymnia</i>		
Atranorin	2.86 \pm 0.11	4.07 \pm 0.69*
Physodalic acid	15.9 \pm 3.26	69.5 \pm 3.27***
Ergosterol	0.56 \pm 0.02	0.62 \pm 0.07 ^{ns}
<i>Xanthoria</i>		
Parietin	5.83 \pm 1.27	12.5 \pm 1.90**
Ergosterol	0.71 \pm 0.03	0.75 \pm 0.04 ^{ns}

Data are means \pm SDs ($n = 5$). *, **, *** indicate significant difference at 0.05, 0.01 and 0.001 level of Student's *t*-test in comparison with respective control. *ns* non-significant. *a.* acid, *ald.* aldehyde

this study) but it can only hardly be considered as the only evidence of AR toxicity and further studies are needed.

We have also measured tissue water content in *Tillandsia* and it was considerably high, being 87.4 and 86.9% in control and AR-treated ones, respectively. These data are similar to hydroponically cultured chamomile (ca. 92%) and suggest high water potential of leaf tissue (close to zero; see Martin et al. 2004 for details). It was really found that many epiphytic plants have high osmotic potential (slightly negative) which was explained by existence of "hydrenchyma" tissue allowing them to overcome drought periods (Martin et al. 2004), agreeing with high water content what we observed in the present study. This high osmotic potential also indicates that concentration of solutes will be low and more data were urged (Martin et al. 2004). Our results fit well with the mentioned facts since many compounds such as amino acids, proteins and mineral nutrients were less-accumulated in *Tillandsia* samples in comparison with C₃ plants such as chamomile (Kováčik et al. 2009c, d).

Free amino acids represent pool for different metabolic processes therefore their damage by stress effects may have extensive impact on overall metabolism. We found

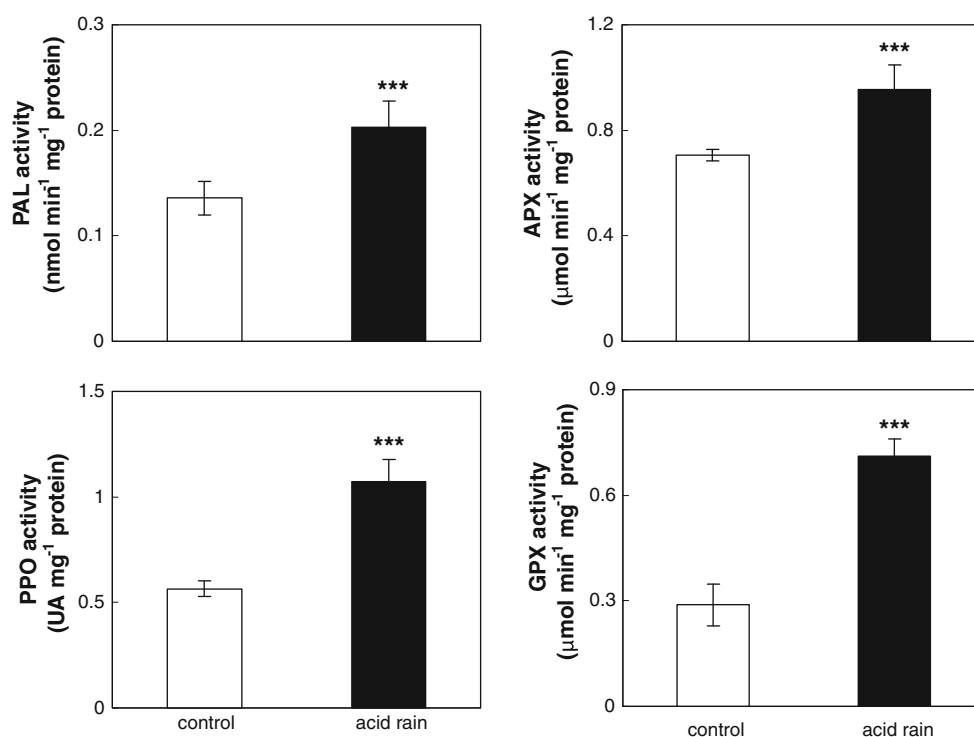


Fig. 3 Activities of selected enzymes (*PAL* phenylalanine ammonia-lyase, *PPO* polyphenol oxidase, *APX* ascorbate peroxidase, *GPX* guaiacol peroxidase) in *Tillandsia* plants after 3 months of exposure

to simulated acid rain. *** indicate significant difference at 0.001 level of Student's *t*-test in comparison with respective control. Data are means ± SDs ($n = 5$)

Table 4 Accumulation of selected mineral nutrients in a studied species exposed to simulated acid rain over 3 months

	<i>Tillandsia</i>		<i>Hypogymnia</i>		<i>Xanthoria</i>	
	Control	Acid rain	Control	Acid rain	Control	Acid rain
K (mg g ⁻¹ DW)	12.3 ± 0.80 a	8.04 ± 0.39 b	3.39 ± 0.36 d	1.93 ± 0.16 e	5.82 ± 0.64 c	4.31 ± 0.56 d
Na (mg g ⁻¹ DW)	5.34 ± 0.36 ab	5.65 ± 0.44 a	4.81 ± 0.31 abc	4.69 ± 0.37 bc	4.26 ± 0.40 c	4.39 ± 0.28 c
Ca (mg g ⁻¹ DW)	5.50 ± 0.35 bc	4.68 ± 0.37 c	10.4 ± 0.79 a	6.45 ± 0.27 b	3.16 ± 0.17 d	1.57 ± 0.24 e
Mg (mg g ⁻¹ DW)	7.24 ± 0.51 a	6.20 ± 0.28 b	1.51 ± 0.23 c	0.25 ± 0.012 e	1.07 ± 0.18 c	0.67 ± 0.062 d
Fe (mg g ⁻¹ DW)	0.30 ± 0.048 b	0.27 ± 0.035 b	0.56 ± 0.048 a	0.55 ± 0.021 a	0.57 ± 0.018 a	0.53 ± 0.015 a
Zn (μg g ⁻¹ DW)	246.5 ± 17.2 a	176.0 ± 17.6 b	122.6 ± 5.0 c	120.4 ± 5.3 c	40.7 ± 3.06 d	38.0 ± 2.11 d
Cu (μg g ⁻¹ DW)	4.58 ± 0.15 b	4.26 ± 0.21 b	6.37 ± 0.15 a	6.09 ± 0.26 a	4.51 ± 0.44 b	4.68 ± 0.26 b

Data are means ± SDs ($n = 5$). Other details as in Table 1

relatively negligible effect of AR on amino acid composition and no similar study is available for deeper comparison. High amount of free amino acids in *Xanthoria* is mainly caused by abundance of glutamate, serving as primary acceptor for glutamine synthetase. Our earlier study showed that sum of free amino acids in terrestrial lichen *Peltigera rufescens* was also high (close to *Xanthoria* in the present study) but *Cladonia mitis* had the lowest amount presently known (Bačkor et al. 2009b) suggesting genetically controlled inter-specific differences since lichens in the present study are both epiphytic, foliose and contain the same photobiont (from the green algal genus *Trebouxia*

sensu lato). However, abundance of free amino acids in *Xanthoria* confirms that it is nitrophytic lichen (Pirintsos et al. 2009) and it also contains higher amount of total nitrogen in comparison with *Hypogymnia* (data not shown). Very low content of proline (in comparison with chamomile, Kováčik et al. 2009a, c) and its slight change in response to AR in all species is another indication of low impact of acid rain-evoked acidification on nitrogen metabolism. In accordance, no extreme decrease in soluble proteins has been found (Table 2). Low proline content in *Tillandsia* is also in accordance with high osmotic potential of epiphytic plants mentioned above since this compound

is a well-known stress-induced amino acid being involved in the mechanisms of osmoregulation (Gajewska and Skłodowska 2005). We also observed strong increase in methionine in *Tillandsia* samples and significance of this observation remains unclear. To our knowledge, these are first data reporting profile of free amino acids in any Bromeliaceae species.

Phenolic metabolites are one of the most abundant organic compounds in plants being involved in many stress responses. At first sight, the highest content of soluble phenols in *Hypogymnia* (Fig. 2) could be evoked by an increase in atranorin and physodalic acid accumulation (Table 3) but we found that only atranorin reacts with Folin-Ciocalteu reagent thus can contribute to increase in soluble phenols in AR-treated samples. In combination with the highest content of flavonoids just in *Hypogymnia* (Fig. 2), this species seems to be potential source of new phenolic metabolites. We note that we measured flavonoids as AlCl_3 -reactive compounds and in this assay, only flavonoids possessing 4-keto and 3- and/or 5-hydroxy group have been suggested to react with AlCl_3 (Deng and van Berel 1998) indicating presence of flavonols. Composition of flavonoids in lichens is not well known and their presence has only occasionally been mentioned (Momoh and Adikwu 2008). Our quantitative data indicate that flavonoids form small part of total soluble phenols and this could be explained by low volume of photobiont cells in lichen's thalli (up to 10% in average). Increase in soluble phenols but decrease in flavonoid accumulation in almost all species suggests that flavonoids do not play important role in tolerance to acid rain. Our data are in accordance with results found in non-epiphytic plants such as *Betula pubescens* where individual phenolics were not considerably affected (Suomela et al. 1998).

In lichens, their specific metabolites could be more essential for responses to AR. For example, increase in parietin in *Xanthoria* (Table 3) was macroscopically visible as intensification in yellow-orange colour and since we cultured samples in the most natural environment (full sun light, see [Materials and Methods](#) section), this indicates AR-evoked increase in sensitivity to light. *Hypogymnia* is a moderately sensitive to SO_2 and heavy metals (Hauck and Huneck 2007) and just physodalic acid was found to increase in thalli transplanted to areas with elevated pollution (Białońska and Dayan 2005). Our data fit well with this finding and strong induction of physodalic acid accumulation was observed in AR-treated *Hypogymnia* thalli (Table 3), representing first direct evidence of this process. Substances in *Hypogymnia* including physodalic acid have also other important functions such as the regulation of metal uptake (Hauck and Huneck 2007) therefore their role in metal homeostasis in the present study may be plausible (Table 4). Ergosterol is a main component of fungal

plasma membrane (Tarhanen et al. 1999) and decrease in its concentration indicates damage to overall lichen's vitality (Bačkor et al. 2009a). Our data showed that despite decrease in K^+ content in lichens (Table 4) suggesting damage to membrane integrity, amounts of ergosterol did not decrease but showed tendency to increase (although differences were not strong enough to be significant, Table 3). Similar discrepancy has been found in lichen *Bryoria fuscescens* exposed to acidity (pH 3, H_2SO_4 , Tarhanen et al. 1999) therefore AR-evoked K^+ decrease is not related to changes in ergosterol accumulation and further studies are needed.

It was surprising to find that soluble phenols and flavonoids were present in low quantities in *Tillandsia* samples since it would be expected that such sun-loving plant will contain higher amount of UV-absorbing compounds (such as flavonoids) in comparison with vascular plant (Kováčik et al. 2009b). This paradox may be explained by its morphology and xerophytic leaves with almost white surface (highlighted in its species name—*albida*, Fig. 1) thus, increasing reflectance of sun rays. Decrease in individual phenols is also visible at the level of detected phenolic acids (we again note their substantially lower accumulation in comparison with chamomile) and only vanillin has increased in response to AR (Table 3). This is a well-known antioxidative compound (Kováčik et al. 2009a) then different responses even within the same group of phenols are presumed thus contributing to slight increase in total soluble phenols. This increase correlates with enhancement of phenylalanine ammonia-lyase (PAL) activity which we recoded in AR-exposed *Tillandsia* samples (Fig. 3). Increase in polyphenol oxidase activity could also contribute to modulation of soluble phenols. Despite prolonged exposure to AR, *Tillandsia* plants also showed elevated activities of peroxidases (APX and GPX), which are important components in removal of excess H_2O_2 , thus leading to unchanged level of ROS (Table 1). We note that APX activities were even higher in comparison with chamomile leaves (Kováčik et al. 2009c) indicating significance of this enzyme at least in this *Tillandsia* species. Other enzymes showed similar or slightly lower activities in comparison with chamomile leaves, indicating that despite considerably lower amount of proteins (ca. 20-times in comparison with chamomile), enzymatic machinery has similar effectiveness.

Macronutrients were more depleted by AR in comparison with micronutrients (Table 4). Especially K^+ was abundant in *Tillandsia* in comparison with lichens and it decreased in all three species. More expressive decrease in Ca and Mg in lichens (if expressed as % of control) could be ascribed to different morphology since minerals may be more readily eluted from damaged thalli in comparison with tissue structure of *Tillandsia* leaves. A study by Pyatt

et al. (1999) has shown that in naturally growing *Tillandsia usneoides* and lichen *Parmotrema praesorediosum*, selected metals and sulphur were bio-accumulated and the accumulation of certain heavy metals increases with age of the *T. usneoides*.

In conclusion, comparison of vascular (*Tillandsia*) and non-vascular (lichens) epiphytic taxa showed that sensitivity to AR differed depending on evaluated parameter. Extensive decrease in chlorophyll amount in *Tillandsia albida* does not match well with absence of visible damage and slight decrease in Mg content suggesting specificity of this assay and further ecotoxicological studies are needed. Among lichens, *Xanthoria* showed higher tolerance in comparison with *Hypogymnia* (at the level of pigments, ROS, mineral nutrients), suggesting that it could be useful for long-term biomonitoring. Accumulation of free amino acids and proteins were not considerably damaged and there exist no data in these or similar species which we could discuss in details. Overall, lichens seem to respond to AR by synthesis of specific metabolites while in *Tillandsia*, enhanced enzymatic activities (including peroxidases we measured) may contribute to low oxidative stress as visible from unaffected ROS accumulation.

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