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Searching for native tree species and respective potential biomarkers for future assessment of pollution effects on the highly diverse Atlantic Forest in SE-Brazil

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ABSTRACT

This study summarizes the first effort to search for bioindicator tree species and respective potential biomarkers for future assessment of potential mixed pollution effects on the highly diverse Atlantic Forest in SE-Brazil. Leaves of the three most abundant species inventoried in a phytosociological survey (*Croton floribundus*, *Piptadenia gonoacantha* and *Astronium graveolens*) were collected in four forest remnants during winter and summer (2012). Their potential bioindicator attributes were highlighted using a screening of morphological, chemical and biochemical markers. The leaf surface structure and/or epicuticular wax composition pointed the accumulator properties of *C. floribundus* and *P. gonoacantha*. *C. floribundus* is a candidate for assessing potential accumulation of Cu, Cd, Mn, Ni, S and Zn. *P. gonoacantha* is a candidate to monitor polycyclic aromatic hydrocarbons. Increased levels of secondary metabolites and decreased antioxidant capacity in leaves of *A. graveolens* may support its value as a bioindicator for oxidative pollutants by visible dark stipplings.

1. Introduction

The expansion of land-use for human interests is considered a major cause of biodiversity loss in terrestrial ecosystems. This loss is due to both the ecosystem fragmentation and environmental contamination by diverse xenobiotic substances (Giam et al., 2010; McKee et al., 2004; Pérez-Vega et al., 2012; Sawidis et al., 2011).

In southeast region of Brazil, the most developed and richest in the country, intense fragmentation of the Atlantic Forest domain as well as the environmental contamination by pollutants have been promoted by the disorderly and intense land occupation by metropolitan conglomerations, industrial centers and extensive agricultural lands (Domingos et al., 2003; Emberson et al., 2001; Freitas et al., 2010; Groeneveld et al., 2009; Lira et al., 2012; Ribeiro et al., 2009). Although the biodiversity loss due to human interference is likely in SE-Brazil, appropriate methodological protocols for assessing potential pollution effects on the Atlantic vegetation remnants are needed.

Biomonitoring methods, which are based on the measurement of selected responses (referred here as biomarkers) in bioindicator species, have long been used in the Northern Hemisphere for estimating adverse effects caused by environmental pollution on forest ecosystems (Ferretti et al., 2010; Fräinzle, 2003; Markert et al., 2003; Rautio et al., 2010). Many lichens, mosses and higher plant species have been employed as bioindicators (Harmens et al., 2013; Kefauver et al., 2014; Llop et al., 2012; Noth et al., 2013; Paoletti et al., 2009; Remon et al., 2013; Sawidis et al., 2011). Among the standardized biomarkers measured in such species are the visible leaf injury after microscopic validation and xenobiotic substance accumulation in different organs, such as heavy metals or polycyclic aromatic hydrocarbons (PAH) (Benham et al., 2010; Beramendi-Orosco et al., 2013; Desalme et al., 2013; Fräinzle,

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2003; Markert et al., 2003; Simonich and Hites, 1994; Weiss et al., 2003). The passive biomonitoring commonly conducted in the Northern Hemisphere, which is based on selected responses of native species in the forest ecosystem under evaluation, has high ecological relevance. However, its quality depends on: 1) representativeness of the observed bioindicator species; 2) selection of relevant biomarkers; 3) degree of objectivity, reliability and validity (Fräinzle, 2003).

We may assume that these successful biomonitoring protocols may also be applicable in SE-Brazil to estimate the potential effects of environmental pollution on remnants of the Atlantic vegetation. However, it is also plausible to ask whether these protocols are applicable to monitor complex mixtures of air pollutants in a forest ecosystem with high tree species diversity. In addition, the native tree species have distinct individual architectures, leaf morphologies, anatomic and biochemical characteristics and possibly tolerance levels against oxidative stress caused either by air pollution, conferred by their genetic properties, as also highlighted by Bussotti (2008), based on a critical overview. For example, the biochemical pathways involved with the production of primary and secondary metabolites and antioxidants of ascorbate-gluthatione cycle in distinct cell organelles may modulate the appearing and intensity of visible leaf injury, the most useful biomarker for biomonitoring the potential effects of oxidant pollutants, such as ozone, on forest ecosystems (Iriti and Faoro, 2008). Under such circumstances, our hypothesis is that the choice of bioindicator trees native to Atlantic Forest should be based not only on the representativeness in the forest ecosystem but also on a comparative screening of morphological, chemical and biochemical markers.

Thus, the present study aimed to: 1) search for the most abundantly occurring tree species from different functional groups within the Atlantic Forest remnants in SE-Brazil; 2) describe the range of morphological (leaf dimensions and tissue thickness), chemical (chemical wax composition and leaf accumulation of PAHs and inorganic elements) and biochemical (antioxidants, primary and secondary metabolites and compounds that indicate lipid peroxidation at cell level) markers of the selected species from polluted and rural forest remnants to highlight their different potentials for a future monitoring of the present air pollution mixture; 3) search for potential biomarkers among those analyzed in each species for assessing future pollution effects on the highly diverse Atlantic Forest, focusing on: 3a) PAH filtering capacities determined by physical (structural) and chemical properties (wax composition) of leaf surfaces; 3b) nitrogen, sulfur and heavy metal leaf accumulation; 3c) biochemical markers related to oxidative stress.

2. Material and methods

2.1. Description of study area

The search for native bioindicator trees was conducted in forest remnants located in the Metropolitan Region of Campinas (MRC), the second most important economical center of São Paulo State (Fig. 1).

The predominant climate in the MRC is Cwa (humid subtropical zone with dry winter and hot summer), according to Koeppen's classification (Alvares et al., 2014). It is characterized by one hot and rainy season between October and March and one dry season between April and September (see climate diagram included in Moura et al., 2014a). The predominant winds are from southeast to northwest.

The concentration of atmospheric pollutants in the MRC is influenced by the predominant weather conditions and the diversity of pollution emission sources. The maximum hourly concentrations of PM_{10}, SO_2, NO_2 and O_3 registered from 2010 to 2012 in a monitoring station located in Paulinia (Fig. 1) were 561 $\mu g\ m^{-3}$, 174 $\mu g\ m^{-3}$, 150 $\mu g\ m^{-3}$ and 251 $\mu g\ m^{-3}$, respectively (Fig. 2; CETESB, 2013). The average (av) hourly concentrations of primary pollutants from 2010 to 2012 (data included in Fig. 2) tended to be higher during the dry seasons ($avPM_{10} \pm SD = 42.0 \pm 30.8\ \mu g\ m^{-3}$; $avNO_2 = 30.0 \pm 21.7\ \mu g\ m^{-3}$; $avSO_2 = 6.7 \pm 11.3\ \mu g\ m^{-3}$) than during the wet seasons ($avPM_{10} = 25.7 \pm 19.3\ \mu g\ m^{-3}$; $avNO_2 = 18.9 \pm 13.3\ \mu g\ m^{-3}$; $avSO_2 = 4.7 \pm 8.0\ \mu g\ m^{-3}$); in contrast, ozone tended to increase during rainy seasons ($avO_3 = 57.7 \pm 35.8\ \mu g\ m^{-3}$) when compared to the average hourly levels measured during the dry months ($avO_3 = 45.6 \pm 35.8\ \mu g\ m^{-3}$). In addition, wind circulation during a single day (SSE and SE in early morning and evening, SSE, SE and NE in the morning and NNE, SSE, N, SSW and S in the afternoon) and the flat topography favor the mixture of pollutants in the whole MRC (Boian and Andrade, 2012; Tresmondi and Tomaz, 2004).

The natural vegetation in the MRC is classified as semideciduous forest, a subtype of the Atlantic Forest domain (Oliveira-Filho and Fontes, 2000). It is characterized by a number of species that partially lose their leaves during the dry season in response to water shortage. The semideciduous Atlantic Forest is currently fragmented into small residual areas (Fig. 1), mostly isolated from each other (Ribeiro et al., 2009).

The study was developed in four forest remnants (Fig. 1), which are located in: Campinas (CA; 233 ha), the largest urban-industrial center at MRC; Paulínia (PA; 190 ha), which contains an important industrial center composed of chemical, agrochemical and petrochemical industries; and Holambra (HO; 35 ha) and Cosmópolis (CO; 200 ha), which are typical rural cities. All the forest remnants are surrounded by extensive agricultural areas (mainly sugarcane cultivation), and located in different directions and distances from the industrial center of Paulínia. The forest remnants are growing on Oxisols. The granulometry of soils varied from sandy clay loam in Paulínia, Holambra and Cosmópolis to clay in Campinas, which offers the best buffering capacity against environmental impact (Lopes et al., 2015).

2.2. Screening of tree species

The field study started with a phytosociological survey to identify the most abundant tree species in the forest remnants, using the modified Gentry transect method, with adaptations according to the protocols proposed by ICP-Forest (Ferretti et al., 2010; Rautio et al., 2010). Sixteen parallel transects (50 m × 4 m, length × width, each), starting 100 m from the forest edge and distant 20 m each other, were installed in each forest remnant (3200 m^2 inventoried per forest remnant). All trees within each transect area and with a circumference at breast height (CBH) ≥ 30 cm were collected and identified. Total density (number of trees per species/hectare), total basal area (sum of basal area of trees per species/hectare), relative density (number of trees per species/total number of trees inventoried) and relative basal area (basal area of trees per species/total basal all trees inventoried) were estimated according to Curtis and McIntosh (1950).

2.3. Biomarker screening to establish the bioindicator potential of the selected three tree species

2.3.1. Morphological markers

The leaf external morphology was described by collecting three fully expanded leaves from approximately 10 trees per species in each forest remnant. Single leaf area (cm^2) and leaf dry mass (g) were determined and specific leaf area (cm^2 g) was estimated.

Five samples with 1 cm^2 were obtained in the intercostal region

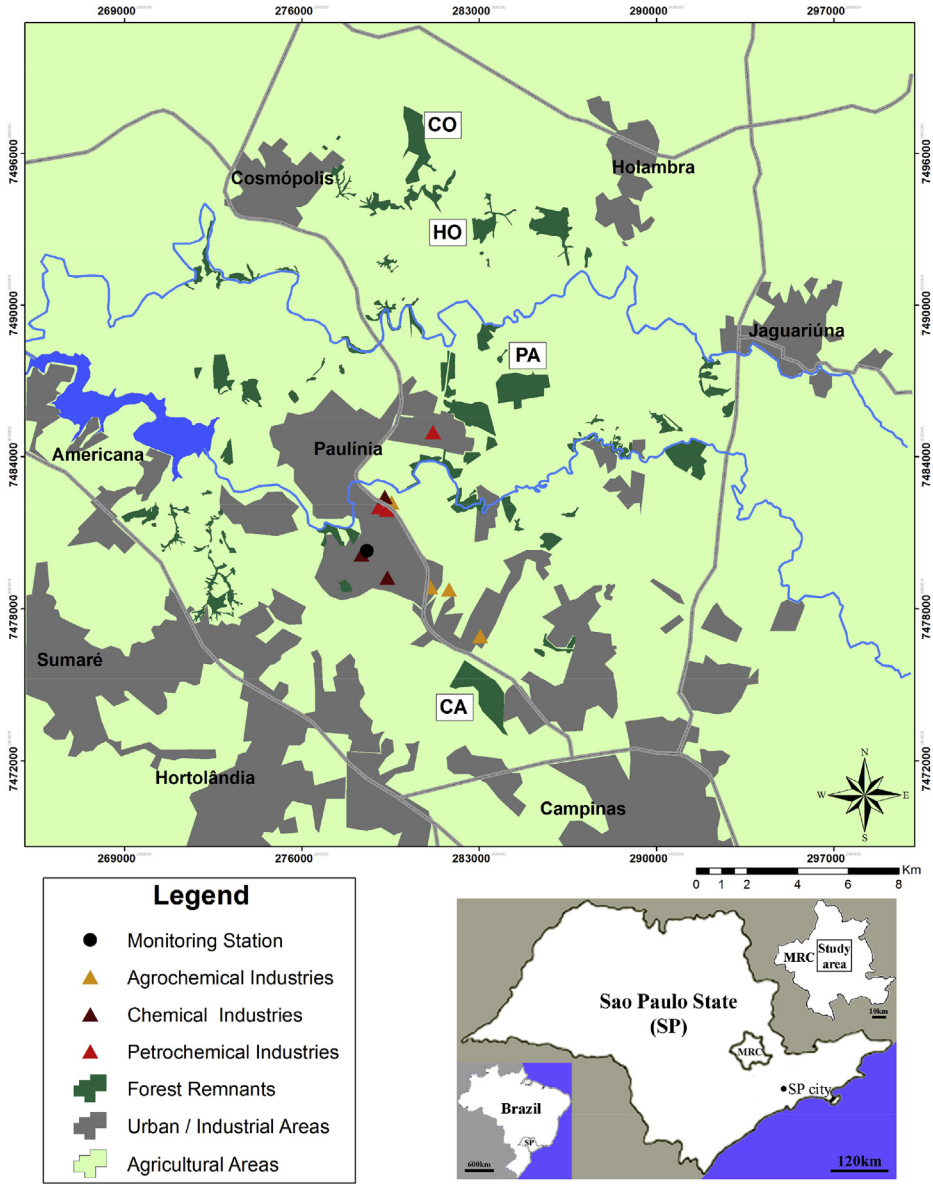

Fig. 1. Representantion of Semideciduous Atlantic Forest remnants, major air pollution sources and monitoring station of climate and air quality in part of the Metropolitan Region of Campinas, São Paulo, Brazil. CA, PA, HO and CO acronyms indicate the forest remnants surveyed.

of five leaves from five trees of each species collected on the northern edge of each forest remnant to measure tissue thickness and describe the characteristics of leaf surfaces, according to methods summarized in Table 1.

Wax structure on the leaf surfaces was described by SEM in two intact leaves (dried at room temperature) per species and forest remnant sampled in the summer/2012 (N = 08 per species). Portions of leaves containing abundant dust particles were avoided to better describe the wax structure.

2.3.2. Chemical markers

Leaf samples from four to five branches fully exposed to the sunlight of four to six trees per species were collected during the winter and summer periods/2012, in two distinct locations in the edge of each forest remnant (exposed to air pollution). After collection, the leaf samples were combined to one sample per species for each side, forest remnant and season (N = 16 per species). Aliquots of these leaf samples were immediately frozen at −20 °C to determine the polycyclic aromatic hydrocarbon (PAH) contents and the remaining leaves were oven-dried and milled to determine nitrogen (N), sulfur (S) and heavy metals (Cd, Cu, Mn, Ni, Pb and Zn, which are markers of particulate matter in the study region).

Epicuticular wax were extracted from leaves of five trees per species collected during the winter and summer in three forest remnants (CA, PA and CO; Fig. 1) (N = 30 per species). The leaves were dried at room temperature and their total wax was extracted by immersion in dichloromethane, weighted and expressed as μg cm^2 of leaf area, following methods described in Furlan et al. (2006). Wax composition was analyzed by GC/MS. Relative percentages of wax constituents were estimated.

PAHs in frozen leaves were extracted and analyzed employing an HPLC (fluorescence detector; 275 nm: extinction and 395 nm: emission), as described by Rinaldi et al. (2012). PAHs with 2–3

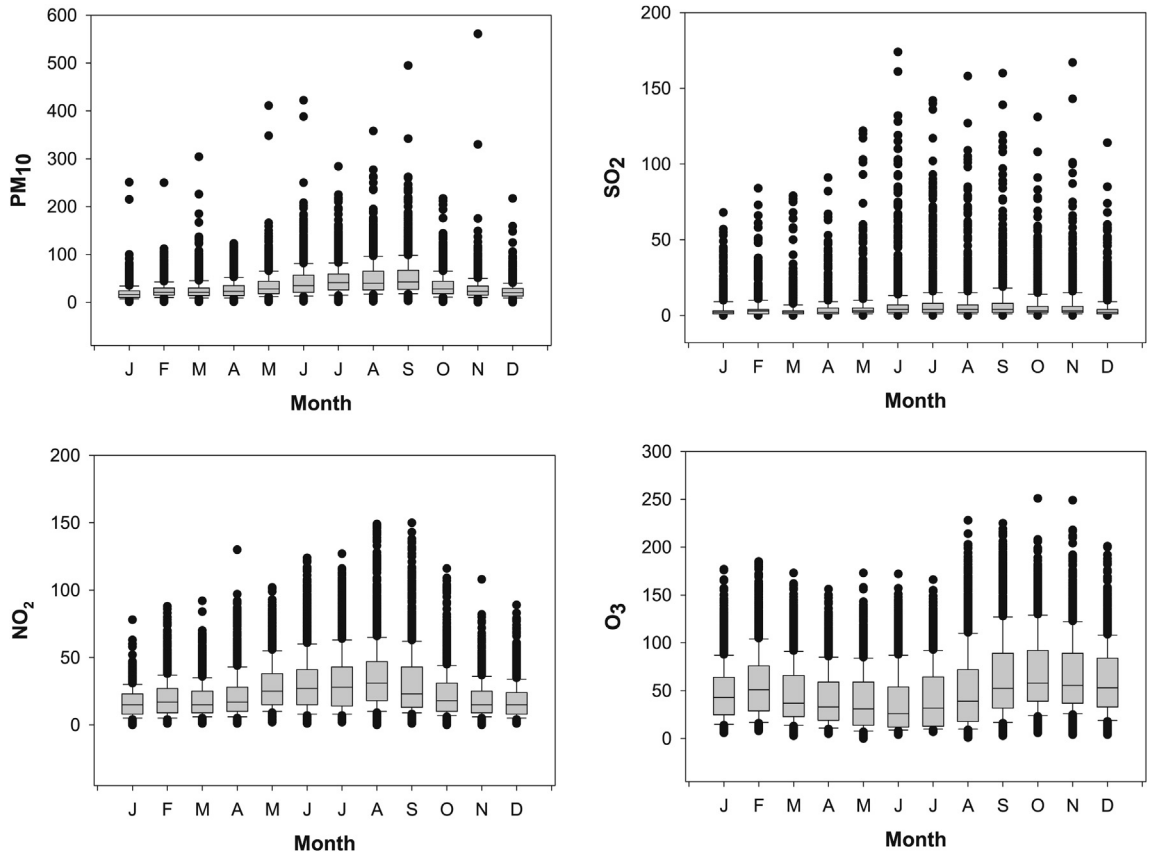

Fig. 2. Box plot representation of hourly concentrations (in $\mu g.m^{-3}$) of air pollutants registered every month from 2010 to 2012, at the monitoring station located in the city of Paulínia, belonged to the Metropolitan Region of Campinas (SE Brazil). Source of data: São Paulo State Company of Environmental Sanity (available in www.cetesb.sp.gov.br). Gray boxes delimit the 25th to 75th percentiles and error bars the 90th and 10th percentiles of the hourly value dataset; horizontal lines within the boxes indicate the medians; round and dashed symbols indicate outlying hourly values.

aromatic rings (naphthalene, acenaphthene, fluorene, phenanthrene, anthracene and fluoranthene) and 4-5-6 aromatic rings (pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenz[a, h]anthracene and benzo[g, h, i]perylene) were analyzed.

The dried and milled leaf samples were acid digested to determine the N and S concentrations using Kjeldahl and turbidimetry methods, respectively, according to Malavolta et al. (1997). The leaf

Table 1
Summary of methods employed for analyses of morphological and biochemical markers in leaves of *A. graveolens*, *C. floribundus* and *P. gonoacantha* sampled in remnants of Atlantic Semideciduous Forest studied in the Metropolitan Region of Campinas (SE Brazil).

Biomarkers	Quantification technique	Reference
Morphological markers		
Tissue thickness	Light microscope (Olympus BX53); Image Pro Express 6.3 software	Feder and O'Brien (1968)
Sections stained with toluidine blue 1%		
Leaf surface (qualitative analyses)	Scanning electron microscope (Philips XL)	Ascensão et al. (1999)
Standard methods		
Biochemical markers		
Antioxidants in frozen leaves (−80 °C)		
Ascorbic acid (AsA, DHA)	HPLC (UV/vis detector)	Dias et al. (2011)
Glutathione (GSH, GSSG)	Spectrophotometry (412 nm)	Israr et al. (2006)
Ascorbate peroxidase (APX)	Spectrophotometer (290 nm)	Nakano and Asada (1981)
Glutathione reductase (GR)	Spectrophotometer (412 nm)	Monteiro et al. (2011)
Primary metabolites in dry and ground leaves		
Total Sugars	Spectrophotometry (490 nm)	Dubois et al. (1956)
Starch	Spectrophotometry (490 nm)	Amaral et al. (2007)
Secondary metabolites in dry and ground leaves		
Total tannins	Spectrophotometry (520 nm)	Sandre et al. (2014)
Condensed tannins	Spectrophotometry (550 nm)	Sandre et al. (2014)
Total flavonoids	Spectrophotometry (420 nm)	Sandre et al. (2014)
Total phenols	Spectrophotometry (760 nm)	Sandre et al. (2014)
Lipid peroxidaton in frozen leaves (−80 °C)		
Hydroperoxide conjugated diene (HPCD)	Spectrophotometry (234 nm)	Levin and Pignata (1995)
Malondialdehyde (MDA)	Spectrophotometry (535 and 600 nm)	Buege and Aust (1978)

Table 2
Family, ecological group, absolute density, relative density (RD), basal area, relative basal area (RBA) of the six most abundant tree species found in the four remnants of Atlantic Semideciduous Forest studied in the Metropolitan Region of Campinas (SE Brazil), as well as sum estimations (Σ) for these species per forest remnant. Range = minimum and maximum values estimated in the forest remnants (N = 4); F = frequency (number of forest remnants in which each species occurred).

Tree species	Family	Ecological groups[a]	Absolute density N ind.ha^{-1}		RD %	Basal area m^2 ha^{-1}		RBA %	F
			Mean	Range		Mean	Range		
Astronium graveolens Jacq.	Anacardiaceae	Non-pioneer	130	119–150	10.2	3.26	2.00–4.97	6.2	4
Metrodorea stipularis Mart.	Rutaceae	Non-pioneer	59	0–187	5.3	1.91	0–5.72	3.9	2
Inga sessilis (Vell.) Mart.	Fabaceae	Pioneer	37	0–69	2.7	1.27	0–2.56	2.7	3
Piptadenia gonoacantha (Mart.) J. F. Macbr.	Fabaceae	Pioneer	62	31–112	5.1	5.82	4.61–7.55	11.4	4
Mabea fistulifera Mart.	Euphorbiaceae	Pioneer	22	0–50	1.6	0.76	0–1.53	0.8	2
Croton floribundus Spreng.	Euphorbiaceae	Pioneer	64	25–94	4.8	1.82	1.21–2.73	3.7	4
Forest remnants (Σ estimations for the six most abundant species)									
Campinas (CA)			1150		22.3	60.9		28.7	
Paulinia (PA)			1256		35.8	41.6		19.6	
Holambra (HO)			1200		28.6	57.6		27.2	
Cosmópolis (CO)			1575		34.8	51.9		24.5	
Average			1295		30.3	53.0		25.0	

[a] According to the definition of Swaine and Whitmore (1988).

concentrations of heavy metals were determined using an ICP-MS after digestion with Aqua Regia.

2.3.3. Biochemical markers

Six to ten total expanded leaves from five branches fully exposed to the sunlight of four to six trees per species were simultaneously collected in the northern edge of three of the selected forest remnants (CA, PA and CO; Fig. 1), during the winter and summer/2012. Sampling replications were conducted over eight consecutive days per season, in two times of the day (10 h and 13 h). After collection, the leaf samples per tree were combined to one sample per species for each time of the day, sampling day and forest remnant (N = 96 per species). These leaf samples were placed in vials and immediately frozen in liquid nitrogen for the following analyses, according to methods briefly described in Table 1: a) Antioxidants: ascorbic acid (reduced-AsA and oxidized-DHA forms); glutathione (reduced-GSH and oxidized-GSSG forms); ascorbate peroxidase (APX); glutathione reductase (GR); b) primary metabolites: water-soluble sugars and starch; c) secondary metabolites: total and condensed tannins, flavonoids and total phenols; c) Injury indicators at membrane level (lipid peroxidation): hydroperoxide conjugated diene (HPCD) and malondialdehyde (MDA).

2.4. Preparation of results and statistics

The results of all biomarkers quantitatively analyzed were described by general averages, medians and ranges between the minimum and maximum values obtained for each composed leaf sample, which were adopted as replicates to emphasize the plasticity of the most important characteristics of each species for biomonitoring purposes. Significant differences among tree species were identified by non-parametric ANOVA, based on the ranking of individual values (Kruskal Wallis method), followed by Dunn pairwise test. The only exception occurred for the tissue thickness data, which were analyzed by parametric ANOVA (F test) followed by a Student-Newman-Keuls pairwise test because normality and equal variances were reached.

3. Results

3.1. Screening of tree species

Six tree species were the most abundant inventoried in the forest remnants (Table 2). These species were represented by 1295 ind.ha^{-1} on average (ranging from 1150 ind.ha^{-1} in Campinas to 1575 ind.ha^{-1} in Cosmopolis), representing 30.3% of the total number of trees with CBH $\geq$ 30 cm sampled (average RD; Table 2). These species also totalized 53.0 m^2 ha^{-1} of basal area on average (41.6 m^2 ha^{-1} in Paulínia to 60.9 m^2 ha^{-1} in Campinas), representing 25% of the Σbasal area estimated for all trees surveyed (average RBA; Table 2). *Astronium graveolens* Jacq., *Croton floribundus* Spreng. and *Piptadenia gonoacantha* (Mart.) J. F. Macbr were the most numerous tree species inventoried, representing 20.1% and 21.3% of the total density and basal area per hectare

Table 3
Means, medians and range between minimum and maximum dimensions and tissue thickness of leaves of *A. graveolens*, *C. floribundus* and *P. gonoacantha* sampled in the four remnants of Atlantic Semideciduous Forest studied in the Metropolitan Region of Campinas (SE Brazil).

Morphological markers	*A. graveolens*			*C. floribundus*			*P. gonoacantha*		
	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range
Dimensions[a] *(N = 40)*									
Area (cm^2)	194	185 a	105–386	102	99 b	46–175	52	51 c	27–79
Dry mass (g)	1.51	1.41 a	0.62–3.17	0.98	0.95 b	0.39–1.79	0.56	0.54 c	0.36–0.86
Specific area (cm^2 g^{-1})	139	128 a	85–270	106	101 b	63–172	95	99 b	35–133
Tissue thickness (μm)[b] *(N = 5)*									
Adaxial epidermis	13.3 a	13.0	11.9–15.6	15.6 a	13.8	13.3–19.6	12.4 a	13.2	10.7–13.6
Abaxial epidermis	6.8 b	6.6	5.6–8.4	9.6 a	9.5	8.3–11.0	8.1 ab	8.0	7.4–9.1
Mesophyll (pp + sp)	109.4 b	112.6	82.5–130.8	134.3 a	134.8	117.7–143.7	88.2 c	97.9	54.9–113.4
Palisade parenchyma (pp)	74.3 a	74.2	11.9–15.6	82.1 a	83.4	75.5–87.4	42.6 b	50.3	22.2–52.9
Spongy parenchyma (sp)	35.5 b	34.6	30.7–44.4	52.2 a	53.2	49.3–54.1	45.6 b	49.7	35.5–53.4

Distinct letters indicate significant differences among tree species for each parameter; $p < 0.05$.
[a] ANOVA on ranks (Kruskal–Wallis test) followed by Dunn pairwise test.
[b] Parametric ANOVA (F test) followed by Student-Newman-Keuls pairwise test.

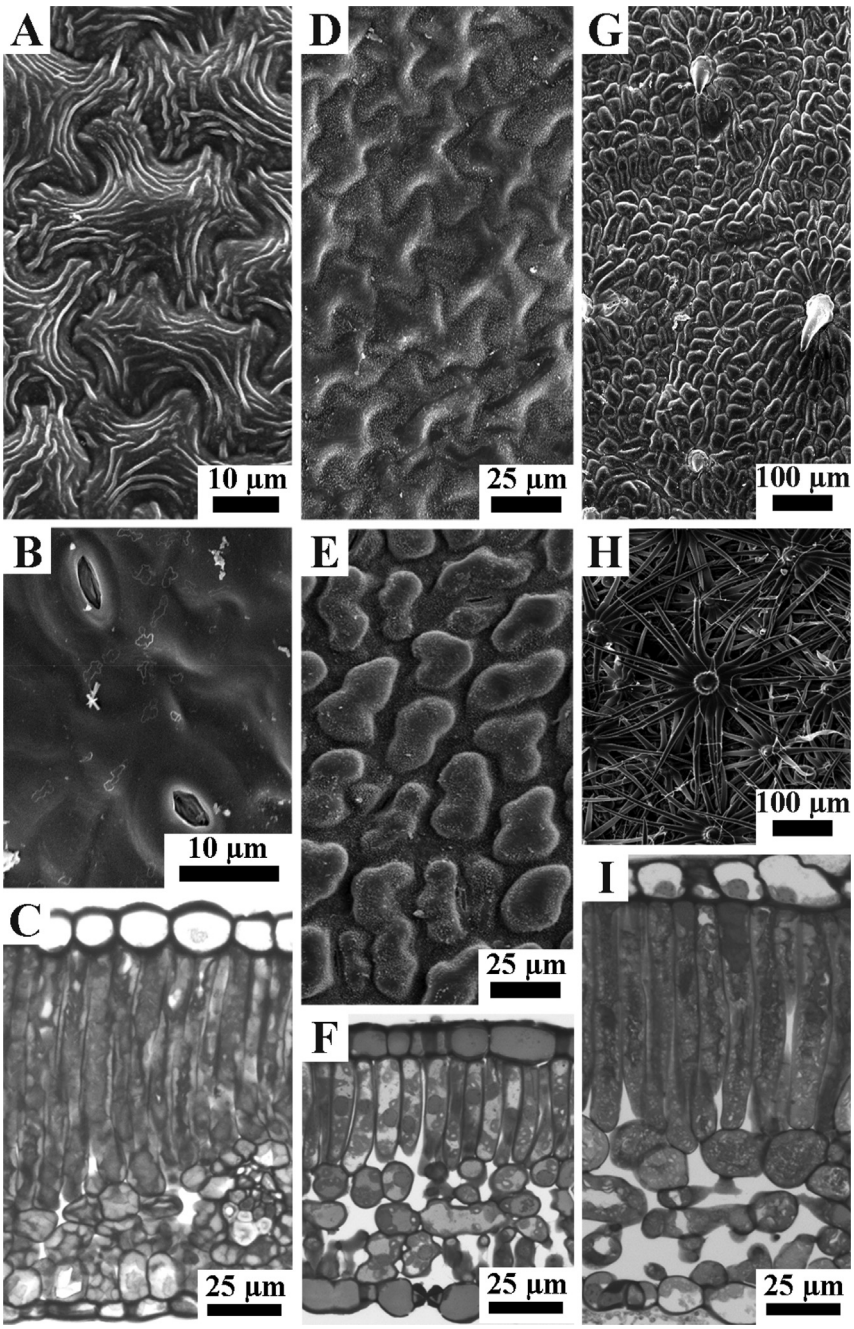

Fig. 3. Leaf surfaces (A, D, G – adaxial surfaces; B, E, H – abaxial sufaces) and transversal sections (C, F, I) of the most abundant tree species found in remnants of Atlantic Semideciduous Forest studied in the Metropolitan Region of Campinas (SE Brazil). A–C) *A. graveolens;* D–F) *P. gonoacantha*; G–I) *C. floribundus*.

respectively and occurred in all four forest fragments (Table 2). *A. graveolens* is a non-pioneer species and *C. floribundus* and *P. gonoacantha* pioneer species, following definition proposed by Swaine and Whitmore (1988).

A. graveolens, *C. floribundus* and *P. gonoacantha* were finally selected to be assessed as potential passive bioindicators, due to their frequencies, abundances and different ecological groups in the forest remnants.

3.2. Morphological markers of selected tree species

The leaf area and dry mass of *A. graveolens* were significantly higher than those of the other species. The leaves of *C. floribundus* were also larger in area and dry mass than those of *P. gonoacantha.* However, the specific leaf area was only significantly increased in *A. graveolens* compared to the other species (Table 3).

The three species differed in epidermal cell shape, features of the cuticle and epicuticular wax, position of stomata in relation to the ordinary epidermal cells and the presence and distribution of trichomes. *A. graveolens* leaves were glabrous and characterized by an adaxial surface covered by cuticular ridges (Fig. 3A) and an abaxial surface containing epidermal cells with slightly sinuous anticlinal walls, a smooth cuticle and stomatal antechamber slightly projected above the neighboring cells (Fig. 3B). The epicuticular wax on the adaxial surface was less apparent in the leaves of *A. graveolens* than the other species (Fig. 3A–B).

Table 4
Means, medians and range between minimum and maximum values of cuticular wax constituents, total polycyclic aromatic hydrocarbons (Σ PAHs) and element accumulation in leaves of *A. graveolens*, *C. floribundus* and *P. gonoacantha* sampled in the four remnants of Atlantic Semideciduous Forest studied in the Metropolitan Region of Campinas (SE Brazil). NI = Non-identified compounds.

Chemical markers	*A. graveolens*			*C. floribundus*			*P. gonoacantha*		
	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range
Cuticular wax (μg cm^2) *(N = 30)*	10	7 b	0–30	17	13 a	0–50	15	6 b	0–60
% Alcohols	6	4 b	1–41	12	10 a	3–54	11	11 a	4–20
% Carboxilic acids	7	7 c	2–13	11	9 b	4–26	21	19 a	8–42
% *n*-Alkanes	30	27 b	11–54	47	47 a	15–78	22	17 b	4–60
% Steroids	19	18 a	4–33	–	–	–	3	3 b	1–14
% Triterpenes	27	26 ab	7–50	21	15 b	9–47	32	32 a	10–58
% NI	9	8 a	2–29	4	4 b	1 – 13	9	8 a	4–21
Σ PAH (ng g^{-1}) *(N = 16)*	220	210 b	79–456	164	162 b	83–300	335	215 a	119–894
% 2–3 rings	72	81 a	18–89	71	71 a	44–91	64	66 a	41–89
% 4-5-6 rings	28	19 a	11–82	29	29 a	9–56	36	34 a	11–59
Element accumulation *(N = 16)*									
N (g kg^{-1})	28.8	28.5 a	18.1–39.8	30.5	32.0 a	17.2–42.7	30.8	30.3 a	16.5–44.2
S (g kg^{-1})	1.5	1.6 b	0.9–3.2	1.9	1.9 a	1.2–3.4	2.0	2.0 a	1.1–3.0
Cd (μg g^{-1})	0.02	0.02 b	0.01–0.02	0.03	0.03 a	0.01–0.11	0.02	0.02 ab	0.01–0.03
Cu (μg g^{-1})	16.4	12.8 a	9.8–49.0	11.7	11.5 a	7.8–17.4	13.5	6.8 b	4.1–93.5
Mn (μg g^{-1})	52.6	43.0 c	23–202	865	760 a	307–1968	338	298 b	105–610
Ni (μg g^{-1})	0.44	0.4 b	0.2–0.7	1.38	1.0 a	0.7–3.3	1.19	1.1 a	0.6–2.7
Pb (μg g^{-1})	0.30	0.27 a	0.15–0.63	0.34	0.32 a	0.21–0.63	0.41	0.34 a	0.21–0.86
Zn (μg g^{-1})	13.1	12.6 b	8.1–20.7	22.1	20.3 a	15.4–33	16.2	14.2 b	11.1–43.1

Distinct letters indicate significant differences among tree species for each parameter; $p < 0.05$ (ANOVA on ranks – Kruskal–Wallis test, followed by Dunn pairwise test).

P. gonoacantha leaves were also glabrous and characterized by both leaf surfaces having a smooth cuticle covered by warty wax particles and epidermal cells with sinuous anticlinal walls (Fig. 3D–E). Slightly convex cells were present in the adaxial surface (Fig. 3D), convex cells were present in the abaxial surface (Fig. 3E), and stomata were present only in the abaxial epidermis with slightly sunken guard cells (Fig. 3E). The leaf adaxial surface of *C. floribundus* was defined by a smooth cuticle apparently devoid of visible surface wax deposits, epidermal cells with straight or sinuous anticlinal walls and scattered uniseriate trichomes (Fig. 3G); its abaxial leaf surface was covered by stellar non-secretory trichomes densely arranged at different heights (Fig. 3H).

A. graveolens leaves were more compact (Fig. 3C) and had less intercellular spaces than *P. gonoacantha* and *C. floribundus* (Fig. 3F and I, respectively). The transversal leaf sections revealed that the thickness of the adaxial epidermis was similar in all three species. However, *C. floribundus* showed a significantly thicker abaxial epidermis, mesophyll, palisade and spongy parenchyma than *A. graveolens* and *P. gonoacantha*. The mesophyll and palisade parenchyma in the *A. graveolens* leaves were also thicker than in the *P. gonoacantha* leaves (Table 3).

3.3. Chemical markers of selected tree species

A significantly higher content of total leaf wax was observed in the leaves of *C. floribundus* than in the leaves of *A. graveolens* and *P. gonoacantha*. The wax composition also differed among tree species. Hydrocarbons (*n*-alkanes) and triterpenes were important constituents of the wax of mature leaves when considering the three species. Significantly higher proportions of steroids were

Table 5
Means, medians and range between minimum and maximum values of biochemical biomarkers in leaves of *A. graveolens, C. floribundus* and *P. gonoacantha* sampled in three remnants of Atlantic Semideciduous Forest studied in the Metropolitan Region of Campinas (SE Brazil). Total ascorbic acid (AsA + DHA) reduced/total ascorbic acid ratio (AsA/AsA + DHA), total glutathione (GSH + GSSG), reduced/total glutathione ratio (GSH/GSH + GSSG), ascorbate peroxidase (APX), glutathione reductase (GR), hydroperoxide conjugated diene (HPCD), malondialdehyde (MDA).

Biochemical markers	*A. graveolens*			*C. floribundus*			*P. gonoacantha*		
	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range
Antioxidants *(N = 96)*									
AsA + DHA (mg gDM^{-1})	2.6	2.5 a	1.0–5.7	1.1	0.87 c	0.24–4.9	1.6	1.5 b	0.54–4.2
AsA/AsA + DHA	0.48	0.33 b	0.07–0.99	0.62	0.64 a	0.14–0.98	0.43	0.35 b	0.10–0.89
GSH + GSSG (nmol gDM^{-1})	37.7	35.5 b	12.7–99.3	54.1	49.2 a	20.5–110.8	37.6	33.6 b	21.2–212.8
GSH/GSH + GSSG	0.79	0.82 a	0.40–0.99	0.66	0.70 b	0.21–0.97	0.72	0.75 b	0.32–0.98
APX (nmol min^{-1} mg^{-1}prot)	10.7	5.6 b	2.3–60.0	18.8	14.9 a	3.7–68.1	21.7	17.4 a	4.9–94.7
GR (μmol min^{-1} mg^{-1}prot)	11.3	6.8 b	2.1–79.3	32.4	26.0 a	9.8–81.3	32.6	29.4 a	7.9–94.3
Primary metabolites *(N = 96)*									
Water-soluble sugars (mg gDM^{-1})	94.7	75.7 a	39.0–485.6	96.8	84.1 a	44.5–494.8	60.3	48.4 b	7.9–289.2
Starch (mg gDM^{-1})	67.0	62.8 b	22.4–130.7	78.3	71.9 ab	34.1–177.9	80.4	80.3 a	29.5–167.6
Secondary metabolites *(N = 96)*									
Total phenols (%)	2.66	1.90 a	0.25 – 8.70	1.70	1.26 b	0.17–6.92	1.81	1.62 ab	0.26–5.86
Total tannins (%)	3.74	3.71 a	0.20–11.37	2.20	1.63 b	0.22–8.80	2.64	2.52 b	0.32–8.55
Condensed tannins (%)	1.69	1.52 a	0.04–5.31	1.61	1.53 a	0.10–5.03	0.68	0.46 b	0.00–3.75
Total flavonoids (%)	0.90	0.86 a	0.23–2.04	0.46	0.41 b	0.17–0.98	0.53	0.50 b	0.19–1.21
Lipid peroxidaton *(N = 96)*									
HPCD (mg g^{-1}DW)	35.6	30.1 a	5.8–113.1	14.7	11.65 b	4.0–41.3	19.1	21.1 b	2.3–45.9
MDA (TBARS nmol g^{-1}FW)	27.5	26.7 b	10.7–71.7	31.3	30.5 b	12.6–75.9	38.7	37.1 a	14.3–97.7

Distinct letters indicate significant differences among tree species for each parameter; $p < 0.05$ (ANOVA on ranks – Kruskal–Wallis test, followed by Dunn pairwise test).

found in the leaf wax of *A. graveolens*, of *n*-alkanes in the leaf wax of *C. floribundus* and carboxylic acids in the leaf wax of *P. gonoacantha* (Table 4).

P. gonoacantha retained significantly greater amount of ΣPAHs in their leaves in comparison with the other species. In addition, the accumulation of PAHs with 2–3 aromatic rings in the leaves of all three species appeared to be proportionally higher than compounds with 4-5-6 aromatic rings (Table 4).

The leaf concentrations of N and Pb were similar in the three tree species. *C. floribundus* presented the highest leaf concentrations of Mn and Zn. Significantly higher leaf contents of S and Ni were found in *C. floribundus* and *P. gonoacantha* than in *A. graveolens*, which only accumulated significant high levels of Cu in comparison to *P. gonoacantha* along to *C. floribundus*. This last species also contained significantly higher levels of Cd in the leaves than *P. gonoacantha* (Table 4).

3.4. Biochemical markers of selected tree species

Significantly higher concentrations of total ascorbate (AsA + DHA), higher GSH/GSH + GSSG ratios and lower activities of APX and GR were measured in the leaves of *A. graveolens* than in those of *C. floribundus* and *P. gonoacantha*. However, the ascorbate redox state, indicated by the reduced/total ascorbic acid ratio (AsA/AsA + DHA), and the levels of total glutathione (GSH + GSSG) were more elevated in the leaves of *C. floribundus* when compared to the antioxidant indicators measured in the leaves of the other species (Table 5).

The primary metabolites (water-soluble sugars and starch) in *A.* graveolens differed significantly from those measured in *P. gonoacantha*. The concentrations of water-soluble sugars (glucose + sucrose + fructose) were higher and of starch were lower in the leaves of *A. graveolens* compared to those measured in *P. gonoacantha* (Table 5).

The contents of secondary metabolites also distinguished the native tree species. The total phenol contents were similar in *A. graveolens* and *P. gonoacantha* and significantly higher in *C. floribundus*. The total contents of tannin and flavonoids were significantly lower in the leaf samples of *P. gonoacantha* than in the other species. *A. graveolens* and *C. floribundus* showed higher levels of condensed tannins in comparison to *P. gonoacantha* (Table 5).

The highest content of HPCD was found in the leaves of *A. graveolens* and of MDA and in the leaves of *P. gonoacantha*, in comparison to the other two species (Table 5).

4. Discussion

The first criterion recommended by Fräinzle (2003) for a qualified passive biomonitoring constituted the first challenge in the present study: screening of the most representative bioindicator trees. The protocols of the ICP-Forests (Ferretti et al., 2010; Rautio et al., 2010) were not completely applicable concerning the representativeness of native bioindicators due to the high biodiversity in the Brazilian Atlantic Forest, as highlighted by Lira et al. (2012) and Ribeiro et al. (2009), and the low number of adult trees per species. Gentry (1988), when describing plant community diversity in 226 locations on environmental and geographical gradients using the transect method, proved that the most abundant species in four Atlantic Forest sites located in SE Brazil contributed only with 4.4–16.5% of the total individuals sampled, similar to the representativeness of the most numerous species established in our survey. These estimations contrasted with the high relative density (between 25.8 and 74.3%) of the most abundant species in European forests (Gentry, 1988). In addition, the six tree species abundantly found in the forest remnants studied represented a small proportion of the species diversity compiled by Gentry (1988) and Phillips et al. (2002) in Atlantic Forest locations in SE Brazil (121–158 plant species with diameter at breast height > 2.5 cm). This species richness differed by one order of magnitude from that described for the European sites studied by these authors (5–21 plant species with similar DBH).

In addition, three factors should be taken into account for the selection of reliable and valid biomarkers of the most abundant tree species suitable for future assessment of pollution effects on the Atlantic Forest remnants, based on the second and third criteria recommended by Fräinzle (2003). First, although pollution-induced injury on native tree species may occur throughout the year due to adequate climatic conditions for plant growth, the intensity of injury may not be uniform throughout the year due to the strong seasonality of both air pollutants and climate, as inferred by Moura et al. (2014a), based on 11 years of O_3 and climate dataset from the monitoring station located in Paulinia city (the same indicated in Fig. 1). Second, the biomarker variations may have represented integrated plant responses to a mixture of gaseous and particulate pollutants from diverse anthropogenic sources distributed in the same region, in association with climatic oscillations, as described by Boian and Andrade (2012) and Tresmondi and Tomaz (2004). Third, the biomarkers analyzed differed among tree species.

The three most numerous tree species belong to distinct taxonomic groups, with inherent characteristics that were potentially useful for future biomonitoring of effects associated with specific pollutant categories, even under the complex mixture of air contamination and seasonal climate.

The epicuticular wax composition and leaf surface structure were more important to define the bioaccumulation properties of the tree species than their leaf area dimension or specific area, as also commented by Bakker et al. (2000) and Jouraeva et al. (2002), regarding PAH accumulation and Rossini-Oliva and Mingorance (2004) regarding metal accumulation.

Experimental studies, such as those conducted by Chen et al. (2005) and Schreiber and Schonherr (1992), also showed that PAHs are easily adsorbed on plants that have higher contents of hydrophobic wax compounds, such as *n*-alkanes that were measured largely in *C. floribundus*. However, a greater PAH accumulation on its leaves was not confirmed. *P. gonoacantha* leaves were the most efficient PAH accumulator, despite the fact that their wax was composed by higher proportions of less hydrophobic constituents than hydrocarbons (alcohols, carboxylic acids and triterpenes, totaling 64% of the total wax content on average, compared to *n*-alkanes at 22%). In fact, the direct correlation between the presence of high levels of hydrophobic compounds in the cuticle and the accumulation of PAHs is not easily observed under natural conditions because PAHs may be either adsorbed on the cuticle surface or absorbed within inner tissues as a result of diffusion through wax and cutine and absorption through membranes (Simonich and Hites, 1994; Desalme et al., 2013). Kaupp et al. (2000), Li et al. (2010), Desalme et al. (2013) and Wang et al. (2014) agreed that the first fate involves a probable fast PAH reversion to the atmosphere due to photolysis and volatilization. Therefore, the high hydrophobicity of *n*-alkanes found abundantly in *C. floribundus* leaves may have conditioned the PAH adsorption in the outer cuticle layer, thus favoring photodegradation and volatilization and resulting in lower PAH accumulation than expected in this species. The epicuticular wax of *P. gonoacantha*, containing proportionally higher contents of less hydrophobic compounds, would permit a higher PAH mobilization within inner leaf tissues, possibly decreasing losses by volatilization and photodegradation. In addition, numerous small leaflets arranged in parallel and next to each other (cf. Fig. 7B included in Moura et al., 2014a), thinner leaves with relatively more air space in spongy parenchyma and

prominent warts of wax densely distributed on their surfaces are other specific morphological characteristics of *P. gonoacantha* that might enhance its ability to retain PAHs.

By contrast, *C. floribundus* was a candidate for future assessment of potential effects of some toxic elements contained in the particulate matter or present in the soil, such as heavy metals, by measuring their concentrations in the leaves. This hypothesis was raised with basis not only on its high potential for accumulating Cu, Cd, Mn, Ni, S and Zn in the leaves, but also on the high content of epicuticular wax and dense layer of stellar trichomes on leaf surfaces, which seemed to facilitate the capture of particulate matter and elemental accumulation, as mentioned by Hwang et al. (2011) and Saebo et al. (2012). Although *P. gonoacantha* plants accumulated Ni and S in similar levels, *C. floribundus* would supposedly tolerate stronger environmental oxidative stress, attributable to specific structural and biochemical markers (as discussed below), potentially extending its usefulness for future biomonitoring purposes.

The thicker mesophyll and numerous stellar trichomes on the abaxial surface of *C. floribundus* leaves, which hide the stomata, were possible barriers against gaseous pollutant uptake (Evert, 2006). This avoidance mechanism resulted in possible reduction of oxidative injury in *C. floribundus*, which was confirmed by the absence of oxidative microscopic visible injuries in leaves of adult trees living in the forest remnants and saplings fumigated with O_3 (Moura, 2013; Moura et al., 2014a, b).

On the other hand, leaves with ample spongy tissue, which facilitates the diffusion of gases in the mesophyll, are potentially sensitive to air pollutants, such as O_3 (Gerosa et al., 2003). Thinner palisade and thicker spongy layers, resulting in lower palisade to spongy parenchyma ratio, were observed in the leaves a sensitive genotype of *Prunus serotida* (Ferdinand et al., 2000). By analogy, a higher palisade/spongy parenchyma ratio would indicate that *P. gonoacantha* is more sensitive to gaseous pollutants than *A. graveolens* (ratios = 0.9 and 2.0, respectively). However, Moura et al. (2014a) identified higher proportion of visible O_3-like injury in *A. graveolens* leaves than in the foliage of *P. gonoacantha* sampled in the same Atlantic Forest remnants. Thus, *A. graveolens* trees would be more sensitive to the oxidative stress supposedly posed by O_3 than those of *P. gonoacantha* if we consider the occurrence of visible symptoms, contradicting *a priori* the previous expectation. However, Moura et al. (2014b), based on the results of fumigation experiments, observed that the O_3 sensitivity of *P. gonoacantha* may be more characterized by the acceleration of leaf senescence and abscission than by visible symptoms.

In addition, Matyssek et al. (2007) and Bussotti (2008) noted that thicker leaves may have higher antioxidant capacity. Once again, *A. graveolens*, with a compact leaf structure, might be more tolerant than *P. gonoacantha*. In fact, this assumption was confirmed by comparative analysis of their biochemical leaf traits.

Distinct scenarios arose in the ascorbate-glutathione metabolism of each tree species, following arguments of many authors (e.g. Caregnato et al., 2013; Foyer and Shigeoka, 2011; Gill and Tuteja, 2010; Singh et al., 2010).

C. floribundus was the most efficient species against the toxic effects of ROS. Its thick leaves contained low basal levels of total ascorbate, but showed a high oxi-reduction ability (based on AsA/AsA + DHA ratio); increased APX activity, intermediate glutathione levels, expressed as the total concentrations or oxi-reduction ratios, and high GR activity were also observed in the leaves, thus resulting in low lipid peroxidation (indicated by both HPCD and MDA contents). In addition, water-soluble sugars (glucose, sucrose and/or fructose) might have contributed to reinforce the antioxidative system of *C. floribundus*, since they are widely believed to be connected to ROS signaling pathways (Van den Ende and Valluru, 2009).

Despite the high oxi-reduction ability of glutathione in *A. graveolens* trees, low activities of GR, which plays an essential role by sustaining the reduced status of GSH at the expense of NADPH (Gill and Tuteja, 2010; Singh et al., 2010), coincided with the lower oxi-reduction capacity of ascorbate. Under low concentrations of reduced ascorbate, the APX activity, particularly the chloroplastic forms, may be rapidly reduced in the presence of H_2O_2 (Foyer and Shigeoka, 2011; Gill and Tuteja, 2010). These antioxidant imbalances may be enough indications that *A. graveolens* has a lower capacity of scavenging ROS than the other species. *A. graveolens* also showed the highest levels of total tannins and flavonoids, as established in several studies after plant exposure to O_3 (Saviranta et al., 2010). These compounds may function as antioxidants, due to their free-radical trapping properties (Agati et al., 2012; Blonkhina et al., 2003) and regulatory role on the detoxifying peroxidases (Takahama and Oniki, 1992). However, *A. graveolens* showed the highest HPCD levels even with high levels of phenolic compounds. If we consider that HPCD is the first product of lipid peroxidation and MDA is produced subsequently in this process (Lima and Abdalla, 2001), we may assume that tannins, flavonoids and carbohydrates were possible agents in avoiding the propagation of lipid peroxidation into leaf cells of *A. graveolens*, increasing to a certain extent the tolerance against oxidative stress despite the antioxidants from ascorbate-glutathione cycle were low. Interestingly, tannin and flavonoid accumulation, independently whether it promoted or not an enhanced tolerance against oxidative stress, appeared to be the elicitor of the visible dark stipplings observed by Moura et al. (2014a) in leaves of *A. graveolens* trees that grow in the Atlantic Forest remnants, which could be associated with O_3 after microscopic validation (Moura, 2013; Moura et al., 2014b). Similar associations were observed in other tropical tree species by Santos and Furlan (2013) and Sandre et al. (2014).

P. gonoacantha appeared to have a lower capacity of scavenging ROS than *A. graveolens* due to the high MDA contents, although the leaf antioxidants from ascorbate-glutathione cycle were measured in low and similar levels in comparison to those of *A. graveolens*. The main differences between both species were related to the contents of secondary metabolites and APX and GR activities that were lower and enhanced, respectively, in *P. gonoacantha*. Lower contents of water-soluble sugars and elevated contents of starch also observed in *P. gonoacantha* may be respectively related to an increase in respiration or inhibition of photosynthesis (Huttunen and Manninen, 2013; Muneer et al. (2014); Seyyednejad et al., 2011) and to the inhibition of carbon allocation to the roots (Skärby et al., 1998; Thomas et al., 2002).

In view of these findings, visible leaf injury observed in *A. graveolens* plants, the only integrating result of all biochemical disorders, appeared to be the potential biomarker for future biomonitoring of effects of oxidative pollutants, such as O_3, on the semideciduous Atlantic Forest. However, this hypothesis still needs to be carefully checked, based on the wide experience acquired by the ICP-Forests experts on the subject (Bussotti et al., 2003, 2006). The phenotypic plasticity of symptoms within the *A. graveolens* population and the possible occurrence of mimetic visible injury in response to other regional biotic and abiotic stresses will possibly be among the major restrictions for the correct recognition of visible injury in *A. graveolens*.

5. Conclusions

The criterion of representativeness was achieved in terms of the number of trees per abundant species, but not in terms of species richness due to the highly diverse Atlantic Forest. Three potential bioindicator trees (*C. floribundus*, *P. gonoacantha* and *A. graveolens*)

were selected by their representativeness in the forest ecosystem and characterized by a comparative screening of biomarkers. The inherent characteristics of these three most numerous tree species investigated seemed to be useful for a future biomonitoring of potential effects of specific pollutant categories on the Atlantic Forest remnants.

C. floribundus showed to be a candidate for assessing potential effects of toxic elements contained in the particulate matter or present in the soil, such as heavy metals and sulfur, by measuring their concentrations in the leaves. The leaf surfaces with a dense layer of stellar trichomes and high content of epicuticular wax accumulated efficiently Cu, Cd, Mn, Ni, S and Zn. Its capacity to tolerate environmental oxidative stress was attributed to specific structural and biochemical markers.

P. gonoacantha was the most efficient PAH accumulator in the leaves and may be recommended for future assessment of the level of these hydrocarbons on the particulate matter, due to the wax composition on the leaf surfaces that may facilitate absorption within inner leaf tissues and to specific morphological characteristics, such as numerous small leaflets, thinner leaves with relatively more air space in spongy parenchyma and prominent wax warts densely distributed on leaf surfaces.

A. graveolens appeared to be an useful bioindicator tree species for future biomonitoring of the potential effects of oxidative pollutants, such as O_3, by assessing visible leaf injury (dark intercoastal stipplings), which possibly resulted from the following biochemical disorders: low capacity of scavenging ROS by antioxidants from the ascorbate-glutathione cycle and leaf accumulation of secondary metabolites (specially tannins and flavonoids). Specific morphological and biochemical biomarkers measured in *P. gonoacantha* trees detached its higher sensitivity to oxidative stress. However, previous studies revealed that accelerated leaf senescence and abscission may be more pronounced than the visible small stipplings observed in saplings exposed to O_3. Therefore, further investigation is still required to better define the most adequate biomarker of *P. gonoacantha* for biomonitoring oxidative pollutants.

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