GUILHERME CARVALHO ANDRADE

NATIVE LEGUMES FROM THE ATLANTIC RAINFOREST AND THEIR POTENTIAL FOR BIOMONITORING URBAN AIR POLLUTION

Dissertation submitted to Federal University of Viçosa in partial fulfillment of the requirements of the Graduate Program in Botany for the degree of *Magister Scientiae*

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APPROVED: November 27, 2015

Cléberson Ribeiro

Sukarno Olavo Ferreira

Luzimar Campos da Silva
(Advisor)

Dedication

To my nephew Vitor Gabriel, a pleasant arrival
which I shall enjoy more from now on,
after the conclusion of this work.

To the rest of my family and my friends.

Motivated by challenge.

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ABSTRACT

ANDRADE, Guilherme Carvalho, MSc., Universidade Federal de Viçosa, November 2015. **Native legumes from the Atlantic Rainforest and their potential for biomonitoring urban air pollution**. Advisor: Luzimar Campos da Silva.

In Southeastern Brazil, the city of Ipatinga is inserted in the Steel Valley Metropolitan Region, which is characterized by the predominance of steel industry, and also by the presence of one of the largest vehicle fleets in the country. Developing standardized biomonitoring methods with native plant species may be an economically viable option for assessing air quality across extensive urban areas, which usually cannot be achieved by instrumental monitoring due to cost issues. In this sense, the potential for biomonitoring airborne particles was evaluated in *Caesalpinia echinata* and *C. ferrea* aiming to test whether metal accumulation by the plants is related to leaf surface features. Plants were exposed in four urban sites for 90 days. A reference station was installed at Rio Doce State Park, 30 km away from the municipality. After the experimental period, plants were evaluated for trace-metal accumulation. Leaf surface roughness was evaluated in two hierarchical levels, through profilometry and atomic force microsocopy. Epicuticular waxes were characterized chemically through GC-MS and FTIR, and micromorphologically through scanning electron microscopy. Leaf tissue thickness was assessed through optical microscopy. Particle accumulation was higher in *C. echinata*, and was related to a lower roughness given by the epidermal tissue (macro-roughness), lower roughness given by the epicuticular wax deposition pattern (micro-roughness), the micromorphology of waxes in the form of a layer, and the wax chemical composition. The waxes of this species have lower amounts of hydrocarbons and ethers, conferring them a polar nature. These characteristics presumably render the leaf higher wettability, which is usually associated with decreased self-cleaning effect and a consequent increased particle accumulation. In contrast, *C. ferrea* showed reduced accumulation of particulate matter (PM), but a more well-defined response gradient across the exposure sites. Sites were a discriminant factor for cell height on the epidermal tissue, especially on the leaf adaxial surface, such tissue showing reduced thickness in plants exposed at the urban stations. The results suggest the use of *C. echinata* as a bioaccumulator of PM and *C. ferrea* as biosensor of urban pollution.

RESUMO

ANDRADE, Guilherme Carvalho, MSc., Universidade Federal de Viçosa, novembro de 2015. **Leguminosas nativas da Mata Atlântica e seu potencial como biomonitoras de poluição aérea urbana**. Orientadora: Luzimar Campos da Silva.

Localizada no Sudeste brasileiro, a cidade de Ipatinga encontra-se inserida na Região Metropolitana do Vale do Aço, a qual é caracterizada pela predominância da atividade industrial siderúrgica, além da presença de uma das maiores frotas veiculares do país. Desenvolver métodos padronizados de biomonitoramento com espécies vegetais nativas pode ser uma opção economicamente viável para avaliar a qualidade do ar ao longo de grandes extensões de área urbana, o que geralmente não há como ser feito através do monitoramento instrumental devido ao seu alto custo. Neste sentido, o potencial para o monitoramento de partículas foi avaliado em *Caesalpinia echinata* e *C. ferrea* visando testar se o acúmulo de metais pelas plantas está relacionado a feições da superfície foliar. As plantas foram expostas em quatro locais urbanos por 90 dias. Uma estação-referência foi instalada no Parque Estadual do Rio Doce, a 30 km do município. Após o período experimental, avaliou-se o acúmulo de metais-traço pelas plantas. A rugosidade da superfície foliar foi investigada em dois níveis hierárquicos, através de análises de perfilometria e microscopia de força atômica. As ceras foram caracterizadas quimicamente através de CG-EM e por espectroscopia no infravermelho, e micromorfologicamente através de microscopia eletrônica de varredura. A espessura dos tecidos foliares foi avaliada em microscopia óptica. O acúmulo de partículas foi maior em *C. echinata* e se relacionou com uma menor rugosidade dada pelo tecido epidérmico (macrorrugosidade), menor rugosidade dada pela micromorfologia das ceras na forma de um filme fino (microrrugosidade) e com características químicas das ceras. As ceras dessa espécie possuem menores teores de hidrocarbonetos e éteres, o que lhes dá natureza polar. Essas características presumivelmente lhes conferem maior molhabilidade, o que geralmente está associado a um menor efeito auto-limpante e a um consequente aumento no acúmulo de partículas. Em contraste, *C. ferrea* acumulou menos material particulado (MP) mas apresentou um gradiente de respostas mais bem definido ao longo dos diferentes locais de exposição. Os locais foram um fator discriminante para a altura das células do tecido epidérmico, principalmente da face adaxial da folha, tendo este tecido este apresentado valores de espessura reduzidos nas estações urbanas. Os resultados sugerem o uso de *C. echinata* como bioacumuladora de MP e de *C. ferrea* como biosensora de poluição urbana.

TABLE OF CONTENTS

1. Introduction

Steel production is currently responsible for ca. 13% of the Brazilian trade balance (Neves and Camisasca 2013). Despite the economic relevance of the sector, according to the Brazilian National Policy for the Environment, metallurgical industrial activity has a highly polluting potential (Brasil 2001). Steel-making may generate environmental impacts due to the intense use of natural resources such as iron ore, coal and water (Neves and Camisasca 2013).

Whenever a thriving industrial development is not conducted with a sustainable background, extensive damage may be caused to the natural resources of a country. In Brazil, the most famous case of environmental disaster due to such reason is the one of Cubatão city, São Paulo state, which gained international repercussion in the 1980's. The vegetation of the Serra do Mar mountain range was highly impacted by airborne pollutants emitted by the industrial complex of the city, which drastically altered the landscape of the Atlantic Rainforest cover in the place (Klumpp et al. 1994).

Among the several compounds emitted by industrial activity, particulate matter (PM) has received attention in the last years due to its high disease-causing potential (Leiva-Guzmán. et al. 2013) and to the high mortality rates associated with its inhalation (Roberts 2013). PM is categorized according to the size of its particles. Thereby, $PM_{2.5}$ is the fraction of particles with aerodynamic diameter lower than 2.5 µm; PM_{10} is the one whose particles are smaller than 10 µm – which are also called inhalable particles; and TSP represents "total suspended particles", i.e., when one considers particles of all sizes (Ravindra et al. 2001, Yin et al. 2013). PM usually originates from anthropogenic sources, like burning of fossil fuels, mainly from industrial and vehicular emissions (Taiwo et al. 2014), but it may also have in its composition pollen grains, spores and suspended soil particles, among others (Ravindra et al. 2001).

A significant portion of PM is formed by heavy metals (Devi et al. 2014, Li et al. 2014). When in high doses, these elements are toxic to living beings, and may even lead to the development of cancer and respiratory and cardiovascular diseases, among other infirmities, in humans (Kampa and Castanas 2008, Chen and Lippman 2009, Cakmak et al. 2014). Plants can absorb metals and accumulate them within their tissues. This capacity, if on one hand represents and advantage, due to the possibility of using plant organisms in the remediation of soils contaminated with some metallic elements, like iron (Santana et al. 2014), on the other has a negative side. Research has identified cultures of some crop species that were contaminated with these elements (Hu et al. 2014, Rötting et al. 2014, Zhao et al. 2014), which has ultimately resulted in the contamination of humans (Ma et al. 2014).

Structural characteristics are directly related with the sensitivity of plant species to certain pollutants (Silva et al. 2006). The degree of accumulation of PM by a certain plant species, for instance, may depend on features such as its degree of pilosity and the type and amount of epicuticular waxes in its leaves (Sæbø et al. 2012, Barima et al. 2014). Therefore, it is important to use tools that investigate features relating to leaf surface, as well as anatomical ones, in biomonitoring studies. By doing so, one can obtain more precise information on the species responses to the stressing agent and on the characteristics that give them differential susceptibility to urban airborne pollution.

In Minas Gerais state, Southeastern Brazil, the city of Ipatinga, with ca. 200.000 inhabitants, is inserted in the Steel Valley Metropolotian Region, which is characterized by the predominance of the steel industry, forming a base industry pole in the country. The city hosts in its central urban region the facilities of the largest rolled-steel factory in Latin America (Neves and Camisasca 2013). The presence of intense steel-making industrial activity notwithstanding, Ipatinga city has also the eighth largest vehicle fleet in the state and the 102th in the country (Denatran 2015). Since heavy metals are not emitted solely by industrial activity, yet also by vehicular one (Kassomenos et al. 2014; May et al. 2014), the presence of these elements in the urban atmosphere may be due to a mixture of compounds from these two sources. However, it is possible to determine the predominant emission source in a given site by evaluating the ration between different elements, which are called technogenic tracers (Calvo et al. 2013, Titos et al. 2014). The proportion between trace-metals such as Cu, Zn, Pb and Cr in a certain environment, for instance, configures a strong chemical marker of the origin of imissions, so that it allows for the discrimination of the origin of these elements between natural sources or anthropogenic ones (Weiping et al. 2014) as well as among several types of anthropogenic sources (Migliavacca et al. 2012, Khillare et al. 2014, Mohiuddin et al. 2014).

The company monitors the air quality in four neighborhoods in the city on a constant basis. However, although physical-chemical methods like the ones used by the company have been employed systematically in the evaluation of air quality in several cities worldwide (Tsai et al. 2015), their use has been questioned due to the fact that they do not allow the evaluation of the effects on living beings (Divan Junior et al. 2009). Furthermore, the traditional methods of instrumental monitoring are considerably expensive, and due to cost limitations the monitoring stations may occasionally have to be implemented in only a few prioritized sites. The biomonitoring approach, on the other hand, has the advantage of being comparatively much less expensive, besides being independent from a power source (Wittig 1993).

In that sense, plant species have been successfully used as biosensors of different environmental conditions (Volkov and Ranatunga 2006). Aiming at detecting the presence of PM in the atmosphere in order to adopt mitigation measures, several countries in the North hemisphere already monitor such particles using plants, e.g. Belgium (Hofman et al. 2013), Spain (Rodríguez-Germade et al. 2014), Finland (Setälä et al. 2013) and Germany (Rodriguez et al. 2010). In Brazil, however, biomonitoring of these compounds in the atmosphere with plant organisms remains an incipient approach. This is in part due to the knowledge of only a few tropical species which have the potential for such finality (Silva et al. 2006, 2015, Kuki et al. 2008a, 2008b, 2009, Pereira et al. 2009, Neves et al. 2009, Zampieri et al. 2013, Domingos et al. 2015). Thus, the standardization of efficient methods with species from the native local flora, aiming at their further use in biomonitoring programs, configures a particularly relevant approach.

Andrade (2013) has worked with *Caesalpinia echinata* (Lam.) Spreng. and *C. ferrea* Mart. ex Tul. (Leguminosae – Caesalpinioideae) investigating their morphoanatomical responses to simulated acid mist. The two species have different anatomical conformations, particularly regarding the deposition pattern of their epicuticular waxes. His results suggest the susceptibility of both species, but a higher one to *C. ferrea*. Such differential susceptibility may be related to their wax characteristics, among other anatomical features. Thus, deepening the scope of analyses with these species may provide subsidies to indicate their use in the biomonitoring of air quality in the tropical region.

The accumulation of particulate matter and heavy metals by plants is related to characteristics such as leaf shape and size (Räsänen et al. 2013; Huang et al. 2015), pilosity (Mitchell et al. 2010) and wax attributes (Wang et al. 2015). Therefore, two native species from the Atlantic Rainforest with similar leaf morphologies yet with previously known distinct epicuticular wax micromorphologies (Andrade 2013) were chosen to perform a preliminary monitoring of air quality in Ipatinga city, during the wet season of 2014-2015. The aim of this study was to test whether the accumulation of metals by the plants is related specifically to leaf surface features, and also by chemical and micromorphological characteristics of the waxes. A novel approach is presented, consisting on the investigation of leaf surface roughness conferred by two different hierarchical levels, i.e., by the epidermal tissue and by the epicuticular wax deposition pattern. To my knowledge, such an approach has not been so far used. The use of the thickness of leaf tissues as an anatomical biomarker of the exposure was also tested. By doing so, the following hypotheses were addressed: 1) differences in wax morphology and chemical composition between the species would provide them with differential deposition of PM on their respective leaves; 2) the studied species can be used as environmental biomonitors of PM emission.

2. Material and methods

2.1. Plant species and cultivation conditions

The studied species were *Caesalpinia echinata* (Lam.) Spreng. and *Caesalpinia ferrea* Mart. ex Tul. (Leguminosae – Caesalpinioideae), both of which have already demonstrated biosensorial potential to acid mist damage in a previous study (Andrade 2013). Saplings were obtained in the nursery of the Instituto Estadual de Florestas de Minas Gerais (IEF/MG) in Viçosa city, Minas Gerais state, Brazil, and transplanted to 0.8 L plastic pots, which were filled with a mixture of commercial substrate and vermiculite (3:1). Then, all plants stayed in greenhouse for acclimation before proceeding to the field experiment. Plants were considered acclimatized after emitting one new leaf.

2.2. Study site

After the acclimation period, saplings were transported to Ipatinga city (217 km away from Viçosa) and to the Rio Doce State Park (RDSP) (ca. 115 km away from Viçosa and ca. 30 km away from Ipatinga), which is located on the border between three municipalities adjacent to Ipatinga: Dionísio, Marliéria and Timóteo. In Ipatinga, plants were exposed in four sites of the urban region: Bom Retiro (19°30'42.2"S, 42°33'25.5"W), Cariru (19°29'28.8"S, 42°31'43.5"W), Veneza (19°28'20.1"S, 42°31'35.9"W) and Cidade Nobre (19°27'41.0"S, 42°33'37.2"W) neighborhoods (Fig. 1). These four sites were selected for being those whose atmosphere is already under constant monitoring, through analytical methods, by means of automatic monitoring stations.

As a reference station, an exposure rack was installed in the RDSP nursery (19°45'45.5"S 42°37'50.6"W). With an area of 360 km^2, The RDSP is the largest continuous fragment of tropical forest in Minas Gerais state. Since 1944 it is an environmental conservation unit under legal protection. The park was chosen as a reference station for being a well-preserved natural fragment of Atlantic Rainforest that was closest to the studied city.

2.3. Field exposure

In each site, plants were placed in racks according to international standards for air quality control using bioindicators (Arndt and Schluter 1985), at 1.5 m above ground. Plants were put under an automatic irrigation system, in which the pots were connected through a polypropylene wick to a water reservoir, according to the model presented by Arndt et al. (1985). Along the experiment, plants were irrigated with deionized water in order to avoid interference of trace metals. Field exposure took place in the wet season, between 10.09.14 and 01.06.15, totalizing 90 days of exposure, the minimum period considered by some authors for carrying out an analytical monitoring in the context of environmental impact studies (Frondizi 2008). A collection was performed in the field at the end of this 90-day exposure period. Additionally, a collection was also performed with a plant lot that remained under greenhouse conditions in Viçosa city (20°45'28.5"S, 42°52'15.3"W), in 11.07.14.

2.4. Atmosphere chemical characterization and meteorological data

Data on chemical characteristics of the atmosphere in Ipatinga during the exposure period were obtained from the analytical monitoring already performed in the city by its Air Quality Continuous Monitoring Automatic Network. The automatic monitoring stations assess the amounts the TSP (total suspended particles), PM_{10} (inhalable particles with aerodynamic diameter < 10 µm) and $PM_{2.5}$ (inhalable particles with aerodynamic diameter < 10 µm), by beta particle attenuation; SO_2 (sulfur dioxide), by ultraviolet fluorescence; NO_x (nitrogen oxides), NO (nitrogen monoxide) and NO_2 (nitrogen dioxide), by dual cross-flow modulation chemiluminescence; CO (carbon monoxide), by non-dispersive infrared absorption through cross-flow modulation; O_3 (ozone), by ultraviolet radiation absorption; THC (total hydrocarbons), NMHC (non-methane hydrocarbons) and CH_4 (methane), by hydrogen flame ionization detection with cross-flow modulated selective combustion; and BTX (benzene, toluene and xylene), by gas cromatography. Besides, the meteorological parameters wind direction, wind speed, air temperature, rainfall, atmospheric pressure, relative air humidity and global solar radiation are also evaluated. All the above-mentioned variables (pollutants and meteorological parameters) are assessed on an hour-to-hour basis by the stations. The biomonitoring racks were placed in 2 to 3 m from each of the four monitoring stations.

2.5. Metal quantification in plant matter

Inorganic chemical elements that are usually associated with several emission sources were evaluated. The amounts of Cu, Fe, Zn, Mn, Ni, Pb, Cr, Ba, Al, Co and V were assessed since they are regarded as technogenic tracers for steel industry and several types of vehicular emission, among others (Calvo et al. 2013), and the amounts of Ca and Mg were assessed since they are important components of slag (Shen et al. 2009) and could therefore be also used as tracers. Three out of the five repetitions were sampled, being chosen through simple random sampling.

In order to determine the concentrations of Cu, Fe, Zn, Mn, Ni, Pb, Cr, Ca and Mg in the plant biomass, at the end of the exposure period all leaves except those at the youngest and at the two oldest nodes (in the latter case, aiming at preventing soil contamination) were collected, oven-dried at 75 ºC until constant weight and ground in a stainless steel willye-type knife mill (model Star FT 50, American Lab, Charqueada, Brazil). Then, 0.5 g of each sample was digested in 10 mL nitroperchloric solution (nitric acid + perchloric acid, 4:1) (Sarruge and Haag, 1974), heated until 200 ºC and the volume was then adjusted to 25 mL with deionized water. The elements were quantified by atomic absorption spectrometry (spectrometer model 240FS, Agilent Technologies, Santa Clara, USA).

For Ba, Al, Co and V quantification, the dry matter was filtered and then proceeded analysis by inductively coupled plasma optical emission spectrometry (spectrometer model Optima 8300, PerkinElmer, Waltham, USA).

2.6. Assessment of leaf surface roughness

In order to assess any relationship between particle accumulation and leaf relief features, I analyzed the roughness of the leaf surface that is given by the relief of the epidermal tissue (due to characteristics such as sinuosity of anticlinal cell walls and convexity of the outer periclinal wall), which I call here “leaf macro-relief”, as well as the roughness determined by the micromorphology of the epicuticular waxes (which determine the deposition pattern of waxes), evaluating the relief of outer periclinal wall of epidermal cells, which I call here “leaf micro-relief”. Leaf macro-relief was evaluated with an optical profilometer (model Contour GT, Bruker, Billerica, USA), in ca. $2x10^4$

µm^2 fields. Leaf micro-relief was evaluated with an atomic force microscope (model NTEGRA Prima, NT-MDT, Moscow, Russia), in 25 µm^2 fields, using the semi-contact mode, in which the needle tip stays in intermittent contact with the surface. The measurements taken from the leaf surface allowed for the determination of the mean roughness value (Sa), which represents the average deviation between the height measurements obtained, and the root mean square roughness (Sq), which represents the square root of the average squared deviation between the height measurements obtained.

For both analyses, leaf samples were collected from three randomly chosen individuals of the two species in the sapling stage. These individuals had not been exposed to any environmental condition outside the plant nursery. As several leaf surface characteristics such as wettability (which is given by wax chemical (Barnes et al. 1996) and morphological (Neinhuis and Barthlott 1997) features) vary according to leaf age (Fernández et al. 2014), one leaf from each individual was sampled and sampling procedures were standardized accordingly. The third-node leaf was sampled since it was the one collected in the experiment for the scanning electron microscopy, particulate matter accumulation and wax chemical composition analyses.

For the profilometry analysis, fresh leaf samples were collected and placed in plastic pots with wet cotton in other to create a wet chamber and thus preserve cell turgidity. Leaf fragments 16 mm^2 were cut with a razor blade from the mid portion of pinnules, which were taken from the mid-portion of the pinnae, which in turn were from the mid portion of the leaf. Samples were then carefully placed on histological glass slides over double-sided tape and immediately analyzed within the period of three hours (for each species).

For the atomic force microscopy (AFM) analysis, fresh leaf samples were collected and sectioned following the same procedure above, but prior to analysis the glass slides containing leaf fragments were placed in a sealed container with silica gel for 48 hours in order to dehydrate samples.

Three pinnules were sampled per leaf for the macro-relief analysis, and two pinnules for the micro-relief one, one leaf fragment being extracted out of each pinnule. The analyses were performed on both leaf surfaces. In each fragment, measurements were taken in three fields, totalizing nine measurements per species, per individual, per leaf surface, in profilometry, and six measurements in each of these same levels, in the atomic force microscopy.

2.7.Extraction, quantification and identification of wax compounds

2.7.1. Sample preparation

For wax analysis, the Hamilton (1995) method was employed, with modifications. It should be noted that, for this analysis, the whole morphological unit interpreted as leaf, i.e. leaf blade + rachis + pulvinus, was used. The sampling method for this analysis was the compound sample, due to the low amount of waxes present in single leaves of these two species. Thus, one third-node leaf from each individual was collected and gathered in one compound sample per species, per monitored site.

Samples were oven-dried at 75 ºC until constant weight and introduced in Petri dishes with 75 mL dichloromethane for 45 seconds under agitation in ultrasonic bath. This procedure was carried out with extreme care in order to prevent leaf rupture and the consequent release of cellular contents (Ferreira et al. 2005; Guimarães et al. 2009). The obtained extracts were filtered with filter-paper and transferred to 150-mL Erlenmeyers. In order to ensure maximum extraction, the already-processed leaf samples were once again placed in Petri dishes with 75 mL dichloromethane, agitated for 45 s more in ultrasonic bath and filtered again to the same respective Erlenmeyer, resulting a total solution of 150 mL. The solution was transferred to round-bottom flasks and evaporated under water-bath in order to obtain the solid residue (waxes). The evaporation was made in a rotary evaporator (model Rotavapor R-3, Büchi Labortechnik AG, Flawil, Switzerland), at low pressure and at a temperature not higher than 40 ºC. This solid residue was solubilized with small aliquots of dichloromethane (less than 4 mL) and transferred to 4-mL tubes of previously known weight. Dichloromethane was then evaporated in a fume hood with compressed air at room temperature.

2.7.2. Wax mass

The flasks containing solid wax (no solvent) were weighed in an analytical balance, the same used for obtained their sample-free weigh, to obtain wax mass. The amount of waxes was expressed by their mass per unit of leaf dry weight ($\mu g.g^{-1}$).

2.7.3. Infrared spectroscopy

All compound samples of epicuticular waxes were subjected to Fourier-transform infrared spectroscopy (spectrometer model 660 FT-IR, Varian Inc., Palo Alto, USA) through attenuated total reflection (ATR attachment: Pike Technologies, Fitchburg, USA), registered between 4000 and 400 cm^{-1} wavenumber range.

2.7.4. Transesterification and gaseous phase chromatography

Samples were subjected to the transesterification process in order to obtain their respective methyl esters of fatty acids. For that, 2 to 5 mg of waxes were transferred to test-tubes and dissolved in 1 mL of a solution of 2% H_2SO_4 in methanol, boiled in water-bath at 55 ºC for two hours, and then cooled at room temperature. Then, 10 mL hexane was added to the tube and the mixture was agitated in ultrasonic bath. The organic phase was then transferred to another test-tube and washed twice with 10 mL saturated NaCl solution, and concentrated in a fume hood with compressed air at room temperature.

The obtained residue from each compound sample, containing methyl esters and alcohols along with the organic fraction, was dissolved in 100 µL hexane, 1 µL of which was injected in a gas chromatographer coupled to a mass spectrometer. Chromatographic analyses were carried out in a mass spectrometer (model GC-MS QP 2010 Ultra, Shimadzu, Kyoto, Japan) using a Rtx-5MS capillary column (30 m length; 0.25 mm internal diameter; 0.25 µm film thickness) and helium as carrier gas at a 1.60 $mL.min^{-1}$ flow. Temperature in the injector was 290 ºC, with initial temperature of 80 ºC, for five minutes, increasing from 80 to 285 ºC at a 4 ºC min^{-1} ratio. Final temperature was kept at 285 ºC for 24 minutes. The ion source and GC-MS interface temperatures were both 290 ºC. The mass detector operated by electron impact ionization (70 eV) in scan mode at the 30 to 600 Da mass range. Identification of wax compounds was made by means of comparison of the mass spectra with those existing in databases NIST 11 and Wiley 7.

2.8.Micromorphological characterization in scanning electron microscopy

Third-node leaves were sampled for the SEM analysis. Samples were taken from three repetitions, which were chosen through simple random sampling. Leaf blade fragments ca. 6 mm^2 were cut with a razor blade from the mid portion of pinnules, which were taken from the mid-portion of the pinnae, which in turn were from the mid portion of the leaf. Rachis fragments ca. 4 mm long were taken from the rachis mid-region. Fragments were then directly affixed in the field onto stubs previously covered with carbon tape, kept in sealed acetate boxes with silica gel and stored at 9 ºC until observation. Leaves were sectioned so that three leaf blade fragments were sampled for visualization of each leaf surface, from each individual of each species.

Samples were observed in a scanning electron microscope (model JSM-6010 LA, Jeol, Tokyo, Japan) through the detection of secondary electrons to analyze the epicuticular wax micromorphology of each species as well as the morphology of deposited particles. Backscattered electrons were used to highlight particles on the leaf surface (Wang et al. 2015). The deposition patterns of the epicuticular waxes were classified according to Barthlott et al. (1998).

2.9.Micromorphometrical analysis

Quantitative anatomical analyses were performed in order to trace possible effects of the atmosphere composition on the thickness of leaf tissues. Leaf fragments ca. 25 mm^2 were fixed in a solution of glutaraldehyde (2.5%) and paraformaldehyde (10%) in a 0.1 M sodium cacodylate buffer (pH 7.2) (Karnovsky 1965) for a minimum period of 24 hours. This procedure was made in the field.

After the fixation period, samples were dehydrated in an ethyl series and stored in 70% alcohol. For the anatomical processing in light microscopy, samples were then dehydrated in a higher-grade ethyl series, embedded in glycol methacrylate historesin (Historesin, Leica Instruments, Heidelberg, Germany) (Gerrits 1991) and cross-sectioned at 5 µm thickness in a fully automated rotary microtome (model RM2265, Leica Microsystems Inc., Deerfield, USA). Sections were then stained in 0.05 % toluidine blue O in a 0.1 M sodium acetate buffer pH 4.7 (O’Brien et al. 1964, modified) and the histological glass slides were mounted in Permount (Fisher Scientific, Waltham, USA). Photographic documentation was performed in a photomicroscope (model AX70 TRF, Olympus Optical, Tokyo, Japan) equipped with a U-photo system, coupled to a digital camera (AxioCam, Carl Zeiss, Jena, Germany).

Samples were collected in second-node leaves of all five repetitions in each exposure site. Sampling followed the procedure adopted by Andrade (2013), with adaptations. From each leaf (one leaf per individual), three leaf blade fragments were anatomically processed, out of which one histological glass slide was confectioned, five sections being selected out of each slide. One micrograph was obtained in each section, and in each micrograph three measurements were taken for each parameter, totalizing 225 measurements per exposure site, per parameter, per species.

The parameters evaluated in cross section were height of epidermal cells from both adaxial and abaxial leaf blade surfaces, of palisade and spongy parenchymas, mesophyll and leaf blade. Measurements were taken in transects passing through the central region of epidermal cells and out of fields with vascular bundles. Mesophyll thickness was obtained by the sum of the height of the two parenchymas, ad leaf blade one by the sum of the mesophyll and epidermal height at both leaf surfaces.

Measurements were taken with image analysis software Anati Quanti version 2.0 (Aguiar et al. 2007).

2.10. Experimental design and statistical analysis

Experimental design was randomized block, with five repetitions per exposure site (n = five sites) and two species. Data on micromorphometrical analysis and leaf surface roughness was subjected to two-way ANOVAs followed by Tukey's test at 5% probability. Micromorphometrical data, as well as data on metal accumulation, was also subjected to multivariate analyses of variance (MANOVAs). Pearson's correlation analysis was used to evaluate the relationship between the adaxial leaf surface roughness and trace-metal accumulation. Coefficients were considered whenever they were significant in both Pearson's analysis and the two-way ANOVA for metal accumulation. All statistical analyses were performed using softwares R version 3.2.2 and Statgraphics Plus version 5.1.

3. Results

3.1. Meteorological data and atmosphere chemical characterization

During the experimental period, the heaviest weekly rainfall was registered at Bom Retiro (96.0 mm). High maximum temperatures characterized the entire period, ranging between 30 and 40 ºC. Average values of mean temperature were 24.74 ºC at Bom Retiro, 27.11 ºC at Caritu, 26.67 ºC at Veneza, and 26.44 ºC at Cidade Nobre (Fig. 2, left column).

Considering all monitoring stations in Ipatinga city, the predominant wind was the Northeast one. Much occasionally were there discordant patterns. E.g., there were weak, infrequent Southeast winds at Bom Retiro and analogously low, infrequent Northwest ones at Cidade Nobre (Fig. 2, right column).

The dynamics of particulate matter emission in the study sites along the experimental period can be seen in Figs. 3 and 4. The average total suspended particle load for the entire period was the highest at Cariru (40.98 $\mu m.m^{-3}$), followed by Veneza (37.83 $\mu m.m^{-3}$), Bom Retiro (35.36 $\mu m.m^{-3}$) and Cidade Nobre (32.17 $\mu m.m^{-3}$) (Fig. 3A). Inhalable particles, however, showed a different pattern. The highest average load was detected at Cariru (25.52 $\mu m.m^{-3}$), followed by Cidade Nobre (22.74 $\mu m.m^{-3}$), Bom Retiro (22.21 $\mu m.m^{-3}$) and Veneza (17.46 $\mu m.m^{-3}$) (Fig. 4A).

3.2. Metal quantification in plant dry matter

Among the investigated tracers, vanadium was not detected in any samples and, therefore, is not shown in the data summarized in Table 1.

There were significant interactions in the accumulation of Ca, Cu, Fe, Mn, Ni, Pb and Co. *Caesalpinia ferrea* showed higher Ca contents in plants at the reference station in comparison with plants exposed at Cariru. This species accumulated higher Cu contents than *C. echinata*. Increased Cu amounts were detected at Bom Retiro, Cariru and Cidade Nobre. The opposite was observed with Fe, Ni and Pb accumulation. *Caesalpinia echinata* accumulated higher contents of these three metals than *C. ferrea* at Bom Retiro, and higher contents at this neighborhood than at the other sites (with the exception of Pb at Veneza). *Caesalpinia ferrea* showed higher Mn in plants at RDSP than in those exposed at Cariru station, and higher contents than *C. echinata* at the former site. The Ni contents followed the inverse pattern, being higher in plants at Cariru than in those at RDSP. As for Co, *C. ferrea* accumulated higher amounts than *C. echinata* at Cariru and Cidade Nobre. These two sites and Veneza propitiated higher accumulation of the element than Bom Retiro and RDSP in *C. ferrea* (Table 1).

Overall, besides Fe, increasing concentrations along the wind direction were verified with elements Zn, Mn, Ni, Pb and Cr, and particularly higher amounts of Al. Bom Retiro showed the highest contents of these elements on the plant matter of both species (Table 1).

Aiming to explore variation between species and among exposure sites, principal component analysis was also performed with data on elemental quantification, in order to obtain a small number of linear combinations of the 12 metals which account for most of the variability in the data. Three components were extracted, with eigenvalues higher than one. Together they account for 80.24% of the variability in the data.

Considering the two principal components plotted, component 1 was composed by Cr, Ca, Al, Ni, Pb, Fe and Ba, and component 2 by Zn, Mg, Mn, Cu and Co. Calcium, however, was more closely associated with the cluster formed by component 2 than to the one formed by the other metals in component 1 (Fig. 5A).

The two species could be clearly separated by the PCA, forming two well-defined clusters. Associations between the variation of certain metals could also be noticed. Iron and Pb varied together, and so did Al and Ni; Co, Cu and Mg; and Ca, Mn and Zn. Barium varied singly toward *C. echinata* data, and Cr varied singly in the verge between species (Fig. 5B).

The exposure sites could also be clearly separated. The reference station, for instance, formed a well defined cluster that could be well-segregated by component 1, showing the lowest component weights (Fig. 5C).

Most elements varied toward the urban exposure stations. The element that varied more closely toward the reference station was Mg (Fig. 5C).

Both components demonstrated influence on the final result. Component 1, however, separated species among sites, according to pollution level, while component 2 did such separation to a lesser degree. Component 1 seems, therefore, to be a revealer of pollution, whereas component 2 seems to discriminate species (Fig. 5B, C).

3.3. Leaf surface roughness

Leaf macro-roughness was significant in the interaction between the species and leaf surfaces (abaxial and adaxial). *Caesalpinia ferrea* showed higher values than *C. echinata*, and in each species the abaxial surface was the roughest one. The results given by Sa and Sq differed only in the comparison between species regarding the abaxial leaf surface, showing significant difference in the former and a non-significant one in the latter (Table 2; Fig. 6).

As for leaf micro-roughness, a significant difference was detected only between species, with *C. ferrea* showing higher values (Table 2; Fig. 7).

In order to track back relationships within metal accumulation and leaf surface roughness, correlation analyses were made between trace-metal and the two estimators (i.e., mean (Sa) and squared (Sq)) of the types of roughness (i.e., macro and micro). Leaf macro-roughness was positively correlated with Ca (at Cariru), Fe (at Bom Retiro) and Ni accumulation (at the reference site) by *C. ferrea* and negatively so with Cr accumulation (at Bom Retiro) by *C. echinata*. Leaf micro-roughness was positively correlated with Cu accumulation (at Cariru) in *C. ferrea* and Ni (at the reference site) and Pb accumulation (at Cidade Nobre) in *C. echinata*, and negatively so with Mn accumulation (at the reference site) in *C. ferrea* and Fe and Mn (at the reference site, both) in *C. echinata* (Tables 1 and 3).

3.4. Waxes

3.4.1. Quantification

As wax quantification was performed in compound samples, no statistical test could be applied to this data. Analyzing this data in terms of absolute values, however, *C. ferrea* leaves seem to be covered by higher wax amounts (Fig. 8). The results also suggest an increase in the amount of waxes in plants exposed at Bom Retiro, Cariru and Veneza, in comparison with the reference station, the least polluted urban station (Cidade Nobre) and non-exposed plants. This response was more noticeable in *C. ferrea*. Although *C. echinata* plants did show higher values at Bom Retiro, Cariru and Veneza than plants at the reference-station, plants at Cariru showed lower values than the ones at Cidade Nobre and than the non-exposed ones (Fig. 8).

3.4.2. Infrared spectroscopy

Infrared spectrum analysis allowed for the determination of the main functional groups of the studied species in the studied sites. The analysis showed that the epicuticular waxes of *C. echinata* and *C. ferrea* are characterized by a typically-shaped band of stretching of O–H bonds in the 3.600–3.200 cm^{-1} region; bands in the ~1375 cm^{-1} region indicating symmetric deformation of CH_3 groups, in the ~1465 cm^{-1} region indicating scissor-type deformation of CH_2 groups, and very intense bands in the 3000–2840 cm^{-1} region, due to strong stretch of C–H bands, thus pointing to the presence of alkanes in both species; C=O stretch bands in the 1770–1630 cm^{-1} region, indicating the presence of ketones; C–O stretch bands in the ~1050 cm^{-1} region, indicating primary alcohols, and in the ~1150 cm^{-1} region, indicating the presence of tertiary alcohols; and weak stretch bands of C≡C at ~2158 cm^{-1} indicating the presence of alkynes (Figs. 9F and 10F). This constitution suggests that the wax of both species is primarily a mixture of ketones, long-chain n-alkanes and short-chain alcohols. The infrared spectra ascertained the need to proceed transesterification with the samples prior to the gas-chromatography/mass-spectrometry analysis, due to the presence of functional groups with an active hydrogen (alcohols and carboxylic acids) (Jetter et al. 2006).

Comparing samples from different sites, *C. echinata* showed reduced O-H bands in Cariru, RDSP and in non-exposed plants. The infrared spectrum that most resembled that of wax of non-exposed plants was the one from Cariru samples. An accentuation was seen in the fingerprint region in samples from Bom Retiro, Veneza and Cidade Nobre, such accentuation being less intense in Cariru samples (Fig. 9). *C. ferrea* showed reduced O-H bands in plants exposed at Cidade Nobre and RDSP, and in non-exposed plants. The spectrum that most resembled the one of non-exposed plants was the one from RDSP samples; a noticeable resemblance could also be seen with the spectrum of plants exposed Cidade Nobre. An accentuation was seen in the fingerprint region in samples from Bom Retiro, Cariru and Veneza, such accentuation being less intense in Cidade Nobre samples (Fig. 10).

3.4.3. Gaseous phase chromatography

The GC-MS analysis allowed the identification of 37 different compounds in the waxes of *C. echinata* (Fig. 11A, Table 4) and 41 compounds in the waxes of *C. ferrea* (Fig. 11B, Table 5). Contaminants such as phthalate and some benzenic compounds were disregarded. The components in highest proportion in the wax chemical constitution are the ketone 2-nonanone (16.5 %) in the former and the hydrocarbon 2-methyloctacosane (7.6 %) in the latter. Following a corresponding pattern, the waxes of the former are predominantly constituted by ketones, hydrocarbons being the second major component; while the opposite proportion is observed in the latter. The other components in decreasing order of proportion in the wax constitution are: carboxylic acids, alcohols, ethers and esters, in *C. echinata*, and carboxylic acids, alcohols, esters and aldehydes, in *C. ferrea* (Fig. 12).

Such chemical constitution renders the waxes of *C. echinata* more polar than *C. ferrea* ones. In the former, ca. one-third of the total wax composition is made up by little polar compounds (hydrocarbons + ethers), while over half the wax constitution has such polarity feature in the latter (Fig. 13). In *C. ferrea*, hydrocarbons alone determine the wax little polar nature, due to the absence of ethers in the wax composition (Fig. 12).

3.4.4. Micromorphology (scanning electron microscopy)

Caesalpinia echinata has the layer type of deposition pattern of the epicuticular waxes (Fig. 14A, B). In *C. ferrea*, the deposition pattern is in vertical non-ordered scales (Fig. 15A, B). The two species are hypostomatic (i.e., have stomata on the leaf abaxial surface only). The wax deposition pattern is the same on both leaf surfaces, in both species (Fig. 14C, D; 15C, D).

Caesalpinia ferrea epidermal cells are characterized by sharply convex outer periclinal cell walls. This feature determines a somewhat grooved epidermal relief (Fig. 15A, C), characterized by recesses among the cells, in which, on the abaxial surface, the stomata are inserted (Fig. 15D). Besides, the anticlinal wall of the epidermal cells of this species are more undulated that those in *C. echinata* (Fig. 15A, D, and Fig. 14A, respectively).

The rachis of the two species have an irregular relief, showing protuberances and furrows along their surface (Fig. 14E, 15E). However, analogously to the leaf blade, they are different as well at a micromorphological level. In *C. echinata*, it has plenty of hairs distributed evenly across its surface (Fig. 14E, F), and the wax is also deposited as a layer. In *C. ferrea*, it is glabrous (Fig. 15E) and the wax deposition pattern is also in vertical scales. However, the wax scales in the rachis differ from those on the leaf blade. The former are larger and have round-edges (Fig. 15F), while the latter have serrated edges (Fig. 15B).

3.5. Deposited particles

For means of absolute comparison, the scanning electron microscopy study was performed on leaf samples from non-exposed plants and on samples from individuals exposed at Bom Retiro, the site initially presumed as the most-polluted one, based on wind direction. Particles could be seen adhered to leaf surfaces of both species (Figs. 16, 17, 18). More particles were retained onto the leaf blade of *C. echinata* (Fig. 16), but less frequently on the abaxial surface (Fig. 16E-G)). Fewer particles were seen on *C. ferrea* leaves, and, analogously, more frequently on the adaxial surface (Fig. 17). In *C. echinata*, the presence of fungal hyphae was noticeable, on both leaf blade surfaces (Fig. 16A, C, E, G, H) and also on the rachis (Fig. 18A, C, E). The occurrence of hyphae onto *C. echinata* leaf surface was frequently associated with the presence of particles (Fig. 16G, H). Hyphae were not seen in *C. ferrea* leaves, in neither leaf region.

In the rachis, particles were also seen in both species (Fig. 18). In *C. echinata*, particle occurrence was associated not only with hyphae occurrence, but also with the presence of hairs along the entire leaf region (Fig. 18A, B, C, E). Particle occurrence in the rachis of *C. ferrea* was much less frequent (Fig. 18G, H).

3.6. Leaf micromorphometry

The interaction between species and exposure sites was significant for no tissue or leaf region. In the comparison between species, cell height differs in all tissues, *C. echinata* having thicker ones than *C. ferrea*, except for the epidermis of the leaf abaxial surface. As for comparison among sites, which is of particular interest for the present approach, higher thickness on the epidermis of both leaf surfaces was observed in *C. ferrea* individuals exposed at the reference station. The epidermis of the adaxial surface was lower in all urban sites, whereas the epidermis of the abaxial surface was lower in Bom Retiro and Cidade Nobre (Table 6). Also, Tukey’s range test showed that the exposure site was a discriminant factor for thickness of the epidermis of the leaf adaxial surface, such tissue being thicker in plants exposed at the reference station. Plants at Cidade Nobre showed the lowest values, while individuals at Cariru showed the highest ones (Fig. 19).

4. Discussion

The predominance of Northeast winds in the region initially suggests that Cidade Nobre would be the least impacted urban site and that Bom Retiro would be the most impacted one, particularly by gaseous pollutants and, among particulate matter, by the ones with the lowest aerodynamic diameter (PM_{10}) (Marris et al. 2012; Almeida et al. 2015). As PM_{10} are particles with lower deposition rates, they tend to remain in the atmosphere for a longer period of time, and be transported to further distant regions (Riffault et al. 2015). These predictions, however, were only partially met. The site with the highest amounts of TSP was Cariru. This would not be totally unexpected, as its location is at Southeast, also along the windstream. But the second highest amounts were detect at Veneza, only then followed by Bom Retiro and Cidade Nobre. Other possible sources of contamination at Veneza must be investigated. In addition, Cidade Nobre was the most affected site by inhalable particles, after Cariru, followed by Bom Retiro and Veneza. Wind direction renders it little probability of it being affected by steel industry activity. Instead, vehicular emissions may be the predominant factor in this neighborhood.

Caesalpinia echinata showed higher accumulation of Al, Ni, Pb, Fe and Ba, while *C. ferrea* accumulated higher amounts of Mg, Cu, Co, Zn, Mn and Ca. Only Cr could not be segregated by neither principal component. Both PCA and Tukey results seem to suggest some sort of particle selection by the species. It would seem like *C. ferrea* selects Cu and Co particles, while *C. echinata* selects Fe, Ni, Pb, Al and Ba particles. Since the plants were all exposed to the environment under the same experimental conditions, differences in the amount of accumulated elements, at least trace-metals, were not to be expected between the two species. These differences could have presumably arisen either from soil or some mechanism of superficial adhesion of specific types of particles. Siqueira-Silva (2015) investigated the effects of cement dust on native Brazilian plant species. The author performed a controlled experiment varying deposition among leaf, soil, and both leaf and soil. According to him, the harmful effects observed at the cellular and subcellular levels of leaf tissues occurred prior to possible alterations on soil pH and chemical composition, being therefore caused majorly be the contact of these particles with the leaf surface. This led me to discard the first possibility of soil interference. The second one, then, seems most likely. I.e., the nature of the leaf surface of the two species may have favored such particle segregation.

Bom Retiro was the most impacted neighborhood by iron-containing particles. The analysis of heavy metals in particles is a safe method for assessing source-related emissions (Calvo et al. 2013; Almeida et al. 2015; Riffault et al. 2015). Iron particles are usually a product of basic oxygen steel-making and sintering (Almeida et al. 2009; Canha et al. 2012). Although the steelworks and sintering plants are nearer Cariru, the highest iron accumulation in plants exposed on this site was an expected result, as it is in accordance with the predominant wind direction in the region.

The leaf surface of *C. ferrea* is rougher than the one of *C. echinata*, both at the epidermal tissue level and at the outer periclinal epidermal cell wall level. The convexity in the outer periclinal cell wall and the platelet-type of wax crystals are the respective determining factors of these two features. To my knowledge, this is the first study quantitatively assessing leaf blade roughness at more than one hierarchical scale in an integrated approach. A possible reason for the lack of studies with this nature is the limitation of atomic force microscopy analysis with leaf samples. Prum et al. (2012a and b) performed an assessment resembling the one proposed herein, but the evaluations addressed qualitative descriptions and were carried out in the context of insect adhesion to leaf surfaces. Koch et al. (2004) assessed leaf micro-roughness of different plant species with AFM, but not along with macro-roughness, as the aims of the authors in both studies were to investigate the dynamics of wax regeneration. I suggest that future studies assessing the potential of plant species for biomonitoring airborne particulate matter investigate not only leaf surface macro-roughness (which itself is still scarce among published research) but also leaf surface micro-roughness, as they may be both related to the degree of particle retention.

Rougher leaf surfaces, if on one hand propitiate the superficial features that allow higher particle accumulation (Belot and Gauthier 1975), on the other usually confers such surface with higher hydrophobicity (Barthlott and Neinhuis 1997). This usually gives the plant a higher self-cleaning effect, i.e., the capacity of removing adhered particles by rain droplets that fall on leaves (Neinhuis and Barthlott 1997). Leaves with such features, like the ones of *C. ferrea*, are known to be related to lower particle accumulation, since the water droplets remove particles by adhering them to the droplet surface. In contrast, droplets that fall on hydrophilic surfaces, such as that of *C. echinata*, do not remove particles, yet tend to merely rearrange them (Barthlott and Neinhuis 1997).

Notwithstanding, *C. echinata* showed a lower amount of waxes than *C. ferrea*, and its wax constitution is also more polar, due mainly to the presence of comparatively less amounts of hydrocarbons. As a result, the leaf surface of this species has more water-affinity. That, along with its less rough leaf surface, suggests its higher wettability (Barnes et al. 1996; Neinhuis and Barthlott 1998; Wang et al. 2013; Wang et al. 2014). High hydrophilicity represents, among other outcomes, the higher tendency for the development of a water film over the leaf surface (Cape 1996). This frequently predisposes the leaf to be colonized by microorganisms (Juniper 1991). The occurrence of fungal colonization was observed in *C. echinata* samples from plants exposed at Bom Retiro, and quite noticeably deposited particles could be seen associated with them. The development of fungal hyphae on the leaf surface has been recently shown to be another enhancing factor to particle accumulation. Sánchez-López et al. (2015) observed, in several plant species, that the fungal mycelium on leaf surfaces formed an extensive web over the phyllosphere, which then retained more particles.

Although the occurrence of hyphae is usually a natural outcome of high leaf wettability, it is possible that fungal colonization has been increased or even triggered by an effect of urban pollution. In view of the known capability of PM to erode epicuticular waxes (Burkhardt and Pariyar 2014), particles could have promoted abrasion on leaf wax cover, thus enhancing its wettability. According to Räsänen et al. (2014), wax erosion by PM in *Picea abies* led to enhanced hydrophily and, along with decreased stomatal conductance, resulted in increased particle capture efficiency. This could also have ultimately led to higher particle accumulation by *C. echinata* at Bom Retiro, as a result of both decreased leaf self-cleaning capacity and higher leaf colonization by fungi. Yet, the higher amounts of waxes found in plants exposed at the most polluted sites may somewhat contradict this and, at a first glimpse, be paradoxical. Nonetheless, higher particle retention by hyphae due to abiotic stress suggests the generation of a positive feedback effect. I.e., higher effects by particulate pollutants lead to wax abrasion, which enhances leaf wettability and consequently propitiates the establishment of fungal hyphae. The hyphae, in turn, retain more particles over the leaf surface, thus leading to enhanced effects of the abiotic stress, which is then summed to the biotic one (Juniper 1991; Carver and Gurr 2006; Sánchez-López et al. 2015).

The increased wax production in *C. ferrea* may be a stress-avoidance mechanism. The wax layer has been regarded as the first barrier against environmental stressing conditions (Pal et al. 2002; Shepherd and Griffiths 2006). Popek et al. (2013) observed that after three years of exposure up to 40% of the total particles adsorbed to the leaf surface was immobilized within the wax layer, instead of onto it. The largest amount of waxes observed in *C. ferrea* individuals exposed at Bom Retiro, Cariru and

Veneza could have been triggered by the exposure to the more polluted atmosphere of these sites.

Accordingly, *C. echinata* was the species that had more particles adhered to the leaf surface. The results of the analyses performed indicate that differences in the wax chemical nature between the two species may have determined such differential particle adsorption, to the extent that hydrocarbon contents determined hydrophobicity, which is associated with the degree of particle retention (Jetter et al. 2006; Wang et al. 2013). Besides wax composition, its microsculpture is another factor that might have interfered with particle accumulation, as it determined a specifically rough microrrelief. (Neinhuis and Barthlott 1998; Wang et al. 2013; Wang et al. 2015). Macrosculpture of the epidermal tissue should be another influencing factor, as it determined leaf roughness at a larger scale.

A particular behavior inherent to the species biology may also have interfered with the degree of particle deposition. Under high insolation and temperature, both of which characterized several days of the exposure period, *C. ferrea* plants close their pinnules, regardless of the time of the day. Such phenomenon also occurs in *C. echinata* individuals, yet much more discretely. Therefore, not only the leaf blade was collected, but also the rachis. Despite having a lower superficial area, this leaf region remained constantly exposed to atmospheric deposition.

Surface features that may have determined the degree of particle retention on the rachis of the studied species were high pilosity in *C. echinata*, presence of a dense cover of wax crystals in *C. ferrea*, and a macrosculpture relief characterized by protuberances and furrows, in both species. The presence of trichomes is a well-reported feature in the literature known to enhance particle accumulation (Sæbø et al. 2012; Zampieri et al. 2013; Mo et al. 2015). Conversely, a cover of wax crystals confers high hydrophobicity to leaves, which thus ultimately reflects on decreased particle accumulation (Neinhuis and Barthlott 1997; Koch and Ensikat 2008). Surface sculptures such as the grooves on the rachis macro-relief may also be correlated with higher particle capture. In fact, the results on scanning electron microcopy point to a higher particle accumulation on this region than on leaf blade. This interesting result can somewhat sound improbable, but a similar effect has long been reported to the adhesion of spores, by Carter (1965). The author observed that the ascospore of *Euphyta armeniacae* had more efficient impaction on petioles than on stems or flat leaves of apricot. Analogously, despite their morphology, the needle-shaped leaves of conifers are known to have higher rates of particle retention than broadleaf species (Beckett et al. 2000; Free-Smith et al. 2004). In view of that, several pine species are rather commonly used as biomonitors of airborne particles in the North hemisphere (Lehndorff and Schwark 2008; Mori et al. 2014; Przybysz et al. 2014; Song et al. 2015), even having an already established standardized protocol (VDI 2007).

I recommend the quantitative assessment of leaf surface roughness in future studies that investigate the interaction between plant species and particle deposition. Recent initiatives have been found in the literature (Wang et al. 2015), but reports are still scarce. Such a parameter may allow for researchers to better tap into an important leaf surface features that may determine the degree of at least some plant responses to the surrounding atmosphere impacted by urban-industrial activities. I point out, however, that a more interesting approach is to investigate the roughness given in more than one hierarchical level. Not only macro-relief of the epidermal tissue determine leaf roughness, but also the deposition pattern of epicuticular waxes. The former can be easily assessed through profilometry analysis and should be no trouble for researchers who can dispose of an optical profilometer. The latter, however, has to be performed in an atomic force microscope, which has limitations concerning biological samples. The first one is the detection limit, since the height differences must not exceed 6 µm. Therefore, is may not be applicable for extremely rough surfaces (Koch et al. 2008). The second one is the fragility of the epicuticular wax crystals (Koch et al. 2004). The third concerns sample wetness and/or humidity. Drying leaf samples prior to AFM analysis is recommended to obtain quality results.

The epidermis was the leaf tissue that better responded to the environmental conditions in the experiment. The epidermis of both leaf surfaces was affected, but the environmental effects were particularly noticeable on the epidermis of the leaf adaxial surface of *C. ferrea*. Thickness of leaf tissues has been tested and successfully used as an anatomical biomarker of abiotic stress (Sant'Anna-Santos and Azevedo 2010; Pedroso and Alves 2015). However, it must be analyzed with care, as climate variations affect leaf development, and may therefore interfere with leaf responses in terms of tissue thickness to air pollutants, like ozone (Moura and Alves 2014). Since the result exhibited a clear statistical distinction between plants from the control site and those exposed at the urban environment, the micromorphometrical analysis represented a consistent approach to the present study. Epidermal tissue thickness could be seen as a biomarker of urban emissions in *C. ferrea*.

The epidermis is the outermost tissue of the primary plant body and it has evolved in such a way that it allows, among other features, restriction of entry of toxic substances into plant organs (Beck 2010). It seems reasonable to propose that the higher response of this tissue could be related to its direct contact with pollutants, yet this cannot be taken as a rule. The Restinga species *Clusia hilariana*, for example, responded to foliar deposition of solid particulate matter of iron with lower thickness of all leaf tissues, except for the epidermis (Rocha et al. 2014).

Besides being harmful to plant formations, air pollution can also cause serious damage to human health. Since Ipatinga city developed in function of the implementation of its steel-industry park (Neves and Camisasca 2013), monitoring its atmospheric conditions is important to evaluate the air quality in the city, and ultimately promote preventive actions concerning public health and environmental impacts. Recent data from 2011 through 2014 showed that daily annual mean of inhalable particles (PM_{10}) in Ipatinga city has surpassed the limit established by the World Health Organization, of 20 $\mu g.m^{-3}$ (Programa Cidades Sustentáveis 2015). Thus, developing new strategies and approaches to constantly and more broadly assess air quality in the city should be encouraged. In that sense, the biomonitoring technique has some remarkable advantages, like its technological simplicity with which it allows the determination of places with higher levels of heavy metal pollution (Arndt and Schweizer 1991). Thus, studying the possibility of use of species from the local flora aiming at standardizing trustable methods remain an important issue to be explored. Assessing leaf surface features that determine higher particle accumulation seems to lead the way of this type of research.

Both *C. echinata* and *C. ferrea* are widely used as ornamental in Ipatinga city. The former was the one that retained more particles, due to characteristics such as low leaf roughness. Its use as ornamental should be encouraged, as the species may have to potential to form phytobarriers (Sánchez-López et al. 2015), thus helping to clean the urban air from airborne particulate matter (Freer-Smith et al. 2004; Janhäl 2015; Sgrigna et al. 2015). However, landscape design must be thoroughly planned, otherwise the random position of trees could have the opposite effect, actually increasing the accumulation of fine particles in the urban environment (Tong et al. 2015).

Regardless, an important following step to the present research would be to perform a passive biomonitoring using the adult individuals already planted throughout the city. Particularly *C. echinata* showed to be promising for this end and, thereby, evaluating the trees already in the city which are constantly exposed to local pollution would raise complementing information to those herein. Therewithal, *C. echinata* is extensively used as ornamental in several other Brazilian cities (Rocha and Barbedo

2008; Moro and Castro 2015), due mainly to its historical significance, as it is the symbol-tree to the country (von Muralt 2006). Standardization remains one of the main issues with comparability of passive biomonitoring studies (Tarricone et al. 2015). Thus, elaborating standardized approaches with this species, in alignment with international studies, and investigating its potential for passive biomonitoring might allow for future establishment of a monitoring network that could sample several cities across the country and give comparable results, not only with local or national, but also with international standards.

5. Conclusions

Caesalpinia ferrea Mart. ex Tul. showed a more well-defined response gradient in relation to the parameters analyzed herein, like the amount and chemical composition of waxes and epidermis thickness. For that reason, this species could be better explored as a biosensor of urban pollution. Its biosensorial potential deserves further investigation, due to the responses observed in its leaf tissues at the anatomical level. The species, however, retained lower amounts of particles and metals associated with vehicular and industrial emission, due to surface features of its leaves that confer them higher self-cleaning effect, like the higher roughness given both by the convex outer periclinal wall of epidermal cells and for its wax-layer microsculpturing. The opposite effects were observed in *C. echinata* and, for that reason, this species is better recommended for biomonitoring of air pollution as a bioaccumulator. Further studies that might investigate the responses of this species to particulate pollutants under controlled experimental conditions are suggested.

The first hypothesis of the study was not rejected. There was a difference in particle accumulation between the species, and such difference was given by epicuticular wax micromorphology – in the form of platelets in *C. ferrea* and layer in *C. echinata* -, to leaf macro-and micro-roughness and to wax chemical composition. The second hypothesis was partially rejected, since based on the afore-mentioned features only *C. echinata* can be recommended for biomonitoring airborne PM.

These results reported herein demonstrate the importance of aggregating anatomical and micromorphological analyses to studies on the use of plants in biomonitoring airborne pollutants. Light microscopy studies allowed the assessment of micromorphometrical responses and showed consistent results, distinguishing the control site from impacted ones. Scanning electron and atomic force microscopies allowed for a better characterization of leaf surface features that determined particle retention. Such an integrated approach, when taken into consideration, can help to better elucidate the extension of air pollution effects and aid in the secure establishment of parameters for biomonitoring.

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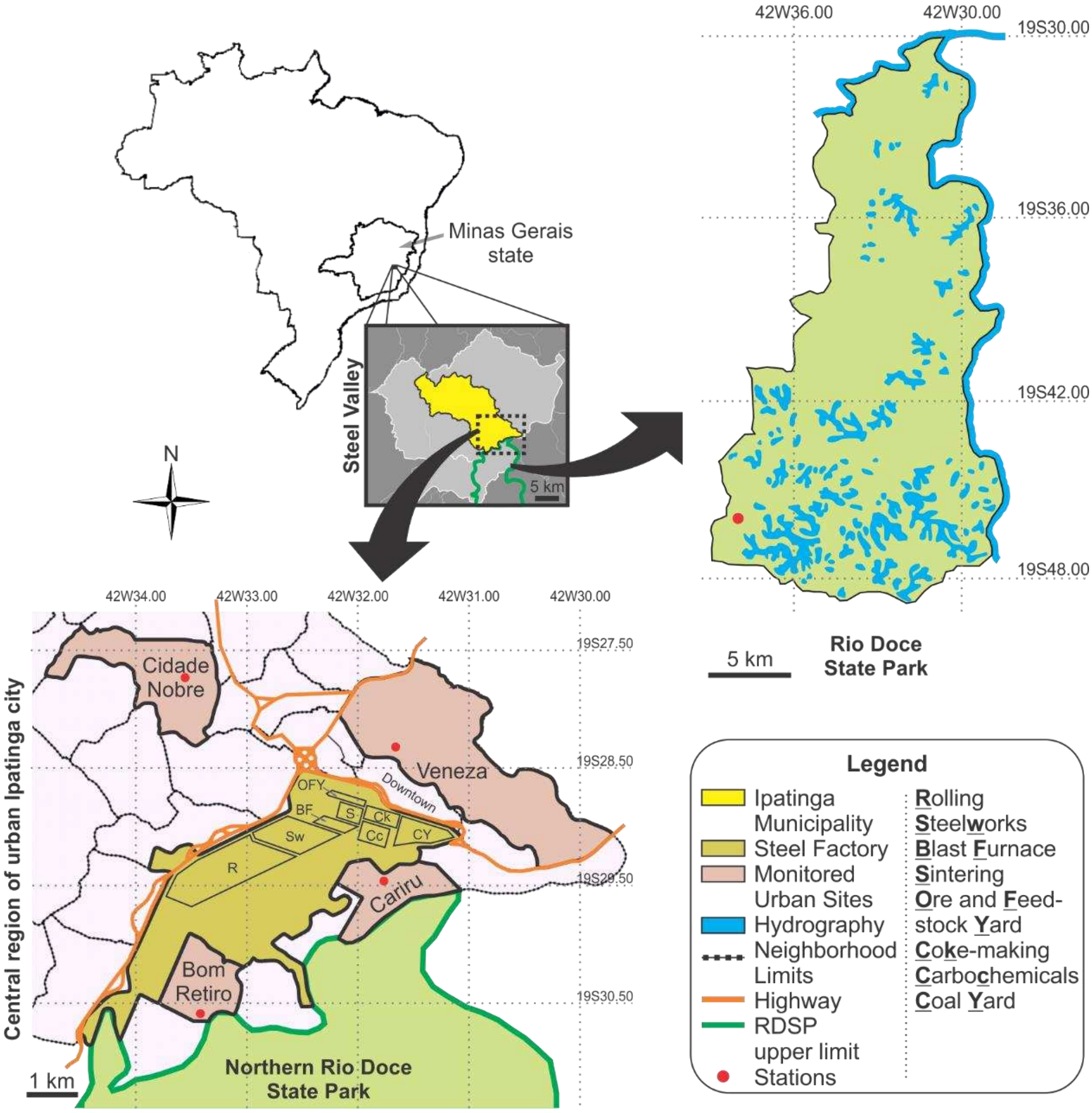

Fig. 1. Study area and sampled sites in Ipatinga city, Southeastern Brazil, located in a metropolitan region called Steel Valley. The sampling sites were four neighborhoods around a steel factory: Bom Retiro, Cariru and Veneza, and a more distant one, Cidade Nobre. A site in the Rio Doce State Park, adjacent to the municipality, was sampled as reference-station.

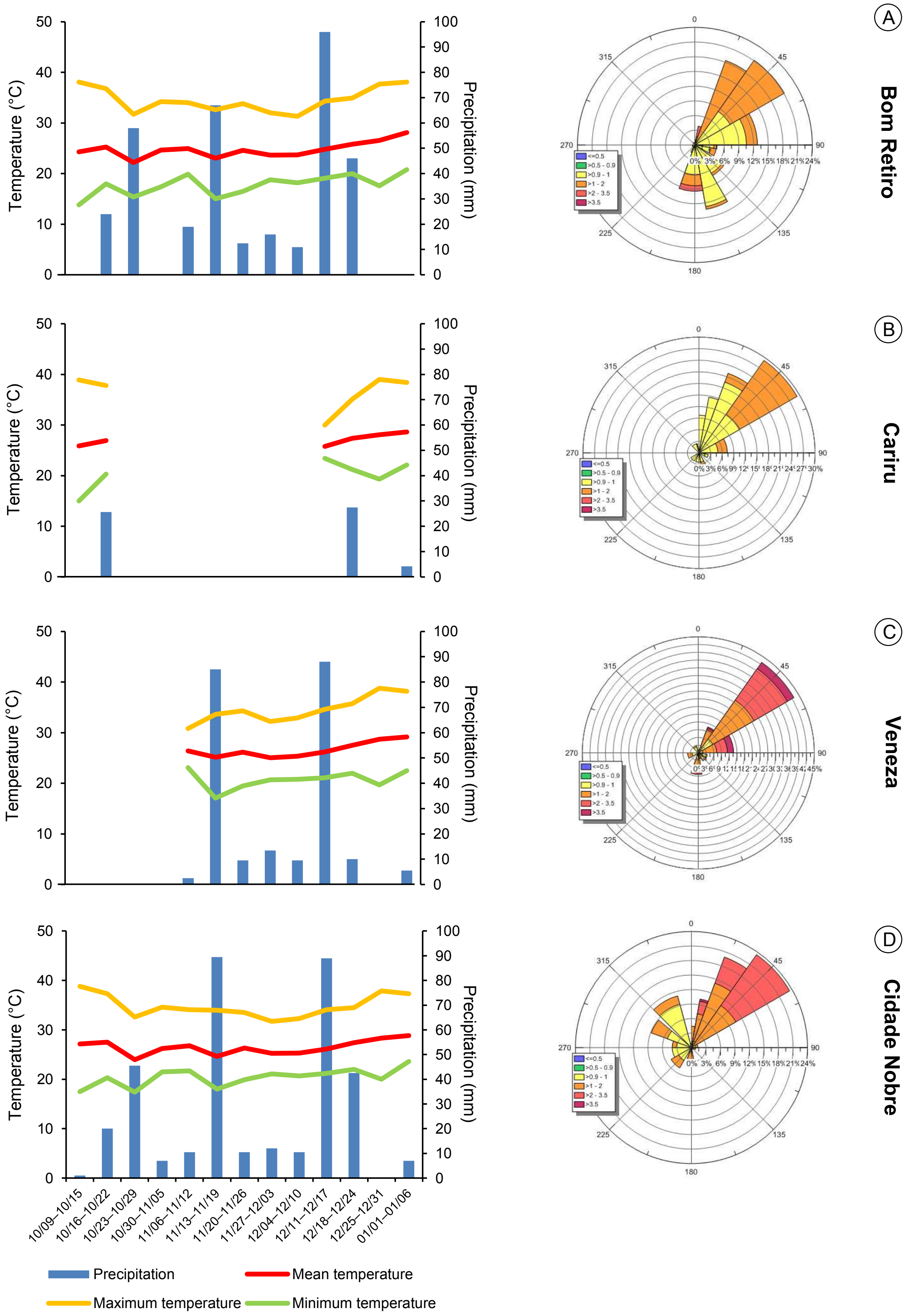

Fig. 2. Meteorological characterization of the studies sites. Climatograms (left column) showing weekly accumulated precipitation (mm) and mean temperature (° C), and windrose charts (right column) showing predominant wind speed and direction in the central region of Ipatinga city along the thirteen-week exposition period. Values not shown correspond to readings not registered by the automatic stations. A: Bom Retiro. B: Cariru. C: Veneza. D: Cidade Nobre.

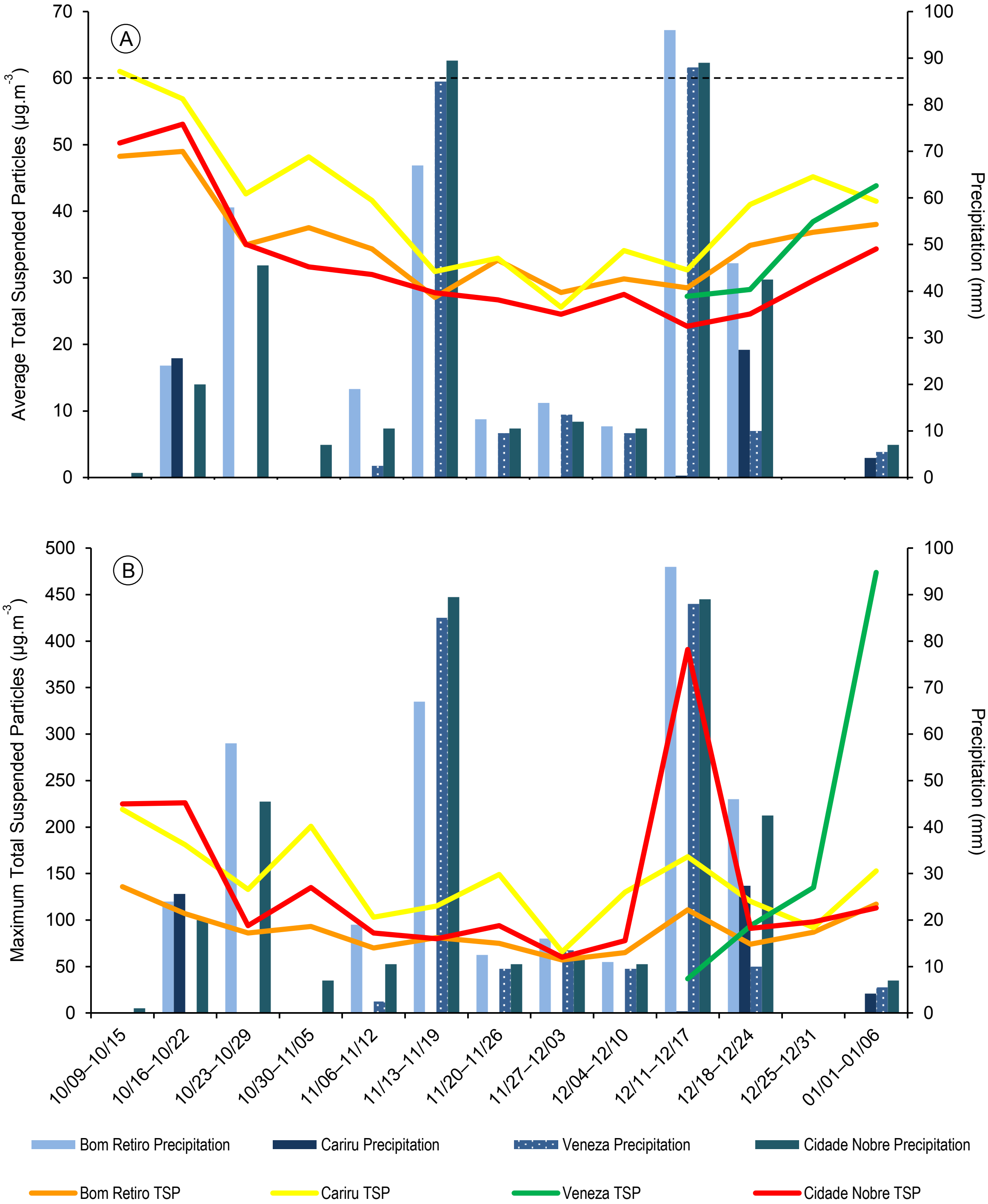

Fig. 3. Weekly geometric means (A) and maximum values (B) of total suspended particles (TSP) in the central region of Ipatinga city and weekly accumulated precipitation (mm) along the thirteen-week exposition period. Values not shown (in the Veneza line) correspond to readings not registered by the automatic stations. Dashed line: Brazilian secondary standard (60 $\mu g.m^{-3}$ annual geometric mean concentration) (CONAMA 1990).

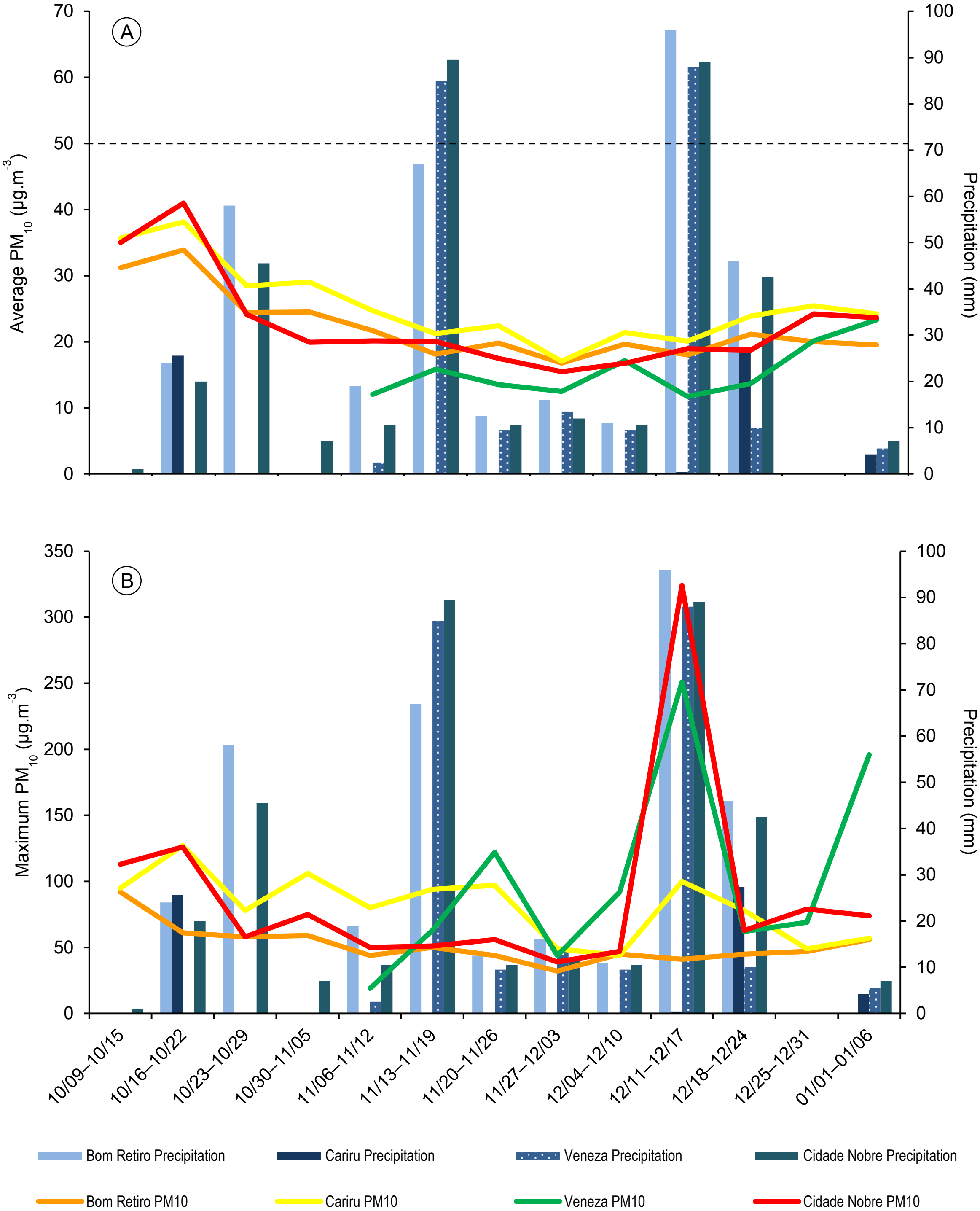

Fig. 4. Weekly arithmetic means (A) and maximum values (B) of inhalable particles (PM_{10}) in the central region of Ipatinga city and weekly accumulated precipitation (mm) along the thirteen-week exposition period. Values not shown (in the Veneza line) correspond to readings not registered by the automatic stations. Dashed line: Brazilian secondary standard (50 $\mu g.m^{-3}$ annual arithmetic mean concentration) (CONAMA 1990).

Table 1. Analysis of variance of trace-metal accumulation in *Caesalpinia echinata* and *Caesalpinia ferrea* leaves after three months of exposure to urban pollution from a steel pole in Southeastern Brazil.

Species (Sp)	Site	Metals▲											
		Ca	Mg	Cu	Fe	Zn	Mn	Ni	Pb	Cr	Ba	Al	Co
Caesalpina echinata	Bom Retiro	1.820 ± 0.175^{Aa}	0.178 ± 0.024^{A}	5.767 ± 0.153^{Ba}	1474.500 ± 597.995^{Aa}	47.133 ± 4.801	545.800 ± 25.982^{Aa}	3.667 ± 0.737^{Aa}	13.000 ± 0.721^{Aa}	3.033 ± 0.289^{a}	3.816 ± 1.233	109.834 ± 51.816^{a}	0.092 ± 0.026^{Aa}
	Cariru	1.640 ± 0.431^{Aa}	0.156 ± 0.009^{A}	5.367 ± 1.365^{Ba}	703.900 ± 297.616^{Ab}	34.067 ± 8.264	458.433 ± 148.647^{Aa}	1.990 ± 0.264^{Ab}	11.100 ± 0.458^{Ab}	1.967 ± 0.305^{ab}	4.601 ± 2.116	57.245 ± 16.741^{ab}	0.116 ± 0.037^{Ba}
	Veneza	1.477 ± 0.216^{Aa}	0.159 ± 0.024^{A}	5.600 ± 0.529^{Aa}	721.267 ± 21.371^{Ab}	28.800 ± 1.819	422.533 ± 17.264^{Aa}	2.000 ± 0.1^{Ab}	11.300 ± 0.360^{Aab}	1.833 ± 0.252^{ab}	4.765 ± 1.343	79.514 ± 13.392^{ab}	0.116 ± 0.014^{Aa}
	Cidade Nobre	1.988 ± 0.213^{Aa}	0.179 ± 0.003^{B}	6.400 ± 1.082^{Ba}	465.900 ± 62.085^{Ab}	38.200 ± 1.778	413.033 ± 25.022^{Aa}	1.800 ± 0.436^{Ab}	10.900 ± 1.212^{Ab}	2.367 ± 0.586^{ab}	2.904 ± 0.028	71.437 ± 3.681^{ab}	0.086 ± 0.028^{Ba}
	Rio Doce State Park	1.647 ± 0.233^{Aa}	0.142 ± 0.027^{B}	4.833 ± 0.586^{Aa}	255.767 ± 20.476^{Ab}	32.517 ± 5.858	328.800 ± 39.392^{Ba}	1.400 ± 0.1^{Ab}	10.267 ± 0.153^{Ab}	1.467 ± 0.416^{b}	2.306 ± 1.418	39.044 ± 8.887^{b}	0.096 ± 0.020^{Aa}
Caesalpina ferrea	Bom Retiro	1.623 ± 0.978^{Aab}	0.303 ± 0.067^{A}	15.167 ± 1.101^{Aa}	633.533 ± 73.559^{Ba}	60.150 ± 15.368	596.600 ± 310.418^{Aab}	1.933 ± 0.115^{Bab}	10.733 ± 0.472^{Ba}	2.433 ± 0.513^{a}	0.398 ± 0.382	67.648 ± 3.148^{a}	0.133 ± 0.027^{Ab}
	Cariru	0.640 ± 0.076^{Ab}	0.186 ± 0.019^{A}	12.033 ± 0.153^{Aa}	364.833 ± 38.317^{Aa}	40.700 ± 3.315	298.833 ± 79.442^{Ab}	2.667 ± 0.252^{Aa}	10.833 ± 0.378^{Aa}	1.933 ± 0.115^{a}	0.389 ± 0.099	60.933 ± 4.902^{a}	0.201 ± 0.027^{Aa}
	Veneza	1.780 ± 0.809^{Aab}	0.232 ± 0.084^{A}	8.167 ± 1.686^{Ab}	335.867 ± 74.423^{Aa}	44.433 ± 5.529	601.367 ± 187.273^{Aab}	2.067 ± 0.153^{Aab}	10.833 ± 0.503^{Aa}	2.500 ± 0.693^{a}	0.793 ± 0.454	67.490 ± 17.660^{a}	0.172 ± 0.0388^{Aa}
	Cidade Nobre	1.463 ± 0.353^{Aab}	0.329 ± 0.081^{A}	11.233 ± 2.892^{Aa}	298.900 ± 9.853^{Aa}	44.117 ± 7.447	657.167 ± 148.806^{Aab}	2.133 ± 0.321^{Aab}	11.033 ± 0.814^{Aa}	2.433 ± 0.115^{a}	0.987 ± 0.622	76.332 ± 19.414^{a}	0.233 ± 0.014^{Aa}
	Rio Doce State Park	2.642 ± 0.824^{Aa}	0.302 ± 0.069^{A}	6.700 ± 2.107^{Ab}	239.267 ± 40.457^{Aa}	52.667 ± 11.481	805.300 ± 274.614^{Aa}	1.533 ± 0.153^{Ab}	10.167 ± 0.305^{Aa}	1.967 ± 0.451^{a}	0.702 ± 0.287	39.145 ± 5.820^{a}	0.094 ± 0.016^{Ab}

Analysis of Variance

SV	DF	Mean Square											
Sp	1	0.05393$^{n.s.}$	0.086941***	192.533***	917630***	1129.15***	187546*	0.05633$^{n.s.}$	2.6403*	0.10800$^{n.s.}$	287463$^{n.s.}$	621.77$^{n.s.}$	0.031948***
Site	4	0.77018$^{n.s.}$	0.006838$^{n.s.}$	20.094***	561481***	279.74**	37278$^{n.s.}$	1.41967***	2.0550**	0.93217**	113996$^{n.s.}$	2105.30**	0.004966***
Sp x Site	4	0.88444*	0.004505$^{n.s.}$	14.206**	145174*	54.79$^{n.s.}$	83121*	1.38300***	1.3853*	0.37050$^{n.s.}$	113928$^{n.s.}$	580.24$^{n.s.}$	0.004688**

Means ± standard deviation. ▲Ca and Mg in dag.kg^{-1}; other elements in mg.kg^{-1}. *** Significant by F test (P < 0.001). ** Significant by F test (P < 0.01). * Significant by F test (P < 0.05). n.s. = non-significant by F test (P > 0.05). Means followed by different letters differ by Tukey's test at 5% probability. Upper case letters compare species in a same site. Lower case letters compare sites in a same species.

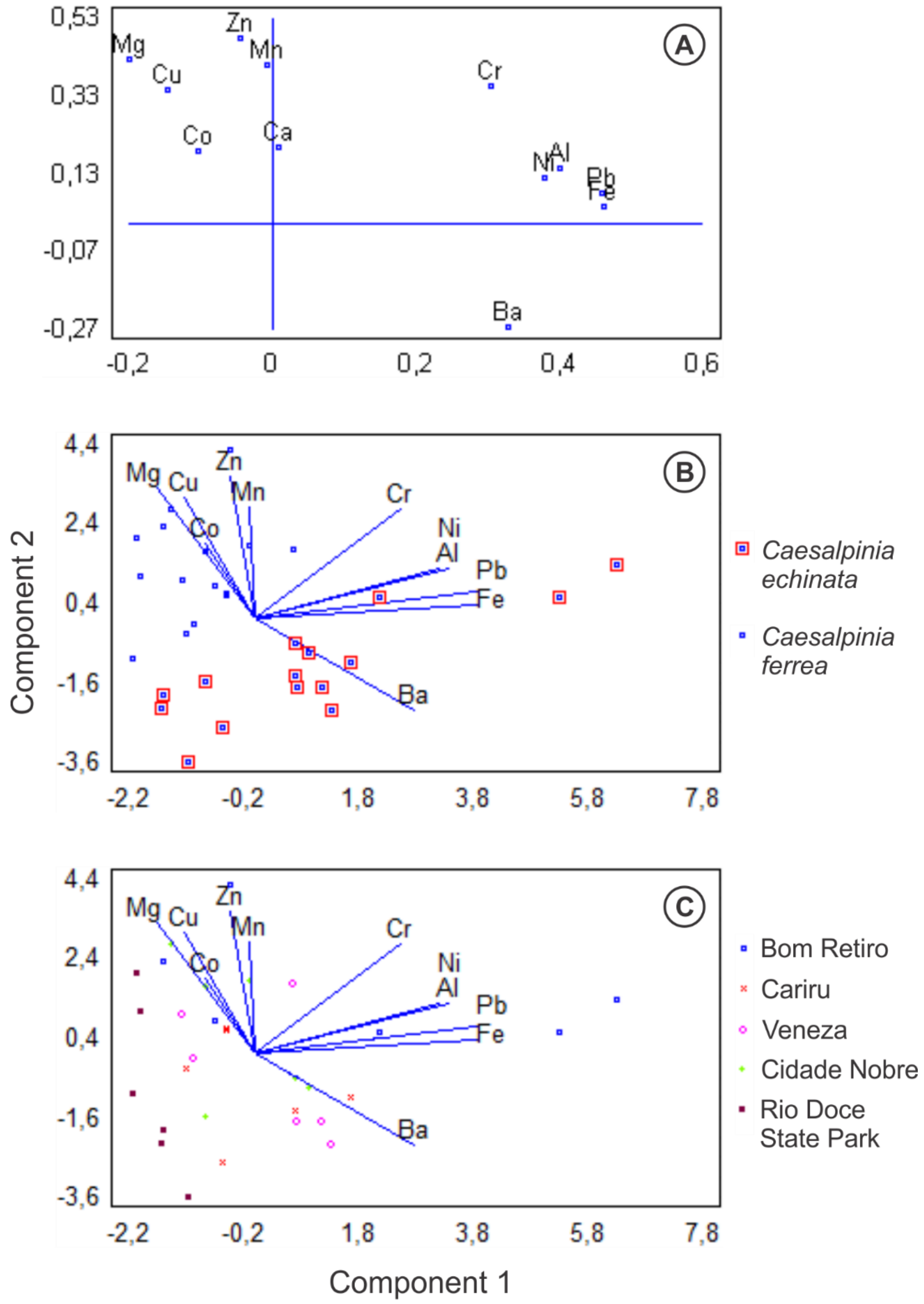

Fig. 5. Multivariate analysis of trace-metal accumulation in *Caesalpinia echinata* and *Caesalpinia ferrea* leaves after three months of exposure to urban pollution from a steel pole in Southeastern Brazil. A: Plot of component weights. B and C: Biplots.

Table 2. Leaf surface roughness given by the leaf micro- and macrorelief, due to the epicuticular waxes and epidermal tissue, respectively.

Species	Leaf surface	Macro-roughness (μm)		Micro-roughness (nm)	
		Sa	Sq	Sa	Sq
Caesalpinia echinata	Adaxial	0.6577 ± 0.2109^{Bb}	0.8288 ± 0.2679^{Bb}	68.1480 ± 4.3017^{Ba}	86.3852 ± 6.2238^{Ba}
	Abaxial	2.5001 ± 0.3307^{Ba}	3.7743 ± 0.4818^{Aa}	50.0364 ± 9.1086^{Ba}	64.4714 ± 11.1439^{Ba}
Caesalpinia ferrea	Adaxial	2.6039 ± 0.3171^{Aa}	3.4059 ± 0.3997^{Aa}	220.2571 ± 28.2445^{Aa}	275.9901 ± 34.3954^{Aa}
	Abaxial	3.2359 ± 0.1256^{Aa}	4.2033 ± 0.1195^{Aa}	267.5347 ± 55.3487^{Aa}	335.2444 ± 74.3694^{Aa}
Analysis of Variance					
SV	DF	Mean Square			
Species	1	5.3949***	6.7777***	102457***	158961***
Leaf surface	1	4.5922***	10.5070***	638$^{n.s.}$	1046$^{n.s.}$
Species x Leaf surface	1	1.0989**	3.4607***	3207$^{n.s.}$	4941$^{n.s.}$

Means ± standard deviation. Sa: mean roughness. Sq: root mean square roughness. *** Significant by F test (P < 0.001). ** Significant by F test (P < 0.01). * Significant by F test (P < 0.05). n.s. = non-significant by F test (P > 0.05). Means followed by the same letter do not differ by Tukey's test at 5% probability. Upper case letters compare species in a same leaf surface. Lower case letters compare leaf surfaces in a same species.

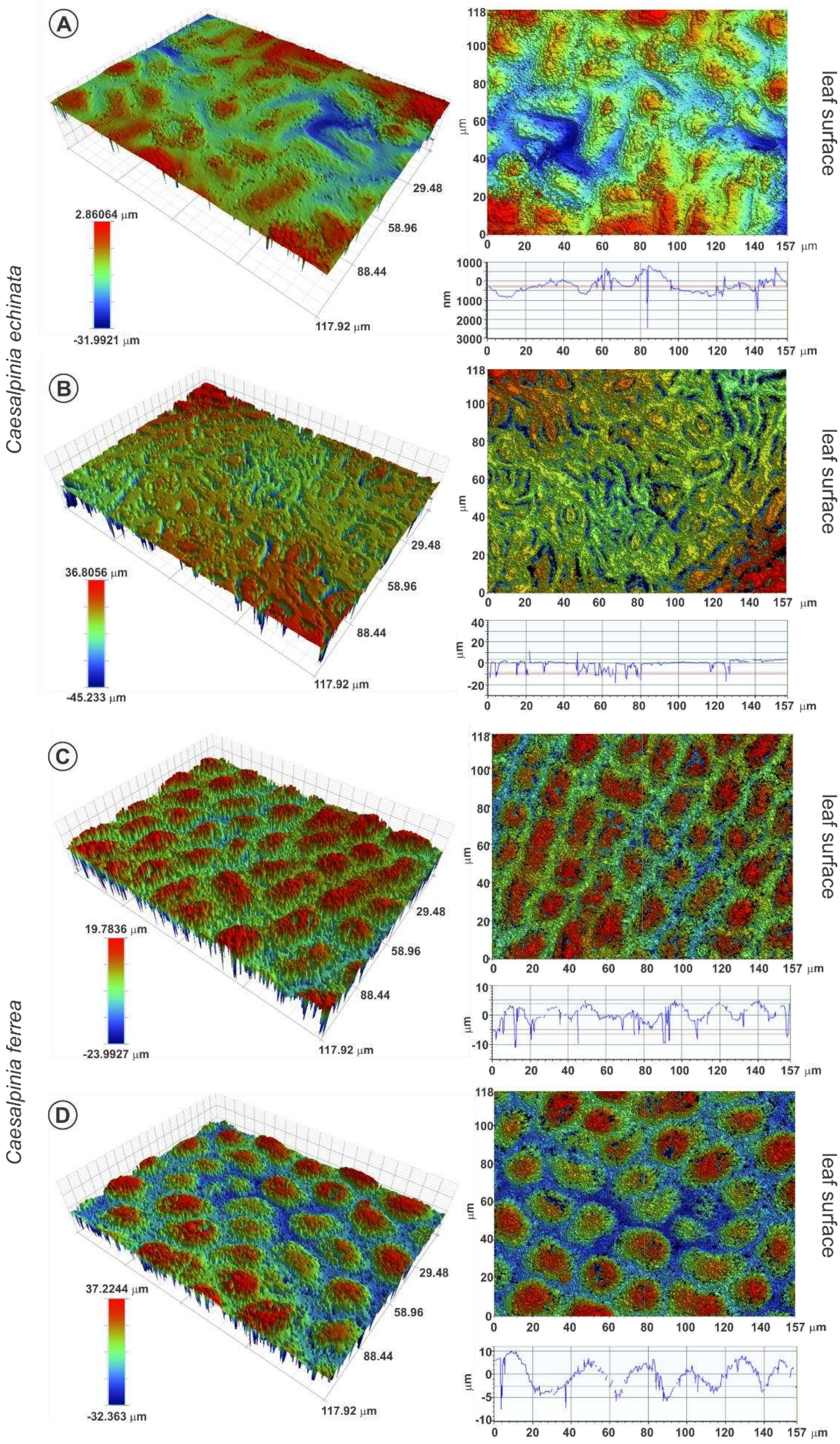

Fig. 6. Epidermal surface mapping of the adaxial (A and C) and abaxial (B and D) leaf blade surfaces of *Caesalpinia echinata* (A and B) and *Caesalpinia ferrea* (C and D). 3D maps (left), 2D maps (top right) and 2D profiles (bottom right). Images were obtained using an optical profilometer and represent the leaf macro-relief, i.e., the one determined by the cell arrangement in the epidermal tissue.

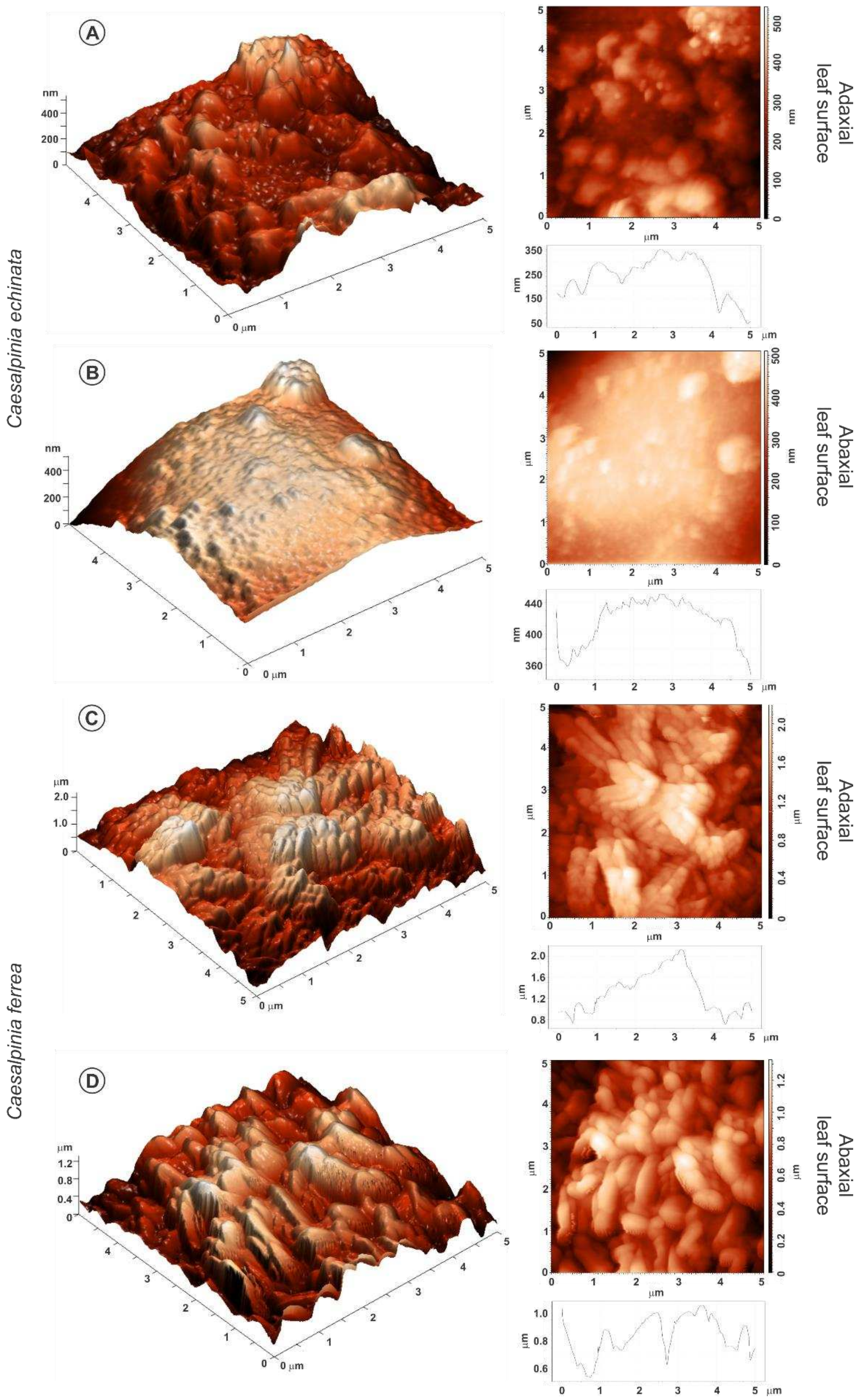

Fig. 7. Outer periclinal cell wall surface mapping of the adaxial (A and C) and abaxial (B and D) leaf blade surfaces of *Caesalpinia echinata* (A and B) and *Caesalpinia ferrea* (C and D). 3D maps (left), 2D maps (top right) and 2D profiles (bottom right). Images were obtained using an atomic force microscope and represent the leaf microrelief, i.e., the one determined by the deposition pattern of epicuticular waxes on the epidermal tissue.

Table 3. Correlation coefficients between macro- and micro-rougnhess (mean and squered ones, both) of the leaf adaxial surface and trace-metal accumulation in leaf dry mass of *Caesalpinia echinata* and *Caesalpinia ferrea* after three months of exposure to urban pollution from a steel pole in Southeastern Brazil.

		Ca	Mg	Cu	Fe	Zn	Mn	Ni	Pb	Cr	Ba	Al	Co
Average macro-roughness													
Caesalpinia echinata	BR	0,96	0,42	-0,93	-0,93	0,99 *	-0,99 *	-0,32	-0,95	*-0,99 **	-0,86	-0,75	0,03
	CA	0,24	-0,64	0,13	0,53	-0,07	0,52	-0,97	0,17	-0,20	-0,08	0,45	0,97
	VZ	0,09	-0,82	0,97	-0,73	0,53	0,50	0,85	-0,96	-0,81	0,98	0,98	-0,89
	CN	-0,99 *	-0,09	0,73	-0,39	-0,30	-0,97	0,99	0,15	0,35	0,99 *	0,41	0,59
	RDSP	0,57	-0,93	-0,62	-0,17	-0,81	-0,28	0,01	0,17	-0,97	0,99 *	0,81	-0,48
Caesalpinia ferrea	BR	0,65	0,06	0,69	0,99	0,49	0,71	0,75	0,87	0,99 *	0,75	-0,41	0,28
	CA	0,98	0,05	0,78	0,91	0,07	0,85	0,43	0,83	0,75	-0,81	0,99	0,96
	VZ	-0,80	0,97	0,89	-0,29	0,83	-0,82	0,93	0,43	-0,18	-0,27	-0,38	-0,32
	CN	0,42	-0,92	-0,87	0,82	0,73	0,55	0,88	0,83	0,75	0,99 *	-0,90	-0,96
	RDSP	-0,96	-0,58	0,40	-0,98	-0,86	-0,84	*0,99 **	0,14	-0,99	0,98	-0,59	0,12
Squared macro-roughness													
Caesalpinia echinata	BR	0,96	0,40	-0,94	-0,94	0,99	-0,99 *	-0,31	-0,95	*-0,99 **	-0,86	-0,74	-0,05
	CA	0,25	-0,63	0,14	0,54	-0,06	0,53	-0,98	0,18	-0,19	-0,07	0,46	0,97
	VZ	-0,08	-0,83	0,98	-0,72	0,52	0,49	0,86	-0,96	-0,80	0,98	0,99	-0,90
	CN	-0,99 *	-0,08	0,72	-0,38	-0,29	-0,97	0,99	0,14	0,34	0,99	0,40	0,60
	RDSP	0,58	-0,92	-0,63	-0,16	-0,80	-0,27	0,002	0,18	-0,97	0,99 *	0,80	-0,49
Caesalpinia ferrea	BR	0,56	-0,04	0,60	*0,99 **	0,39	0,62	0,82	0,92	0,98	0,67	-0,51	0,38
	CA	*0,99 **	0,16	0,70	0,92	0,18	0,90	0,53	0,89	0,82	-0,75	0,99 *	0,93
	VZ	-0,73	0,94	0,84	-0,19	0,89	-0,75	0,96	0,53	-0,07	-0,16	-0,28	-0,21
	CN	0,31	-0,87	-0,92	0,88	0,65	0,46	0,82	0,88	0,82	0,99	-0,94	-0,98
	RDSP	-0,93	-0,49	0,50	-0,95	-0,80	-0,78	0,99	0,25	-0,99 *	0,99 *	-0,49	0,01
Average micro-roguhness													
Caesalpinia echinata	BR	0,33	0,93	0,27	0,15	0,15	-0,04	-0,96	0,22	-0,05	0,44	-0,70	-0,99
	CA	-0,95	-0,80	-0,97	-0,80	-0,99	-0,80	0,13	-0,97	-0,99	-0,99 *	-0,85	-0,16
	VZ	0,99 *	0,54	-0,13	-0,72	0,87	0,89	-0,45	-0,32	-0,63	0,25	-0,08	0,37
	CN	-0,01	-0,99 *	0,73	-0,94	-0,97	-0,28	-0,06	*0,99 **	0,95	0,15	0,93	-0,76
	RDSP	-0,77	-0,42	0,73	-0,99	-0,63	-0,97	*0,99 **	-0,97	-0,29	0,12	0,63	0,83
Caesalpinia ferrea	BR	0,98	0,66	0,98	0,69	0,92	0,99	0,19	0,39	0,81	0,99 *	0,23	-0,37
	CA	0,67	-0,57	*0,99 **	0,46	-0,55	0,34	-0,21	0,32	0,19	-0,99 *	0,69	0,92
	VZ	0,99 *	0,89	0,97	-0,82	0,31	-0,99 *	0,50	-0,21	-0,75	-0,80	-0,87	-0,83
	CN	0,89	-0,96	-0,39	0,30	0,99 *	0,95	0,98	0,31	0,19	0,80	-0,45	-0,59
	RDSP	-0,91	-0,96	-0,24	-0,89	-0,99	*-0,99 **	0,78	-0,49	-0,70	0,67	-0,96	0,71
Squared micro-roughness													
Caesalpinia echinata	BR	0,44	0,96	0,16	0,16	0,26	-0,16	-0,98	0,10	-0,17	0,33	-0,78	-0,97
	CA	-0,96	-0,86	-0,94	-0,72	-0,99	-0,73	0,01	-0,93	-0,99 *	-0,99	-0,79	-0,04
	VZ	0,99 *	0,39	-0,01	-0,80	0,92	0,94	-0,34	-0,43	-0,72	0,36	0,03	0,26
	CN	-0,13	-0,99 *	0,80	-0,97	-0,99	-0,39	0,05	*0,99 **	0,98	0,27	0,97	-0,67
	RDSP	-0,69	-0,52	0,64	*-0,99 **	-0,71	*-0,99 **	0,98	-0,93	-0,40	0,23	0,72	0,76
Caesalpinia ferrea	BR	0,97	0,64	0,98	0,71	0,91	0,98	0,22	0,42	0,82	0,99 *	0,21	-0,34
	CA	0,69	-0,54	*0,99 **	0,48	-0,53	0,37	-0,18	0,34	0,22	-0,99 *	0,71	0,93
	VZ	-0,99 *	0,91	0,98	-0,80	0,34	-0,99 *	0,52	-0,18	-0,73	-0,79	-0,85	-0,82
	CN	0,87	-0,96	-0,41	0,33	0,99	0,94	0,98	0,34	0,22	0,81	-0,48	-0,61
	RDSP	-0,92	-0,95	-0,21	-0,90	-0,99	*-0,99 **	0,80	-0,46	-0,72	0,69	-0,95	0,68

BR: Bom Retiro. CA: Cariru. VZ: Veneza. CN: Cidade Nobre. RDSP: Rio Doce State Park. * Significant correlation ($P < 0.05$).

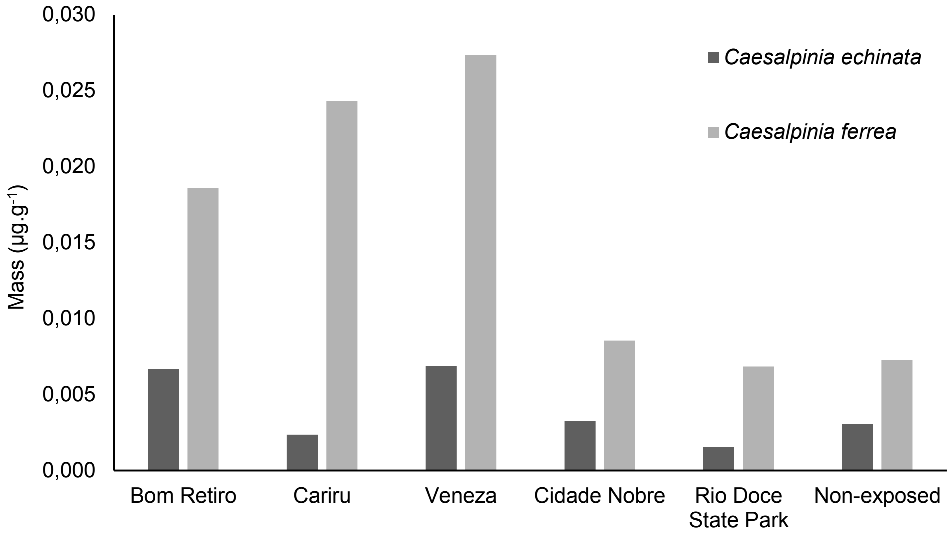

Fig. 8. Amounts of waxes ($\mu g.g^{-1}$) in third-node leaves of *Caesalpinia echinata* and *Caesalpinia ferrea* after three months exposure to urban pollution from a steel pole in Southeastern Brazil.

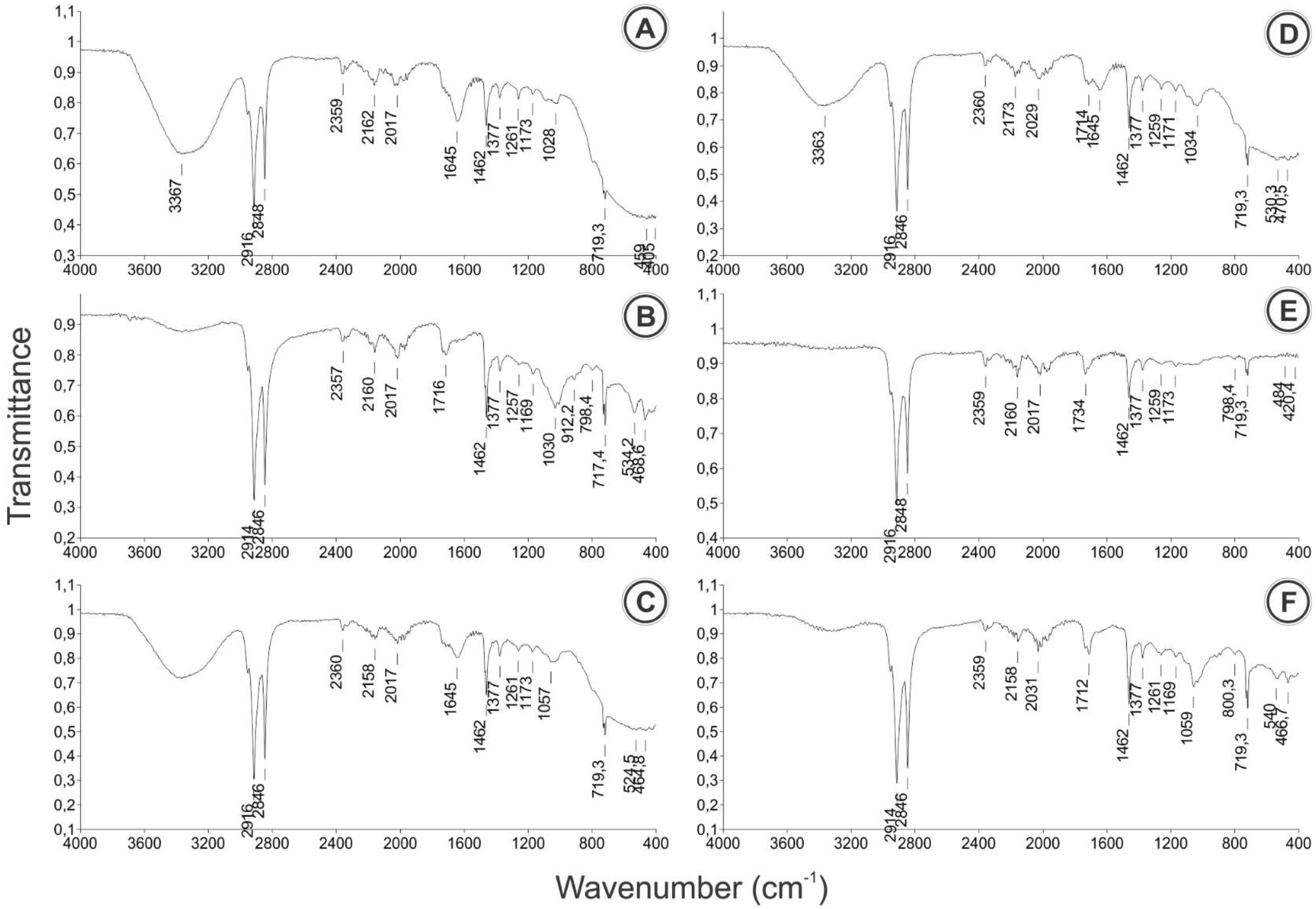

Fig. 9. Infrared spectrometry of the waxes of third-node leaves of *Caesalpinia echinata* after three months of exposure to urban pollution from a steel pole in Southeastern Brazil. A: Bom Retiro. B: Cariru. C: Veneza. D: Cidade Nobre. E: Rio Doce State Park. F: Non-exposed plants.

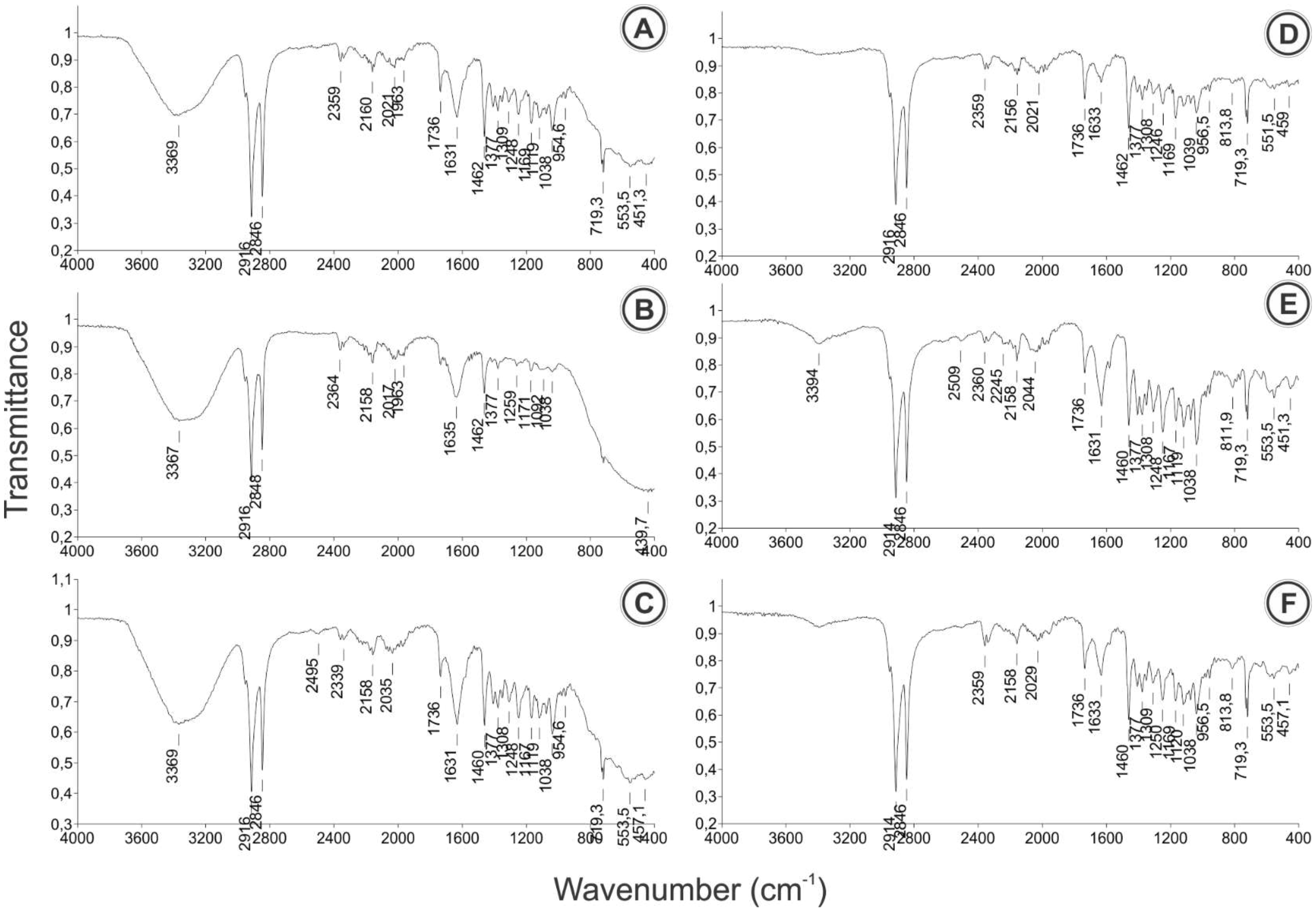

Fig. 10. Infrared spectrometry of the waxes of third-node leaves of *Caesalpinia ferrea* after three months of exposure to urban pollution from a steel pole in Southeastern Brazil. A: Bom Retiro. B: Cariru. C: Veneza. D: Cidade Nobre. E: Rio Doce State Park. F: Non-exposed plants.

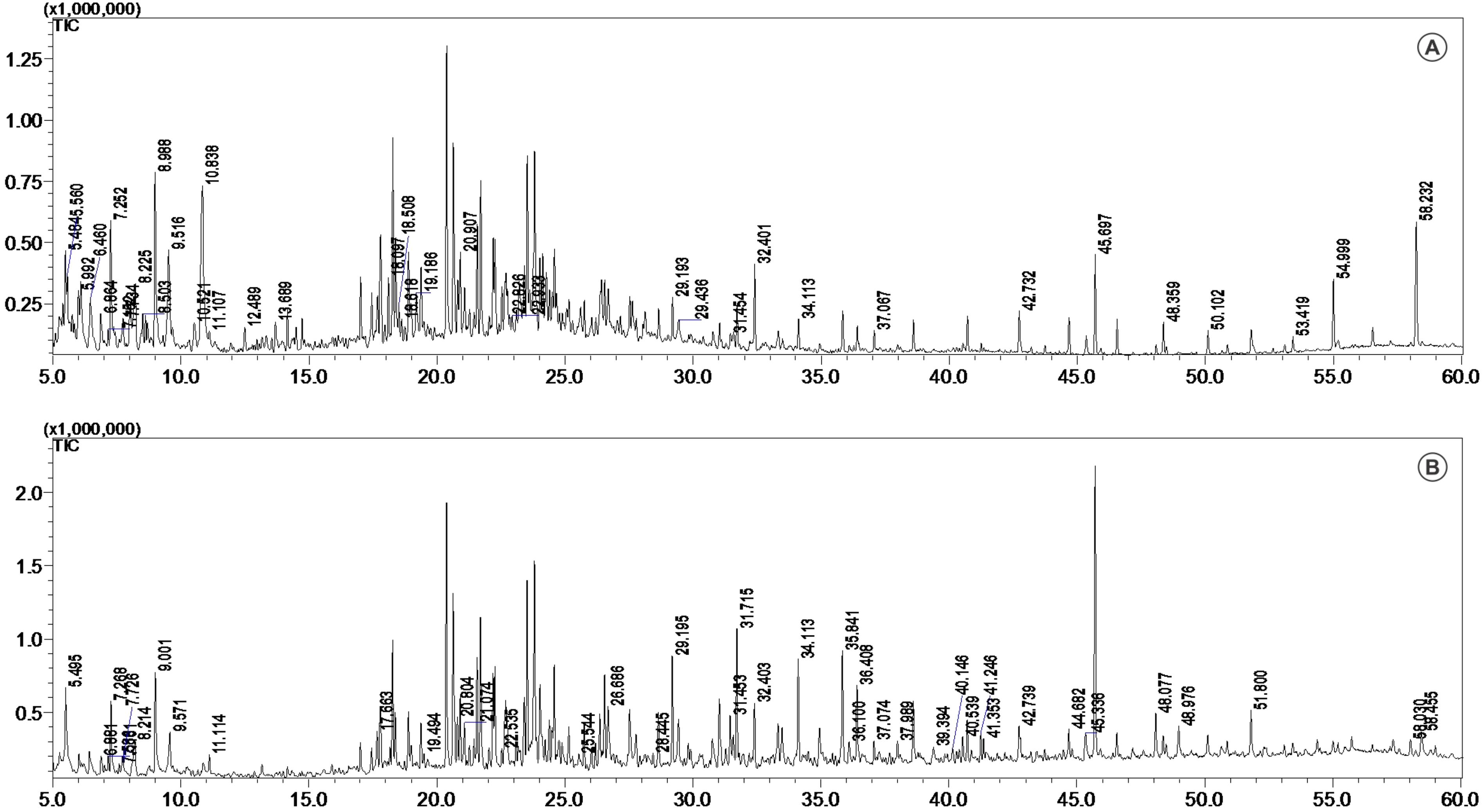

Fig. 11. Mass-chromatograms of the waxes of third-node leaves of *Caesalpinia echinata* (A) and *Caesalpinia ferrea* (B) (non-exposed plants).

Table 4. Chemical composition (%) of the waxes of third-node leaves of *Caesalpinia echinata* (non-exposed plants).

Compound	**Retention time (min)**	**Percentage in wax composition**
3,3-dimethyl-pentane	5.484	4.72
3,7-dimethyl-1-octene	5.560	2.62
7-oxo-octanoic acid	5.992	2.24
4,4,5-trimethyl-1,3-dioxan-5-ol	6.460	1.87
2,5-dihydro-3,5-dimethyl-2-furanone	6.864	0.84
3-Z-hepten-2-ol	7.152	0.64
3-methyl-1,2-cyclopentanedione	7.252	6.00
1,3,9-Trioxaspiro[5.5]undecane	7.734	1.01
2-ethyl-hexanoic acid	8.225	3.48
5,6-dimethyl-decane	8.503	0.85
3-methyl-1,2-cyclopentanedione	8.988	8.51
1-nitro-2-octanone	9.516	6.60
2-tetradecanone	10.521	1.16
2-nonanone	10.838	16.55
1,2-epoxyundecane	11.107	0.20
4-decanone	12.489	1.27
2,6-dimethyl-4-hepten-3-one	13.689	0.92
5,5-dimethyl-3-oxo-hexanoic acid ethyl ester	18.097	2.82
2-methoxy-3-methyl-2-butenoic acid methyl ester	18.508	0.99
2-(2-vinyloxy-ethoxy)-cyclohexanol	18.618	0.80
Cyclobutanecarboxylic acid, 2-octyl ester	19.186	2.61
3-(2-methyl-propenyl)-1H-indene	20.907	4.56
Citronellolepoxid (R oder S)	22.826	0.40
5-Undecanone	22.933	0.43
Eicosane	29.193	1.57
2,6,10-trimethyl-dodecane	29.436	0.80
Homosalate	31.454	0.31
Hexadecanoic acid methyl ester	32.401	3.74
Hexadecane	34.113	1.03
Methyl stearate	37.067	0.73
Heneicosane	42.732	2.10
1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester	45.697	4.79
Nonacosane	48.359	1.15
Tritriacontane	50.102	0.84
2,6,10,14-tetramethyl-hexadecane	53.419	0.18
Tetracontane	54.999	3.28
Dotriacontane	58.232	7.39

Table 5. Chemical composition (%) of the waxes of third-node leaves of *Caesalpinia ferrea* (non-exposed plants).

Compound	**Retention time (min)**	**Percentage in wax composition**
2,3,4-trimethyl-pentane	5.495	4.83
2,5-dihydro-3,5-dimethyl-2-furanone	6.881	0.70
Undec-3-en-2-ol	7.161	0.67
2-hydroxy-3-methyl-2-cyclopenten-1-one	7.268	3.98
Nonanal	7.586	0.40
1-acetyl-1-cyclopropancarbonic acid ethyl ester	7.726	0.67
2-ethyl-hexanoic acid	8.214	3.69
3-methyl-1,2-cyclopentanedione	9.000	6.84
5-hydroxy-4,5-dimethyl-2,5-dihydrofuran-2-one	9.571	2.32
Decanal	11.113	0.58
6-cyclohexyl-dodecane	17.663	2.23
Tetraneurin-a-diol	19.494	0.60
Pentadecane	20.804	2.99
11-isopropylidenetricyclo[6.2.1.0(2,7)]undeca-2,4,6,9-tetraene	21.074	3.32
7-tridecanone	22.535	0.90
8-hexyl-pentadecane	25.544	0.29
2,6,10,14-tetramethyl-hexadecane	26.686	3.83
3,8-dimethyl-decane	28.445	0.62
Heptadecane	29.195	6.43
Homosalate	31.453	3.08
2-methyloctacosane	31.716	7.63
Hexadecanoic acid methyl ester	32.403	3.52
Octadecane	34.113	5.92
4,6-dipropyl-nonan-5-one	35.841	6.18
1-octadecanol	36.100	1.50
Heneicosane	36.408	4.31
Methyl stearate	37.073	1.18
2-methylhexacosane	37.989	0.82
Dotriacontane	39.394	1.14
2-methylhexacosane	40.146	0.60
Eicosanoic acid, 2,3-bis(acetyloxy)propyl ester	40.539	1.18
3-(4-methoxyphenyl)-2-propenoic acid, 2-ethylhexyl ester	41.246	1.54
Eicosanoic acid methyl ester	41.354	1.02
Pentacosane	42.739	2.53
Nonacosane	44.682	1.25
Tetratriacontane	45.336	1.65
Octocrylene	48.077	2.58
Tetracosanoic acid methyl ester	48.976	1.72
Tetracontane	51.800	2.65
4,6-dipropyl-nonan-5-one	58.030	0.71
1-heptacosanol	58.455	1.40

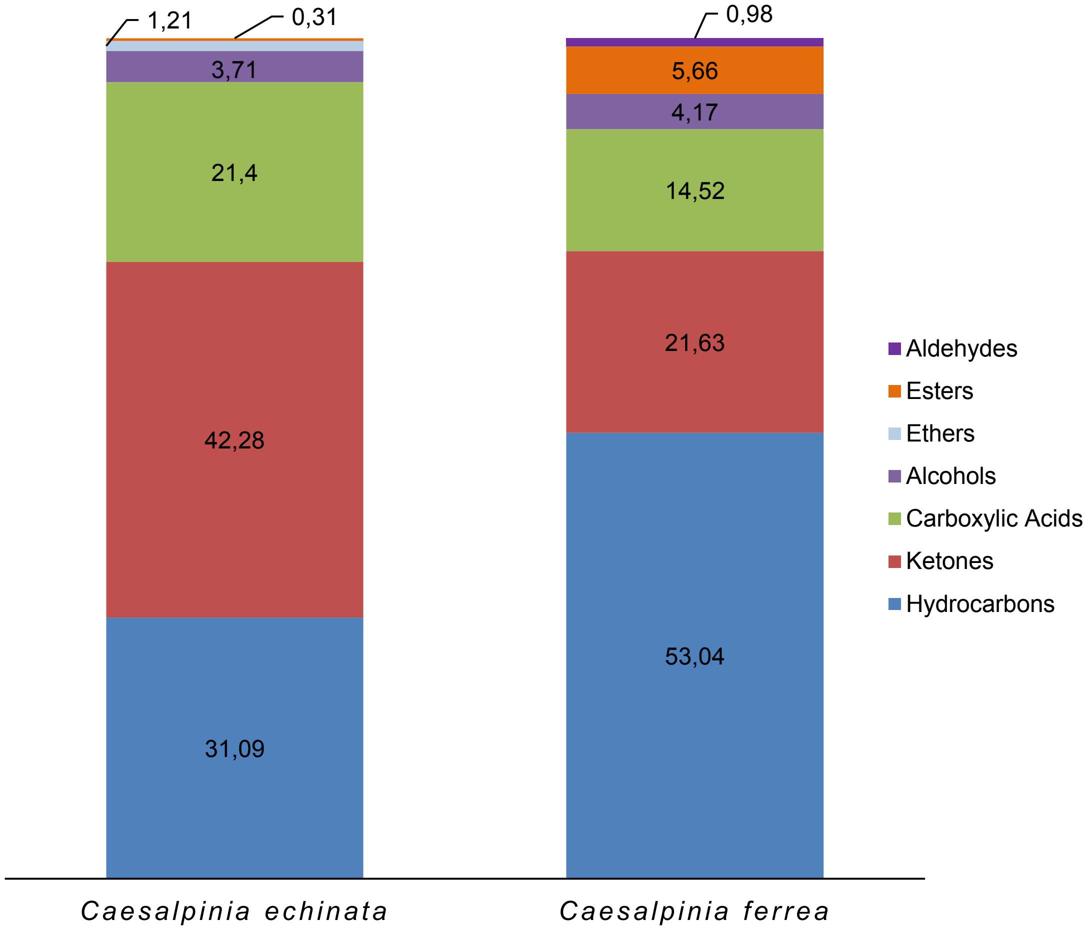

Fig. 12. Groups of compounds (%) in the waxes of third-node leaves of *Caesalpinia echinata* and *Caesalpinia ferrea* (non-exposed plants).

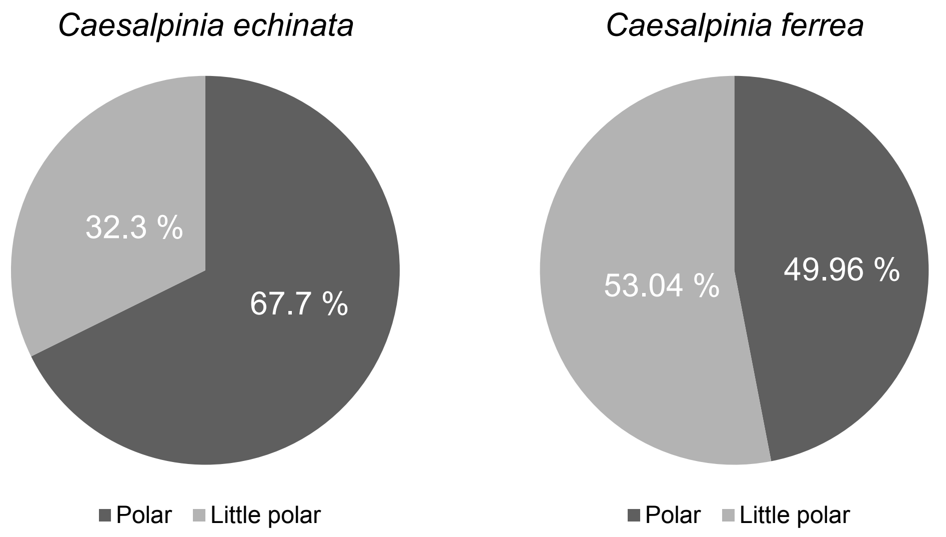

Fig. 13. Percentage of polar and little polar compounds in the waxes of third-node leaves of *Caesalpinia echinata* and *Caesalpinia ferrea* (non-exposed plants).

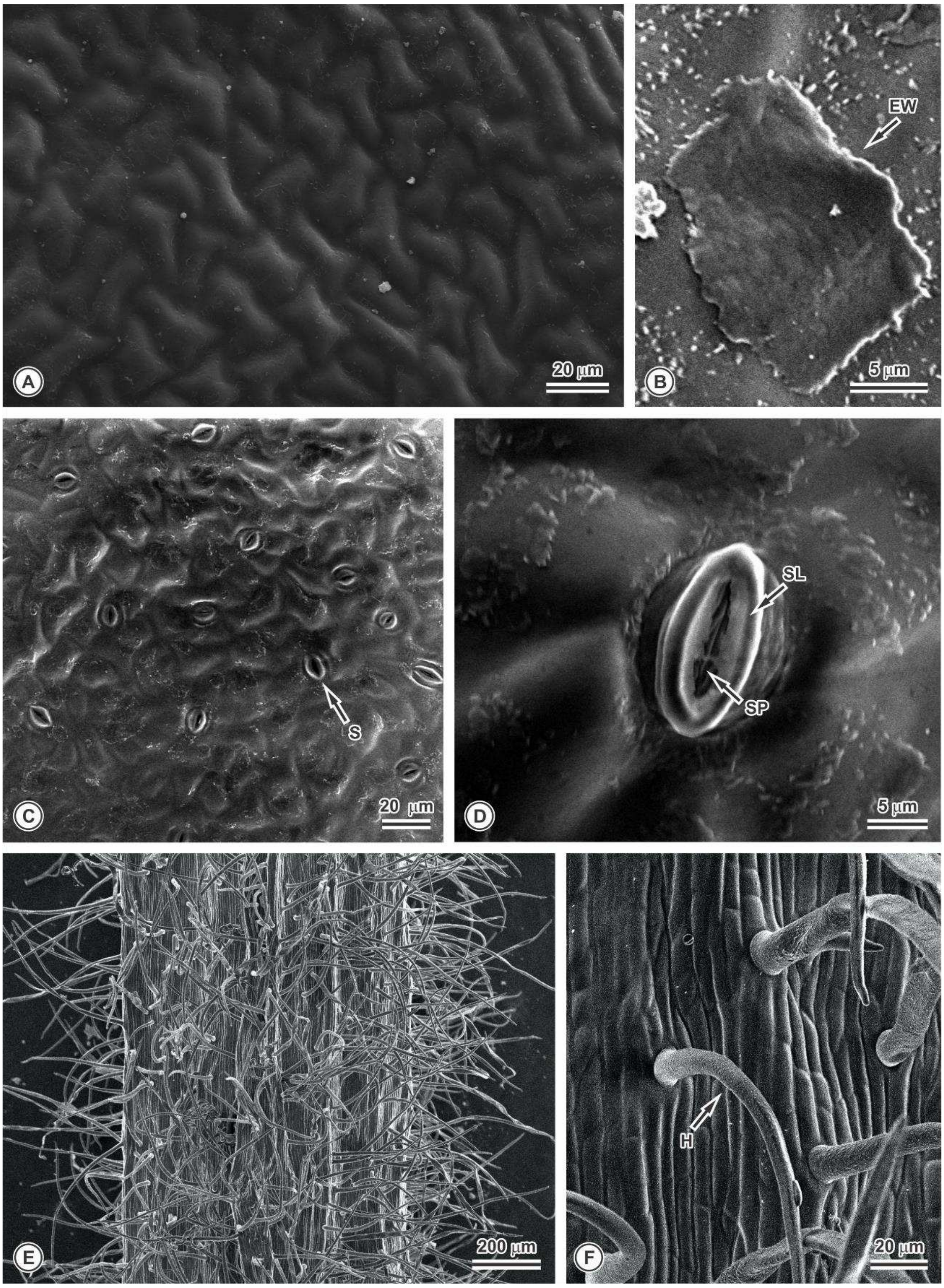

Fig. 14. Leaf surface of *Caesalpinia echinata* (non-exposed plants) in scanning electron microscopy (secondary electron signals). A-B: leaf blade adaxial surface. C-D: leaf blade abaxial surface. E-F: rachis. The epicuticular wax deposition pattern is the film-type (A). This feature can be better noticed in leaf regions where the epicuticular wax layer is ruptured (B). Notice the dense hair cover in the rachis region (E, F). EW: epicuticular wax (fragment). S: stomata. SL: stomatal ledge. SP: stomatal pore. H: hair.

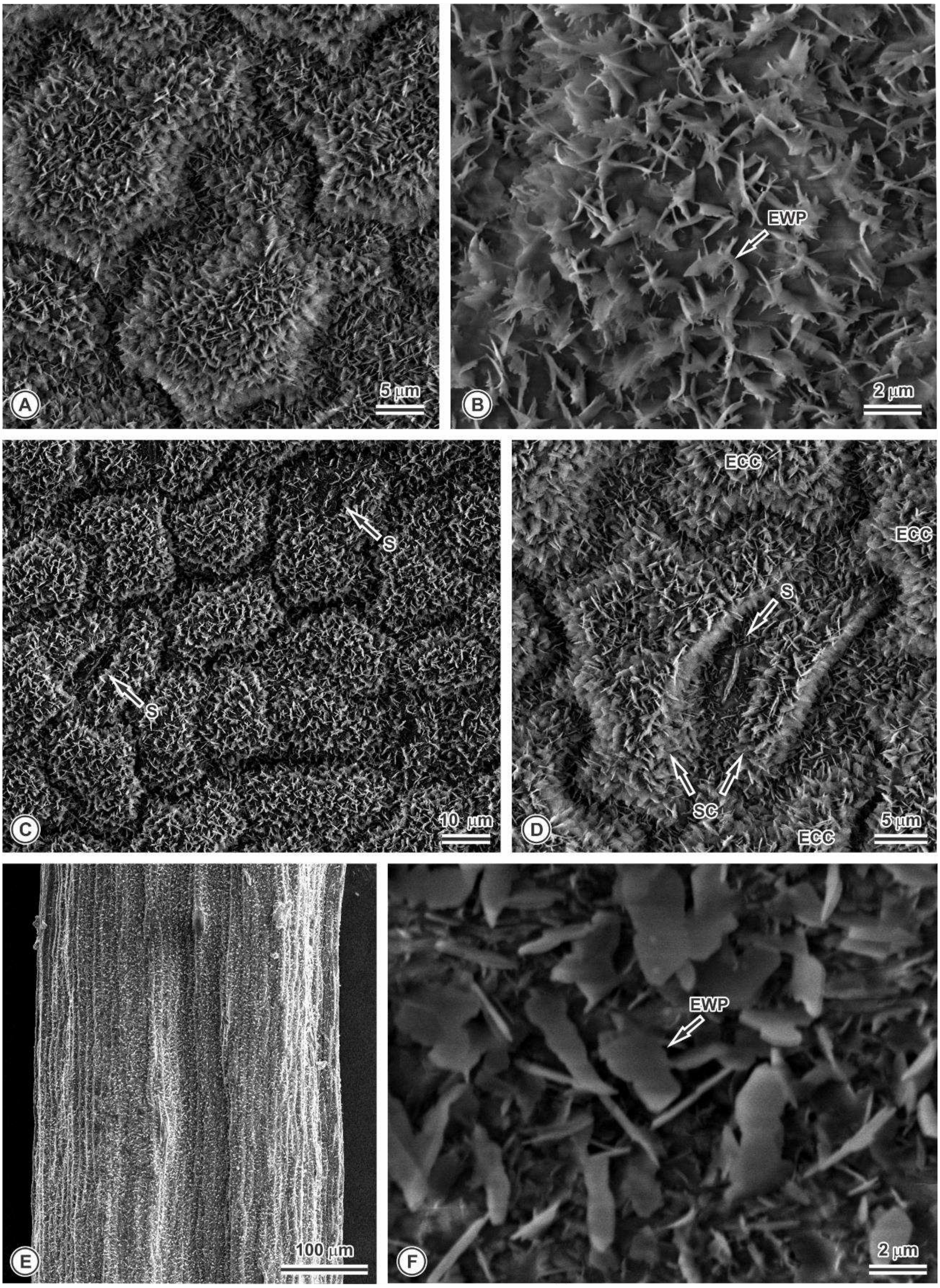

Fig. 15. Leaf surface of *Caesalpinia ferrea* (non-exposed plants) in scanning electron microscopy (secondary electron signals). A-B: leaf blade adaxial surface. C-D: leaf blade abaxial surface. E-F: rachis. The epicuticular wax deposition pattern is the platelet-type (B, F). The rachis of this species is glabrous, but densely covered by epicuticular wax platelets (EWP). S: stomata. SC: subsidiary cells. ECC: epidermal common cells.

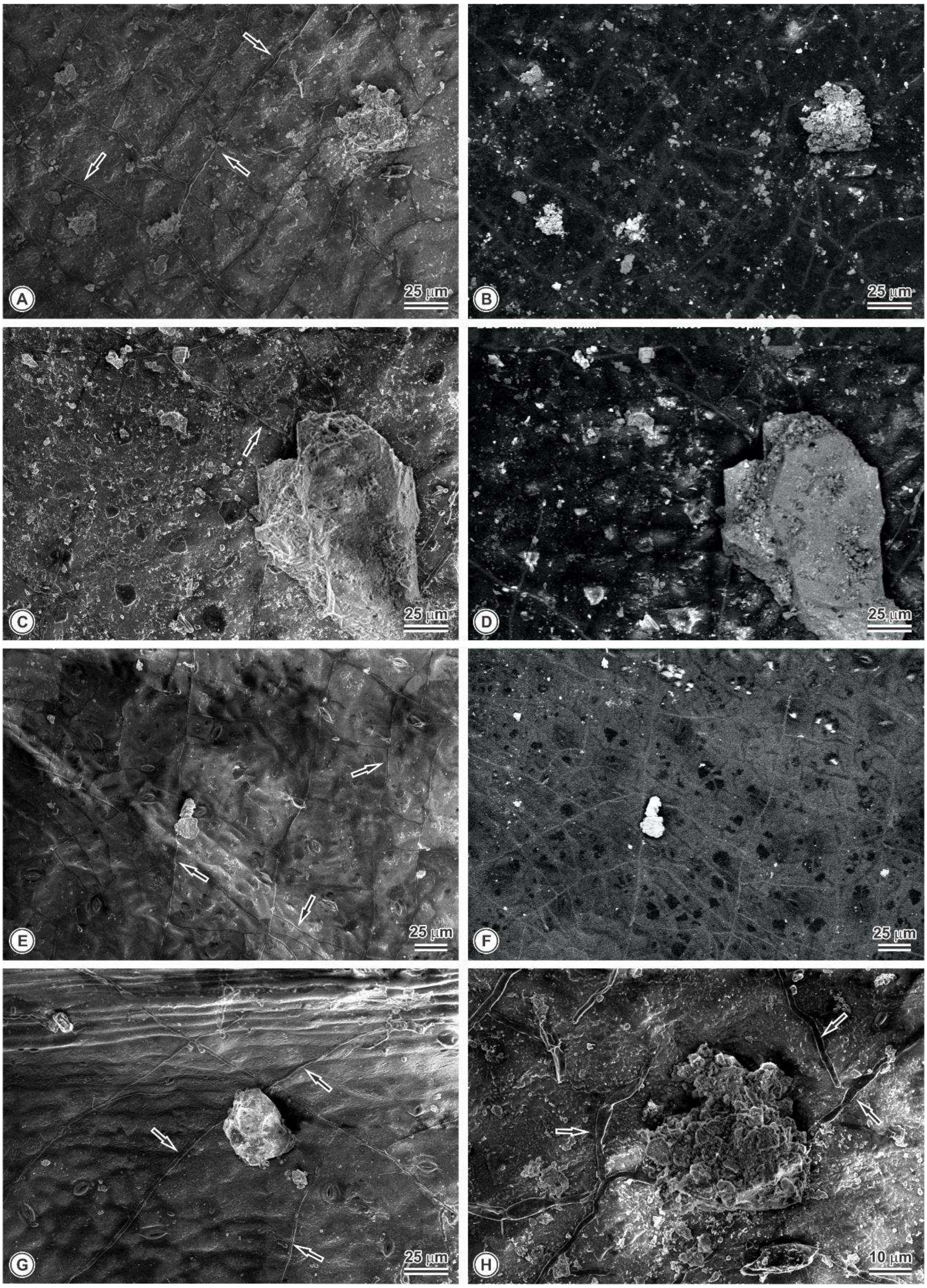

Fig. 16. Particles adhered to the adaxial (A-D, H) and abaxial (E-G) leaf blade surfaces of *Caesalpinia echinata* after three months of exposition in Bom Retiro neighborhood, Ipatinga city, Southeastern Brazil. Micrographs were obtained with scanning electron microscopy, using secondary electron signals (SEI) (A, C, E, G) and back-scattered signals (BSE) (B, D, F, H). A and B, C and D, and E and F are pairwise micrographs from the exact same leaf region. SEI images allow surface and particle microstructural characterization. BSE images allow to highlight particles and assess their cover on the leaf surface. Arrows: fungal hyphae.

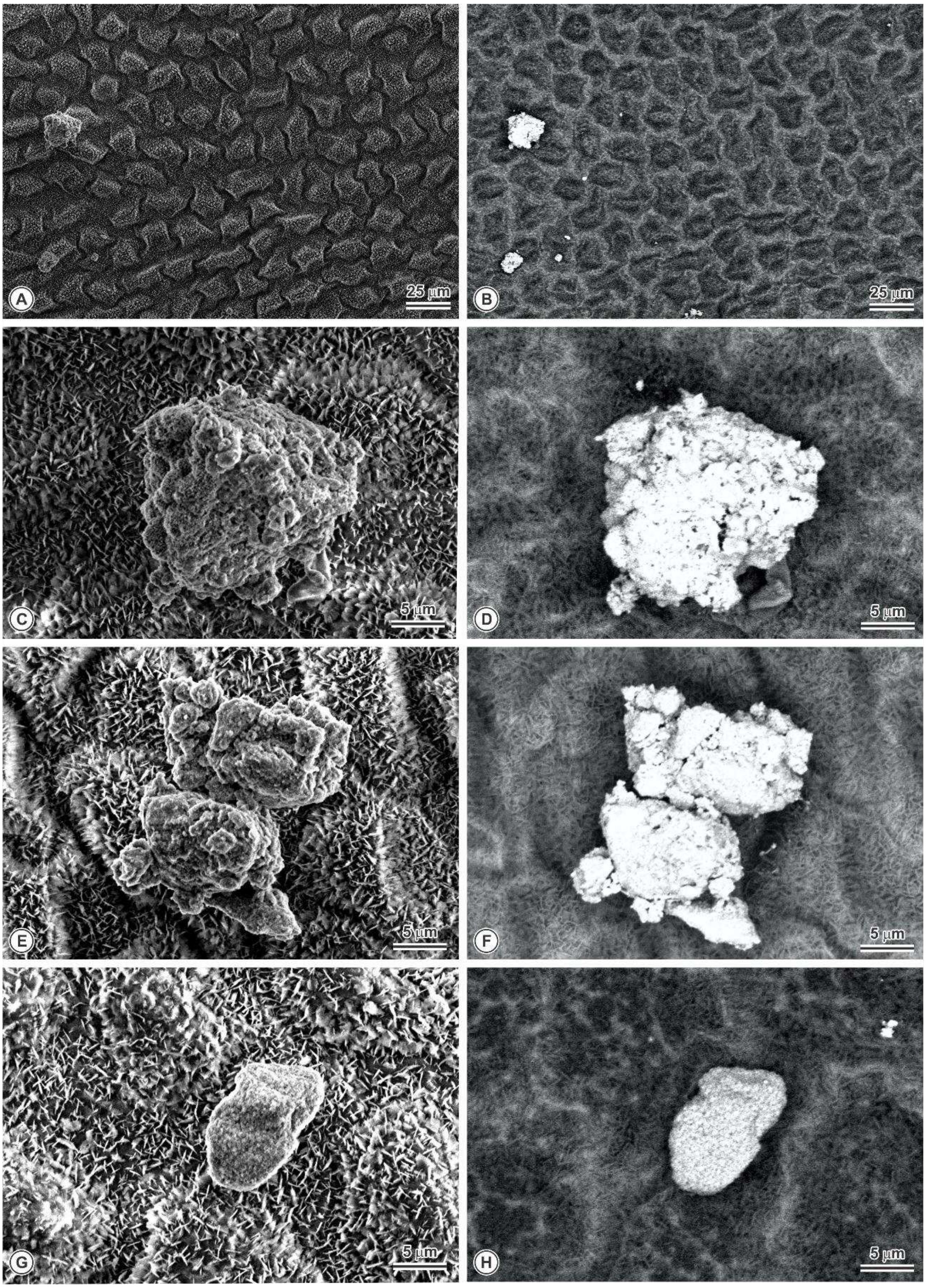

Fig. 17. Particles adhered to the adaxial leaf blade surface of *Caesalpinia ferrea* after three months of exposition in Bom Retiro neighborhood, Ipatinga city, Southeastern Brazil. Micrographs were obtained with scanning electron microscopy, using secondary electron signals (SEI) (A, C, E, G) and back-scattered signals (BSE) (B, D, F, H). A and B, C and D, E and F, and G and H are pairwise micrographs from the exact same leaf region. SEI images allow surface and particle microstructural characterization. BSE images allow to highlight particles and assess their cover on the leaf surface.

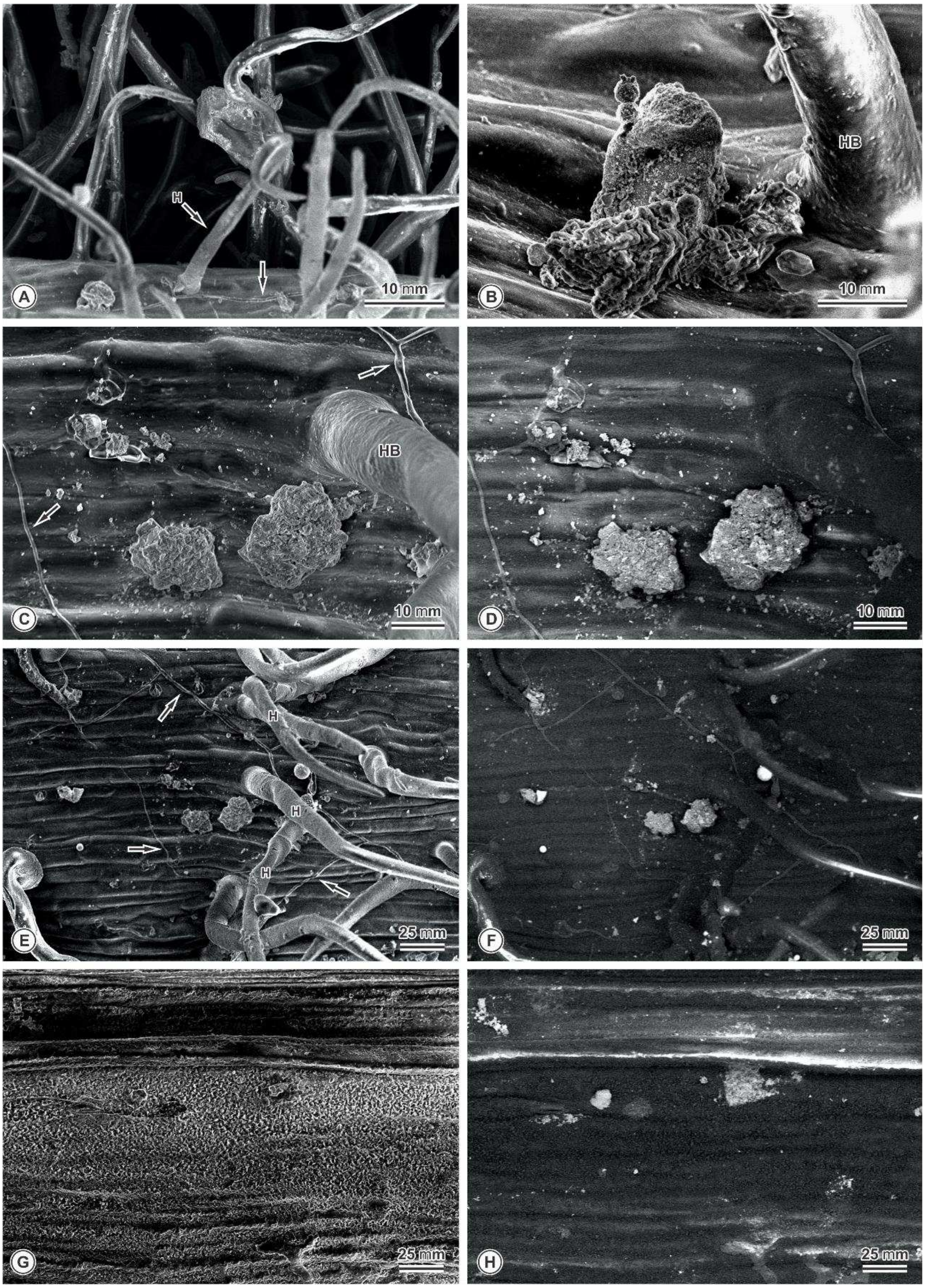

Fig. 18. Particles adhered to the rachis of *Caesalpinia echinata* (A-F) and *Caesalpinia ferrea* (G-H) after three months of exposition in Bom Retiro neighborhood, Ipatinga city, Southeastern Brazil. Micrographs were obtained with scanning electron microscopy, using secondary electron signals (SEI) (A-C, E, G) and back-scattered signals (BSE) (D, F, H). C and D, E and F, and G and H are pairwise micrographs from the exact same leaf region. SEI images allow surface and particle microstructural characterization. BSE images allow to highlight particles and assess their cover on the leaf surface. H: hair. HB: hair base. Arrows: fungal hyphae.

Table 6. Micromorphometry (µm) of second-node *Caesalpinia echinata* and *Caesalpinia ferrea* leaves after three months of exposure to urban pollution from a steel pole in Southeastern Brazil.

Species	Site	Epidermis of Adaxial Surface	Palisade Parenchyma	Spongy Parenchyma	Mesophyll	Epidermis of Abaxial Surface	Leaf Blade
Caesalpinia echinata	Bom Retiro	53.459 ± 1.745[a]	273.049 ± 41.525	174.849 ± 24.859	447.899 ± 56.702	36.241 ± 1.301[a]	537.599 ± 58.062
	Cariru	58.045 ± 5.667[a]	231.646 ± 37.414	195.938 ± 9.398	427.584 ± 28.176	38.720 ± 3.304[a]	524.350 ± 19.302
	Veneza	55.969 ± 1.879[a]	310.832 ± 62.422	192.802 ± 25.290	503.634 ± 71.758	37.920 ± 1.271[a]	597.522 ± 72.010
	Cidade Nobre	53.674 ± 3.180[a]	263.193 ± 49.222	176.369 ± 13.116	439.562 ± 59.890	36.527 ± 0.995[a]	529.762 ± 56.967
	Rio Doce State Park	57.082 ± 1.161[a]	284.368 ± 71.539	189.372 ± 15.284	473.741 ± 84.989	37.566 ± 1.771[a]	568.389 ± 83.404
Caesalpinia ferrea	Bom Retiro	35.637 ± 2.997[b]	88.420 ± 6.362	109.230 ± 5.625	197.650 ± 8.108	36.601 ± 4.317[b]	269.888 ± 12.367
	Cariru	35.437 ± 1.053[b]	88.140 ± 5.371	115.372 ± 17.431	203.513 ± 21.547	40.049 ± 1.239[ab]	278.999 ± 21.947
	Veneza	36.732 ± 2.727[b]	112.284 ± 12.363	131.164 ± 15.198	243.448 ± 25.066	41.172 ± 2.999[ab]	321.352 ± 28.018
	Cidade Nobre	33.871 ± 2.602[b]	105.638 ± 4.870	119.676 ± 13.919	225.314 ± 12.746	39.103 ± 3.955[b]	298.289 ± 13.367
	Rio Doce State Park	41.736 ± 1.668[a]	126.525 ± 41.272	135.034 ± 14.240	261.560 ± 53.788	44.142 ± 2.270[a]	347.438 ± 52.734

Analysis of Variance

SV	DF	Mean Square					
Species	1	3798,560***	299620,133***	42957,949**	569479,527***	83,931**	651426,119***
Site	4	39,393**	3442,076[n.s.]	705,407[n.s.]	5883,365[n.s.]	25,873*	7056,963[n.s.]
Species X Site	4	13,606[n.s.]	1070,885[n.s.]	206,315[n.s.]	1031,582[n.s.]	11,521[n.s.]	1172,846[n.s.]

*** Significant by F test (P < 0.001). ** Significant by F test (P < 0.01). * Significant by F test (P < 0.05). n.s. = non-significant by F test (P > 0.05). Means followed by the same letter do not differ by Tukey's test at 5% probability. Letters compare sites in a same species.

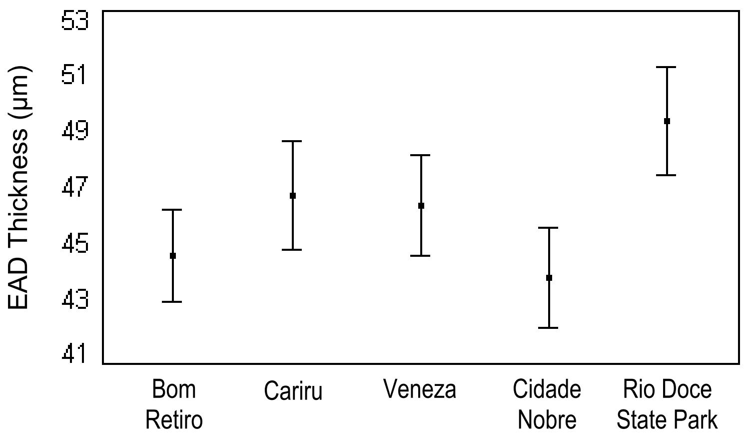

Fig. 19. Means and 95 percent Tukey's honest significant difference intervals of the epidermis thickness of the adaxial leaf surface in *Caesalpinia echinata* and *Caesalpinia ferrea* after three months of exposure to urban pollution from a steel pole in Southeastern Brazil.