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Evaluation of micro-energy dispersive X-ray fluorescence and histochemical tests for aluminium detection in plants from High Altitude Rocky Complexes, Southeast Brazil

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ABSTRACT

The soils developed under High Altitude Rocky Complexes in Brazil are generally of very low chemical fertility, with low base saturation and high exchangeable aluminium concentration. This stressful condition imposes evolutionary pressures that lead to ecological success of plant species that are able to tolerate or accumulate high amounts of aluminium. Several analytical methods are currently available for elemental mapping of biological structures, such as micro-X-ray fluorescence (μ-EDX) and histochemical tests. The aim of this study was to combine μ-EDX analysis and histochemical tests to quantify aluminium in plants from High Altitude Rocky Complexes, identifying the main sites for Al-accumulation. Among the studied species, five showed total Al concentration higher than 1000 mg kg^{-1}. The main Al-hyperaccumulator plants, *Lavoisiera pectinata*, *Lycopodium clavatum* and *Trembleya parviflora* presented positive reactions in the histochemical tests using Chrome Azurol and Aluminon. Strong positive correlations were observed between the total Al concentrations and data obtained by μ-EDX analysis. The μ-EDX analysis is a potential tool to map and quantify Al in hyperaccumulator species, and a valuable technique due to its non-destructive capacity. Histochemical tests can be helpful to indicate the accumulation pattern of samples before they are submitted for further μ-EDX scrutiny.

Key words: Al-hyperaccumulator plants, aluminon, chrome azurol, High Altitude Rocky Complexes, μ-EDX.

INTRODUCTION

The soils of tropical and subtropical regions commonly exhibit acidic properties due to intense leaching, which results in removal of negative charges and retention of compounds containing iron and aluminium (Echart and Molina 2001). About 30 % of the world's soils are acidic, with pH ≤ 5.5, presenting low levels of organic matter and base saturation, and high levels of exchangeable aluminium (Al^{3+}) (Hartwig et al. 2007). In Brazil,

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soils derived from granitic and gneiss rocky outcrops of the Serra da Mantiqueira are associated with high Al^{3+} contents (Benites et al. 2007).

High soil acidity increases aluminium availability, which, in turn, can affect Al-sensitive plants causing both root growth inhibition and thickening of root epidermis (Ciamporová 2002, Vitorello et al. 2005). Low nutrient availability combined with high aluminium concentrations in soil solution act as an important environmental stress agent (Larcher 2000, Grime 2001). The tolerance to high Al^{3+} concentrations is an ecological attribute that permits the occupation of a site qualified as inappropriate for Al-sensitive species (Jansen et al. 2002a). Some plants living in these environments can accumulate more than 1000 mg kg^{-1} of Al in their tissues, being called hyperaccumulator species (Baker 1981). Hyperaccumulator plants have mechanisms of aluminium resistance, such as synthesis of chelator agents and turnover of roots and leaves which have already reached high levels of aluminium (Cuenca and Herrera 1987, Cuenca and Medina 1990).

Melastomataceae, Rubiaceae, Asteraceae, Vochysiaceae and Myrtaceae contain a great number of taxa whose life histories are related to soils presenting high Al contents (Chenery and Sporne 1976, Haridasan 1988, Jansen et al. 2000, 2002a, b). Lycopodiaceae and Pteridaceae were previously described as pertaining to the aluminium accumulator families (Church 1888, Olivares et al. 2009). The chelation mechanism of these species is based on Al^{3+} retention by organic acids in celular compartments such as cell wall and vacuole (Taylor 1991, Delhaize and Ryan 1995, Shen et al. 2002).

The study of metal distribution patterns in plant tissues can clarify accumulation processes in tolerant species, simultaneously quantifying and mapping the content of chemical elements (Moradi et al. 2010). Methods commonly used for mapping studies in biological tissues are based on μ-EDX or Synchrotron radiation X-ray fluorescence (West et al. 2009, Majumdar et al. 2012), micro-proton-induced X-ray emission (Lyubenova et al. 2012), and also laser-based methods such as laser ablation inductively coupled plasma mass spectrometry (Guerra et al. 2011, Qin et al. 2011) and laser induced breakdown spectroscopy (Galiová et al. 2007, Santos Jr. et al. 2012, Piñon et al. 2013).

X-ray fluorescence analysis (XRF) is a fast and non-destructive method which has several applications (Saisho and Hashimoto 1996, West et al. 2009). Although the common detection limit of the XRF technique ranges from mg kg^{-1} to % ww^{-1} (Saisho and Hashimoto 1996), it can be successfully applied to hyperaccumulator species tissues (Memon et al. 1981, Cuenca et al. 1991, Robinson et al. 2003, Broadhurst et al. 2004, Berazain et al. 2007, Turnau et al. 2007, Tolrà et al. 2011). In order to perform quantitative measurements, calibration can be done by the linear relationship between the intensity of X-ray emission of a target element and its concentration previously determined by a reference method. The quality of the XRF data can be evaluated by the correlation between the XRF intensity and the reference values using figures of merit such as linear correlation factor, standard error of prediction (SEP), confidence intervals and bias (Paltridge et al. 2012).

Applying histochemical and chemical techniques for detection of compounds of interest, such as potentially toxic metals, can complement the data obtained from X-ray microanalysis, since they are useful for locating discrete quantities of chemical elements in biological tissues (Pearse 1972, 1988, Krishnamurthy 1998). Several reagents are used in Al histolocalization, such as Hematoxylin, Aluminon, Chrome Azurol, Pyrocatecol and Azurine (Baker 1962, Denton et al. 1984, Clark and Krueger 1985, Haridasan et al. 1986, Cotta et al. 2008).

In this context, this study aimed to combine μ-EDX analysis and histochemical tests to quantify aluminium in plants from High Altitude Rocky Complexes, characterizing, furthermore, the main sites for Al-accumulation in shoots.

According to our knowledge this is the first study reporting aluminium detection in hyperaccumulator plants from High Altitude Rocky Complexes using both μ-EDX apparatus and histochemical tests.

MATERIALS AND METHODS

Study Area

Serra do Brigadeiro State Park

The Serra do Brigadeiro State Park is located in Araponga (State of Minas Gerais, Brazil, 20°40' S and 42° 26' W) in Serra da Mantiqueira massif. According to Köeppen, the climate is mesothermal (CWb). The mean annual rainfall and air temperature are about 1500 mm and 15 °C, respectively (Benites et al. 2001).

The High Altitude Rocky Complexes are located in the highest points of the Serra da Mantiqueira massif, in areas above 1500 m, being associated with igneous and metamorphic parent materials (Vasconcelos 2011). The vegetation cover is related to soils with high aluminium saturation and low calcium and magnesium contents (Benites et al. 2001).

Studied Species

Ten plant species were sampled: *Marcetia taxifolia, Lavoisiera pectinata*, *Tibouchina heteromalla* and *Trembleya parviflora* (Melastomataceae); *Baccharis trimera* and *Eremanthus erythropappus* (Asteraceae); *Nanuza plicata* and *Vellozia variegata* (Velloziaceae); *Myrsine umbellata* (Myrsinaceae) and *Lycopodium clavatum* (Lycopodiaceae). Samples of three individuals of each species were collected. Soil and vegetation classification of sampling sites are described in Table I (B.V. Tinti et al., unpublished data).

TABLE I
Soils and vegetation types studied at the Serra do Brigadeiro State Park.

SOIL	SPECIES	FAMILY	VEGETATION	SOIL	$[Al^{3+}]$ *
1	*Lavoisiera pectinata* Cogn.	Melastomataceae	Monodominant wet scrub Lavoisiera	Dystric Humic Cambisol	1.3
	Eremanthus erythropappus (DC.) MacLeish	Asteraceae	Dwarf forest /scrub	Spodic Sapric Organosol	3.7
	Marcetia taxifolia (A. St.-Hil.) DC.	Melastomataceae	Dwarf forest /scrub	Spodic Sapric Organosol	3.7
2	*Myrsine umbellata* Mart.	Myrsinaceae	Dwarf forest /scrub	Spodic Sapric Organosol	3.7
	Tibouchina heteromalla Cogn.	Melastomataceae	Dwarf forest /scrub	Spodic Sapric Organosol	3.7
	Trembleya parviflora (D. Don) Cogn.	Melastomataceae	Dwarf forest /scrub	Spodic Sapric Organosol	3.7
	Baccharis trimera (Less) DC.	Asteraceae	Wet grassy field	Dystric Humic Cambisol	1.4
3	*Nanuza plicata* (Mart.) L.B.Sm. & Ayensu	Velloziaceae	Wet grassy field	Dystric Humic Cambisol	1.4
	Vellozia variegata Goethart & Henrard	Velloziaceae	Wet grassy field	Dystric Humic Cambisol	1.4
4	*Lycopodium clavatum* L.	Lycopodiaceae	Scarpment scrub	Folic Organosol	1.4

* Exchangeable Al concentration (cmolc dm^{-3}) at the 0-20 cm horizon.

AL-Localization in Plant Tissues

Samples of stems and leaves from all plant species were fixed using a solution composed of formaldehyde and acetic acid (FAA) 50 % (v v^{-1}), dehydrated through an ethanol series (Johansen 1940) and freehand sectioned with a razor blade. Subsequently, the sections were submitted to a histochemical test using Chrome Azurol (reaction time: 15 minutes). Sections that showed positive reactions, evidenced by the blue color, were subjected to new tests with Chrome Azurol and Aluminon for 30 minutes. For Aluminon test the intense red color is identified as a positive reaction.

Photographs were taken using a light microscope (Olympus AX70TRF, Olympus Optical, Tokyo, Japan) coupled with a U-Photo Camera system (Spot Insightcolour 3.2.0, Diagnostic Instruments inc. New York, USA).

AL-Determination in Plant Material

Determination of total Al concentration using ICP OES

Samples of plant material (n = 10) were oven-dried at 70 °C, until constant weight, and were powdered with the help of a knife mill.

Powdered samples were digested, in triplicate, with nitro-perchloric solution in an electric plate following procedure described by Tedesco et al. (1995). A comparative wet-based decomposition procedure based on nitric acid and hydrogen peroxide using a microwave-assisted digestion was also performed as described by Guerra et al. (2013).

Determination of total Al concentration in acid extracts was performed using inductively coupled plasma optical emission spectrometry with dual-view configuration (Perkin Elmer, Shelton, CT, EUA). Operational conditions of ICP OES measurements were described in Table II.

TABLE II
Operational conditions in ICP OES determinations.

Instrumental parameters	Operational conditions
Generator frequency (MHz)	40
Spray chamber	Cyclonic
RF applied power (kW)	1.3
Integration time (s)	1.0
Plasma gas flow rate (L/min)	15
Auxiliary gas flow rate (L/min)	1.5
Nebulizer gas flow rate(L/min)	0.8
Sampling flow rate (mL/min)	1.5
Al I (nm)	396.152

µ-EDX analysis

Samples which showed higher Al concentration were selected and analyzed using micro-energy dispersive X-ray fluorescence technique (µ-EDX-1300, Shimadzu, Kyoto, Japan). The usefulness of this technique in determination of total Al concentration and mapping studies on the raw sample material was evaluated.

Pellets of powdered plant samples (particle size lower than 250 µm) were prepared after applying 10 t cm^{-2} (Perkin Elmer, Waltham, MA, EUA) pressure during 5 minutes on 0.15 g of dried material. On each pellet 10 points were randomly selected to be analyzed by µ-EDX apparatus. The operational conditions were described in Table III.

A linear regression model was adjusted to correlate Al total concentration determined by ICP OES and µ-EDX intensity (cps μA^{-1}) as recommended by Guerra et al. 2013. By using this linear regression model, it was possible to evaluate the Al distribution on the aerial parts of pre-selected plants. Samples of leaves, with or without the stem, were oven dried at 70 °C and fixed on paper supports using adhesive tape. For each plant species, different parts of leaves and stem were analyzed and, for each part, five points were randomly selected.

Statistical Analysis

The data was analyzed using analysis of variance (ANOVA), followed by Tukey test at 5% signifi-

TABLE III
Operational conditions in µ-EDX analysis.

Instrumental parameters	Operational conditions
Measuring principle	X-ray fluorescence spectrometry
Measuring method	Energy-dispersive X-ray analysis
Working distance (mm)	1.5
Detector	Si(Li) semiconductor
Irradiated diameter (µm)	50
Measurement time (s)	200
Analyzed spectra region (keV)	0.00 – 4.00
Measuring atmosphere	Atmospheric air
X-ray power unit	X-ray tube (Rh target)
Monitored peak	Al (Kα) – 1.49 keV
Channel	Na-Sc
Electric voltage (kV)	15
Electric current (µA)	500

cance level. All analyses were performed using R 2.13 software (R Development Core Team 2006) and were followed by residual analyses to check for the suitability of the models (Crawley 2007).

RESULTS

AL-Localization in Plant Tissues

Among the analyzed species, seven presented negative result for Al presence using Chrome Azurol (*Myrsine umbellate, Eremanthus erythropappus*, *Vellozia variegata*, *Baccharis trimera*, *Tibouchina heteromalla*, *Nanuza plicata* and *Marcetia taxifolia*). Otherwise, three species (*Lavoisiera pectinata*, *Trembleya parviflora* and *Lycopodium clavatum*) showed a positive reaction for Chrome Azurol and Aluminon, which indicates higher Al concentration in the stem or leaf tissues (Figure 1). The positive reaction to Chrome Azurol was noted in primary walls, epidermal cells, parenchyma and phloem. A negative reaction to Chrome Azurol was observed in secondary cell walls including those of xylem vessels and sclerenchyma fibers. Aluminon results showed the same pattern observed for Chrome Azurol.

The reaction in *L. pectinata* was markedly evident in the cuticle layer on both sides of the leaf epidermis cells (Figure 1A-C). In *T. parviflora* a negative reaction to Chrome Azurol was noted in palisade parenchyma cells (Figure 1E) even though a positive reaction to Aluminon had been detected (Figure 1D). A positive reaction to Aluminon was observed around the vascular cylinder in *L. clavatum* (Figure 1G).

AL-Determination in Plant Material

Total Al concentration

The average of the total Al concentration in plants (mg kg^{-1}) was as follows: *Vellozia variegata* (216), *Myrsine umbellata* (297), *Nanuza plicata* (344), *Marcetia taxifolia* (641), *Baccharis trimera* (749), *Eremanthus erythropappus* (1149), *Tibouchina heteromalla* (2000), *Trembleya parviflora* (3878), *Lavoisiera pectinata* (8589) and *Lycopodium clavatum* (9049). The following samples: *L. clavatum*, *L. pectinata* and *T. parviflora* were selected for X-ray fluorescence analysis due the highest Al concentrations observed in these species.

µ-EDX results

Micro-energy dispersive X-ray fluorescence analysis revealed significant differences between the studied species after evaluating the data obtained from Al peak intensities ($F_{2,27} = 14.323$; $p < 0.0001$) at 1.49 keV (Kα line). *L. clavatum* (0.029 cps μA^{-1}) and *L. pectinata* (0.027 cps μA^{-1}) had the highest intensities and its averages were not significantly different ($F_{2,28} = 0.245$, $p = 0.62$) (Figure 2). *T. parviflora* had a mean significantly lower (0.0123 cps μA^{-1}). Linear regression model, adjusted in order to verify the correlation between the total Al concentrations and the intensity values, revealed a high linear correlation factor (r^2 higher than 0.99).

The highest Al concentrations in leaves, obtained by µ-EDX analysis were observed in *L. pectinata* ($F_{2,9} = 31.84$, $p = 0.002$). This result, however,

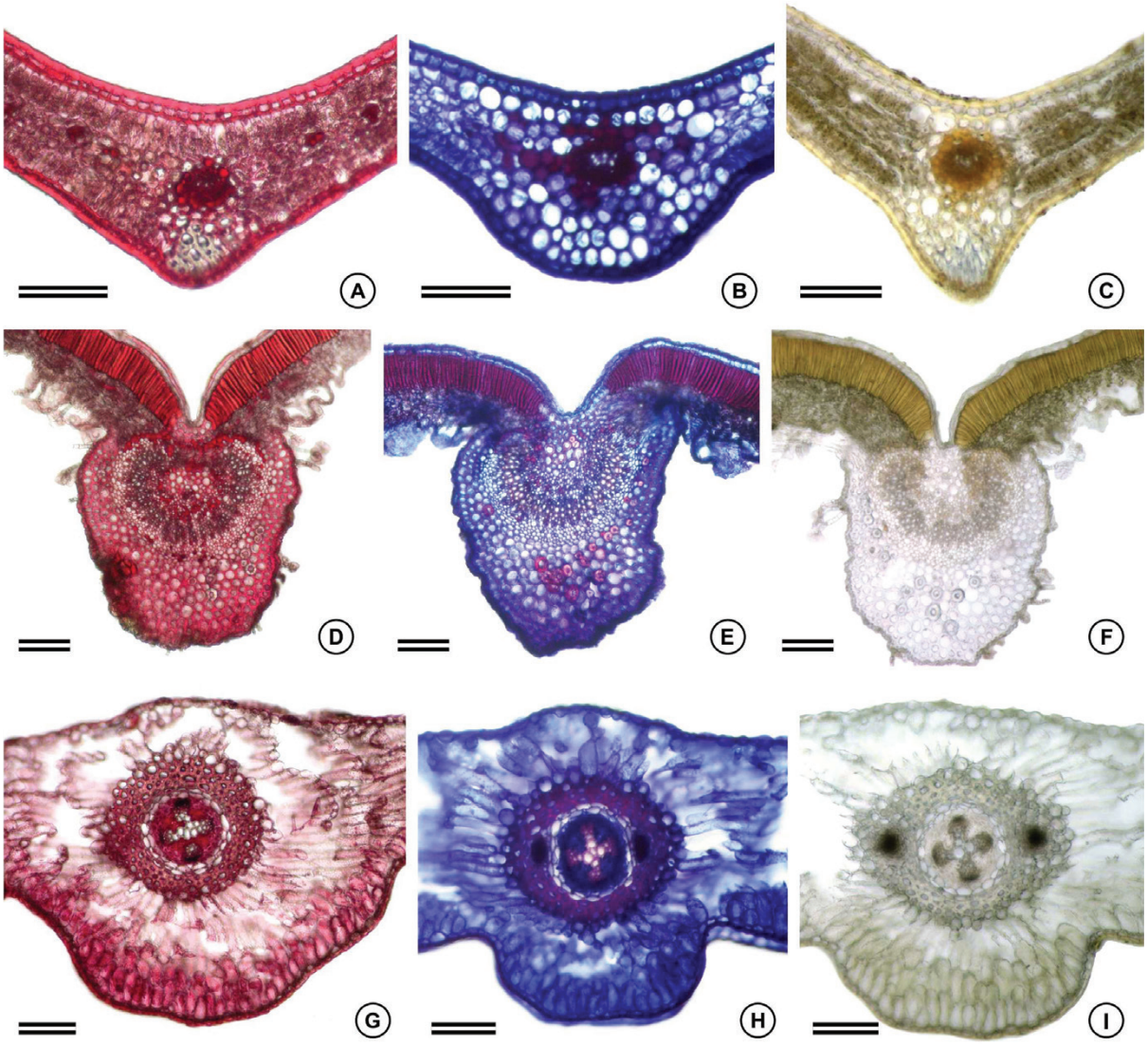

Figure 1 - Cross sections of leaves of *Lavoisiera pectinata* **(A-C)**, *Trembleya parviflora* **(D-F)** and stem of *Lycopodium clavatum* **(G-I)**. **A**, **D** and **G**. Tissues treated with Aluminon. **B**, **E** and **H**. Tissues treated with Chrome Azurol. **C**, **F** and **I**. Blank test. Bars length = 100 µm.

underestimate the concentration of Al in *L. clavatum* when the average concentration in the points were compared with the total Al concentration obtained by ICP OES determination . In *L. pectinata* and *L. clavatum*, there were no differences in Al concentrations among the average concentrations of the analyzed regions and the ICP OES results ($F_{2,14} = 0.51$, $p = 0.61$; $F_{2,29} = 0.06$, $p = 0.81$; respectively). In *T. parviflora* the highest Al concentration was observed in the midvein ($F_{2,32} = 20.00$, $p < 0.0001$) (Figure 3).

DISCUSSION

The occurrence of plant species in soils with high availability of Al^{3+} suggests physiological mechanisms that define them as stress-tolerant (Chenery and Sporne 1976, Grime 2001, Jansen et al. 2002a, Ramírez-Rodriguez et al. 2005). Hyperaccumulator plant species can be found in approximately 45 different families, being an intrinsic characteristic to at least 18 of them (Jansen et al. 2002a).

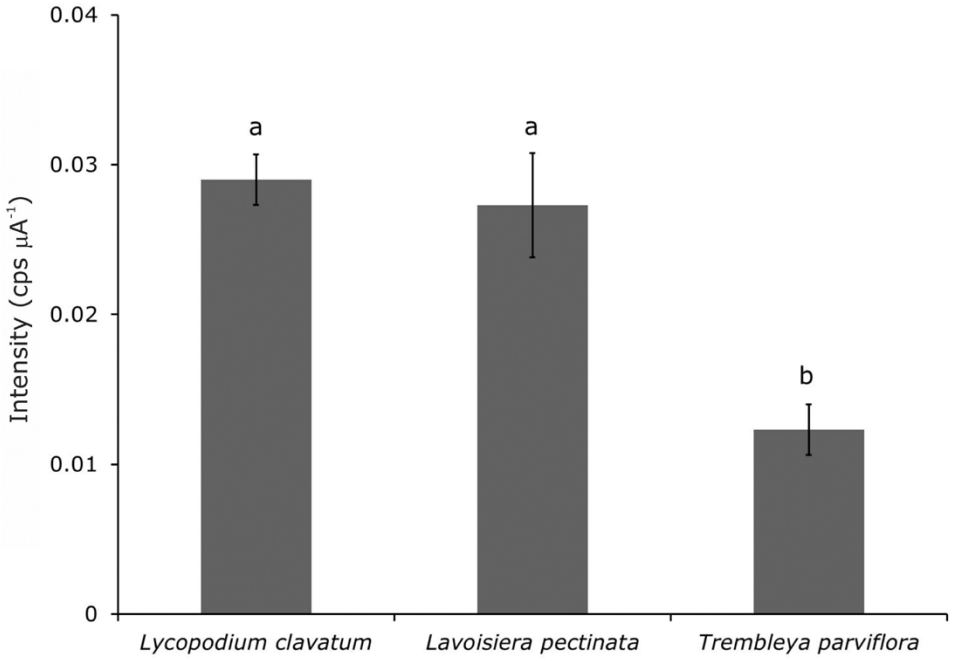

Figure 2 - Micro-energy dispersive X-ray spectrometry data of three different plant species in pellet formats ($F_{2,27} = 14.323$; $p < 0.0001$). Different letters represent significant differences of detected intensities.

Among the sampled species, *Lycopodium clavatum*, *Lavoisiera pectinata*, *Trembleya parviflora*, *Tibouchina heteromalla* and *Eremanthus erythropappus* showed Al concentrations higher than 1000 mg kg^{-1}, which characterize them as hyperaccumulator species (Baker 1981). *Lavoisiera pectinata*, *T. parviflora* and *T. heteromalla* belong to Melastomataceae family, which comprises the highest number of Al-accumulator plant species (Jansen et al. 2002b). The aluminium concentration found in *L. clavatum* in our study was higher than that observed on aerial parts of plants living in high Al-concentration soils by Olivares et al. (2009). According to Haridasan and Araújo (1988), *Eremanthus glomerulatus* shows low aluminium concentration (150 mg kg^{-1}), although other Asteraceae were described as hyperaccumulator species (Geoghegan and Sprent 1996). The low aluminium concentrations observed in *Marcetia taxifolia*, *Nanuza plicata*, *Vellozia variegata*, *Myrsine umbellata* and *Baccharis trimera* could be related to exclusion mechanisms or compartmentalization of this element in underground organs (Jansen et al. 2002a, Kochian et al. 2005, Hartwig et al. 2007).

Some Al-accumulating plants releases organic acids to chelate Al^{3+} or maintain it within cells compartments, such as cell wall or vacuole (Taylor 1991, Delhaize and Ryan 1995, Shen et al. 2002). Among cell wall components, pectates are considered the main linkers to Al^{3+} (Chang et al. 1999, Blamey 2001). The histochemical tests indicated the primary cell walls as preferential sites of aluminium accumulation, possibly due to high content of pectates in relation to xylem secondary walls (Evert 2006).

The intense color of the cuticle and bundle sheath cell walls in *L. pectinata* suggests a feasible mechanism whose function would be the protection of photosynthetic apparatus, as observed for *Erica andevalensis* by Turnau et al. (2007). The positive response observed in chlorenchyma of *L. clavatum* and *T. parviflora* suggests that chloroplasts could have an important role in accumulation of aluminium. High aluminium concentration observed in

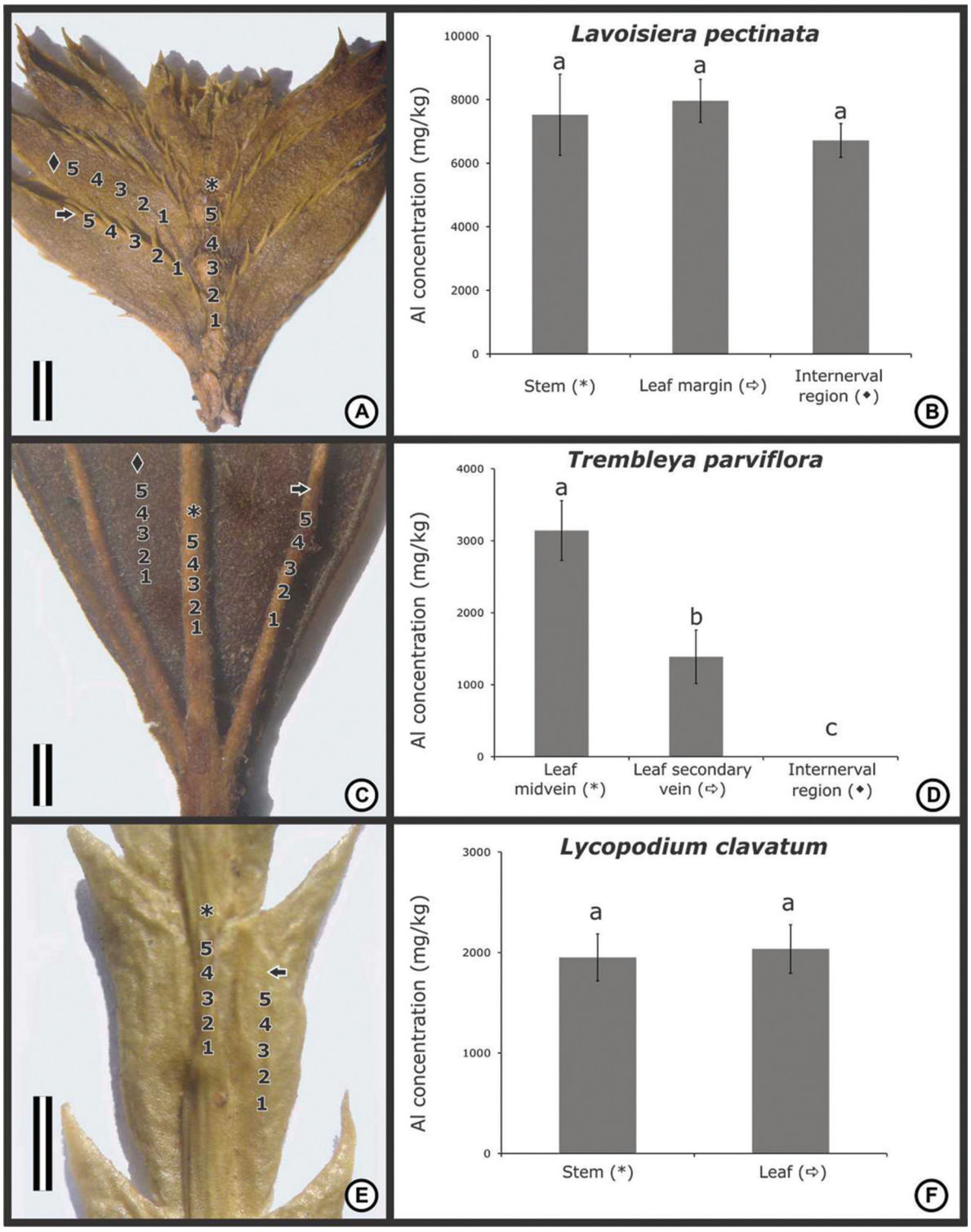

Figure 3 - Aluminium concentration evaluated by micro-energy dispersive X-ray spectrometry in dry leaves of three different plant species ($F_{2,9}$ = 31.84; p = 0.002). Different letters represent significant differences of aluminium concentration. **A-B**: *Lavoisiera pectinata*. **C-D**: *Trembleya parviflora*. **E-F**: *Lycopodium clavatum*. **A**, **C** and **E** indicate the selected points analyzed in leaves and stem. Bars length = 1 mm.

the mesophyll cells of pre-senescent leaves of *Richeria grandis* is possibly stored in the vacuole and chloroplasts (Cuenca et al. 1991).

Tests with Aluminon and Chrome Azurol represent techniques easily applied for preliminary studies, since they indicated, in this study, species with Al concentrations above 3000 mg kg^{-1}. However, the histochemical tests are qualitative methods which are not able to discriminate between different Al concentrations. Aluminon is not a specific reagent for Al^{3+} detection, and the intensity of its reaction can be influenced by other ions such as Fe^{3+} or Be^{2+}, as well as other factors, such as cell type or intracellular pH (Clark and Krueger 1985). These limitations can explain the differences observed among the histochemical tests results.

Data obtained from pellets subjected to μ-EDX analyses were well correlated with the total Al concentrations obtained by ICP OES measurements, exhibiting linear correlation factor higher than 0.99, revealing that μ-EDX method is a suitable tool for Al quantification in plant samples.

The μ-EDX results obtained on dried leaves were consistent with those observed in histochemical tests, which showed a more homogeneous Al distribution in leaves of *L. pectinata* and *L. clavatum*. The low values obtained for Al in *L. clavatum* can be explained by a number of intercellular spaces presented in the leaf and stem of this specie, which can hamper the μ-EDX application due to its small spot size, 50 μm. The differences between the sampling points in *T. parviflora* may be related to higher cell density in leaf veins.

The feasibility of XRF technique on direct determination of elements in plant tissues was already demonstrated in several studies, such as Anjos et al. (2002), Hokura et al. (2005) and Marguí et al. (2009).

Despite all well documented limitations, mainly those related to matrix effects, the XRF technique is a fast and non-destructive method that can be successfully used in simultaneous determination of all target elements without requirement of laborious sample preparation steps (Saisho and Hashimoto 1996, Torok et al. 1998). The use of additional techniques, such as histochemical tests, can be used to complement data obtained from μ-EDX analysis.

CONCLUSIONS

Micro-energy dispersive X-ray fluorescence appears to be a suitable technique to Al quantification in pellets of ground plant tissues as well as for mapping studies in preserved dried leaves. Among the main advantages of this technique, we can mention (i) the low amount of sample required, (ii) its non-destructive capacity and (iii) achievement of reliable data without using expensive and hazardous chemicals.

Histochemical tests can be helpful for screening purposes of main bioaccumulation sites of samples before they are submitted to further μ-EDX scrutiny.

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RESUMO

Solos associados aos Complexos Rupestres de Altitude no Brasil destacam-se pela baixa fertilidade química, baixa saturação de bases e elevados teores de alumínio trocável. Esta condição estressante impõe pressões evolutivas que determinam o sucesso ecológico de espécies capazes de tolerar ou acumular grandes quantidades de alumínio. Vários métodos analíticos são utilizados para mapeamento de elementos químicos em estruturas biológicas, como microfluorescência de raios-X (μ-EDX) e testes histoquímicos. O objetivo do presente trabalho foi combinar a μ-EDX e testes histoquímicos para quantificar

o teor de Al em espécies vegetais de Complexos Rupestres de Altitude, e identificar os principais sítios de bioacumulação de Al. Cinco das espécies investigadas apresentaram concentração total de Al maior que 1000 mg kg^{-1}. As principais hiperacumuladoras de Al, *Lavoisiera pectinata*, *Lycopodium clavatum* e *Trembleya parviflora*, apresentaram reação positiva nos testes histoquímicos com Chrome Azurol e Aluminon. Alta correlação positiva foi observada entre as concentrações totais de Al e as magnitudes de sinal obtidas por μ-EDX. A análise com o uso da μ-EDX mostrou-se uma ferramenta promissora para mapear e quantificar Al em espécies hiperacumuladoras, constituindo uma importante técnica não destrutiva. Testes histoquímicos podem ser úteis na identificação de padrões de acumulação de Al em amostras vegetais antes de serem submetidas a uma minuciosa análise com a μ-EDX.

Palavras-chave: plantas hiperacumuladoras de Al, aluminon, chrome azurol, Complexos Rupestres de Altitude, μ-EDX.

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