



# Leaf Morpho-anatomical Structure Determines Differential Response Among Restinga Species Exposed to Emissions from an Iron Ore Pelletizing Plant

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**Abstract** An iron ore pelletizing plant in southeastern Brazil exposes the tropical coastal ecosystems known as restinga to emissions of dust, iron solid particulate matter, and sulfur dioxide (SO<sub>2</sub>). We aimed to assess the effects of those emissions on the leaf morphology and anatomy of the restinga species *Byrsonima sericea*, *Cordia verbenacea*, and *Psidium guineense* by evaluating visual symptomatology and analyzing the anatomical and micromorphological alterations resulting from exposure. Leaves were collected from individuals growing at two sites: a restinga forest fragment located 800 m away from the pelletizing plant and a restinga conservation unit 20 km away, which served as reference site. In all three species, individuals growing near the pelletizing plant showed necrotic regions on the leaf and foliar micromorphological alterations like turgor loss of epidermal cells, cuticle and epicuticular wax erosion, stomatal obliteration, and rupture and plasmolysis of trichomes. Anatomically, we found cell collapse, cell

hypertrophy, and formation of a wound tissue. *C. verbenacea* showed the most severe visual and anatomical damage, being thus considered the most sensitive species to emissions. Leaf structural features such as uniseriate epidermis, lack of hypodermis, and presence of trichomes contributed to the highest sensitivity of *C. verbenacea*. Our findings reinforce the importance of performing morpho-anatomical studies to elucidate how leaf structure determines differential sensitivity to airborne pollutants in native species.

**Keywords** Particulate iron deposition · Sulfur dioxide · Symptomatology · *Byrsonima sericea* · *Cordia verbenacea* · *Psidium guineense*

## 1 Introduction

Restinga is a sandy coastal biome constituted by quaternary sand deposits (Henriques et al. 1986). It is characterized by high temperatures, elevated irradiance, water and nutrient scarcity, and high salinity levels (Pereira 1990; Scarano 2002; Assis et al. 2004), thus offering extreme conditions of survival for its characteristic fauna and flora. In Brazil, the restinga comprises about 70% of the country coastline, being recognized as a permanent conservation biome (CONAMA 1993). However, off the coast of the southeastern state of Espírito Santo, iron ore pelletizing plants, which process iron (Fe) ore into pellets for exportation, are located near restinga areas. Pelletizing activity is thereby affecting local restinga ecosystems due to the emission of airborne

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pollutants that can alter vegetation dynamics, culminating in decreased biodiversity (Grantz et al. 2003; Silva et al. 2006; Kuki et al. 2008a; Silva et al. 2015; Silva et al. 2017).

Pelletizing plants are sources of iron solid particulate matter (SPM<sub>Fe</sub>) and sulfur dioxide (SO<sub>2</sub>) (Silva et al. 2006; Kuki et al. 2008b; Silva et al. 2015). When deposited onto leaves, SPM<sub>Fe</sub> interferes with the photosynthetically active radiation and may cause stomatal blockage, thereby increasing leaf internal temperature and ultimately leading to alterations in transpiration rates (Kuki et al. 2008a; Pereira et al. 2009). Additionally, anatomical alterations such as epicuticular wax erosion and stomatal damage have been reported to the restinga species *Clusia hilariana* after exposure to SPM<sub>Fe</sub> (Rocha et al. 2014). As for SO<sub>2</sub>, when it reaches the atmosphere it oxidizes to form sulfate ions, which in turn combine with hydrogen atoms in the air to form sulfuric acid, thereafter becoming a component of acid rain (Kuki et al. 2009; Neves et al. 2009). In plants, SO<sub>2</sub> induces intracellular accumulation of H<sup>+</sup>, which promotes cell disturbances like increased membrane permeability (Neves et al. 2009).

Exposure to pollutants emitted by iron ore pelletizing plants can affect growth and development of sensitive species. Such exposure might even eliminate entire plant populations from an impacted area (Silva et al. 2006; Kuki et al. 2008a; Neves et al. 2009). Visual, structural, physiological and biochemical evaluations have been performed to evaluate the effects of SPM<sub>Fe</sub> and SO<sub>2</sub> in restinga species (Silva et al. 2006; Neves et al. 2009; Pereira et al. 2009; Rocha et al. 2014; Silva et al. 2015, 2017). Silva et al. (2006) demonstrated that *Byrsonima sericea* DC. (Malpighiaceae), *Cordia verbenacea* DC. (Boraginaceae), and *Psidium guineense* Sw (Myrtaceae), all of which occur abundantly in the Brazilian restinga, are impacted by iron ore pelletizing plant emissions. The authors, however, failed to correlate the sensitivity of each species to the morpho-anatomical features of the species leaves. Considering that the three species have different leaf morphological features, we hypothesized that their micro-morphological and anatomical traits would determine their responses to pelletizing plant emissions. Thus, we aimed to evaluate the morpho-anatomical alterations caused by such emissions on leaves of *B. sericea*, *C. verbenacea*, and *P. guineense* individuals growing at two sites: a restinga fragment near a pelletizing plant and a conservation unit known as “Paulo César Vinha

State Park,” which served as reference site. We expected that evaluating anatomical and micromorphological parameters would provide us with information on species sensitivity and on the extent of visible damage caused by emissions, as well as with prognostic information prior to the onset of visible injuries.

## 2 Materials and Methods

The sampled species were *Byrsonima sericea* DC. (Malpighiaceae), *Cordia verbenacea* DC. (Boraginaceae), and *Psidium guineense* Sw (Myrtaceae), due to their high representativeness and high frequency in restinga ecosystems and to the fact that their leaves are morphologically different from each other. Voucher specimens were deposited in the VIC Herbarium at Federal University of Viçosa, Brazil, with numbers 27179, 27181, and 27188, respectively.

The field study was carried out in two restinga forest areas at Espírito Santo state, southeastern Brazil. The first area is located 800 m away from the iron ore pelletizing plant. The second area, a conservation unit known as “Paulo César Vinha State Park” (hereafter, PCVSP), is 20 km away from the plant. The weather conditions and levels of airborne pollutants at both sites have been previously reported by Silva et al. (2006).

Three individuals of each species were selected per site. Three branches of each individual were evaluated for visual symptoms, which, whenever present, were photographed with a digital camera (model Cyber-Shot DSC-W310, Sony Corporation, Tokyo, Japan).

Fragments of the leaf median and marginal regions ( $n = 3$ ) were collected from individuals of all three species growing at both sites. Leaves of individuals growing near the iron ore pelletizing plant showed visual symptoms. Samples were fixed in a solution of 4% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer pH 7.0 (Karnovsky 1965, modified), dehydrated in an ethyl series, and embedded in methacrylate (Historesin, Leica Instruments, Heidelberg, Germany). Cross sections 5- $\mu$ m thick were obtained with an automatic rotary microtome (model RM2255, Leica Microsystems Inc., Deerfield, USA) and stained with 0.05% toluidine blue pH 4.7 (O’Brian and McCully 1981). Glass slides were mounted with Eukitt (Eukitt Mounting Medium, Sigma-Aldrich Corporation, St. Louis, USA) and photographed in a light microscope (model AX70RF, Olympus Optical, Tokyo, Japan)

equipped with a U-Photo system and coupled with a digital camera (model Spot Insightcolour 3.2.0, Diagnostic Instruments Inc., New York, USA).

For micromorphological characterization, leaves without visual symptoms were also collected from individuals growing at both sites. Leaf samples were fixed in Karnovsky's solution (Karnovsky 1965) and dehydrated in an ethyl series. Samples were then critical-point dried (equipment model CPD 030, Bal-Tec, Balzers, Liechtenstein), affixed onto stubs and sputter-coated with gold (sputter coater model FDU 010, Bal-Tec). Micrographic documentation was carried out in a scanning electron microscope (model LEO 1430 VP, Carl Zeiss, Jena, Germany).

### 3 Results

#### 3.1 Visual Injury

At the PCVSP conservation unit, *B. sericea* and *P. guineense* had waxy glabrous leaves the latter species having more coriaceous and less waxy leaves than the former (Fig. 1a, b). Leaves of *C. verbenacea*, on the other hand, were membranaceous, rough and less waxy (Fig. 1c). Plants at PCVSP showed no visual symptoms (Fig. 1a–c).

In all species, we found visual injuries on leaves of individuals growing near the iron ore pelletizing plant (Fig. 1d–f). The three species showed brownish necroses on both interveinal and marginal regions of the leaf blade (Fig. 1d–f). In *C. verbenacea*, in addition to necrosis, which overall compromised a large area of the leaf, we also found chlorotic spots spreading throughout the entire leaf blade (Fig. 1f).

#### 3.2 Micromorphological Alterations Preceding Visual Injury

Micromorphological damage was observed on the surface of leaves that macroscopically showed no symptoms (Figs. 2 and 3).

*B. sericea* showed less foliar injuries than the other species. The contour of epidermal cells was not discernible (Fig. 2a). On the abaxial leaf surface, stomata were located in depressions (Fig. 2b). In contrast, near the pelletizing plant, *B. sericea* leaves showed damage such as turgor loss; alteration of epidermal relief (Fig. 2c);

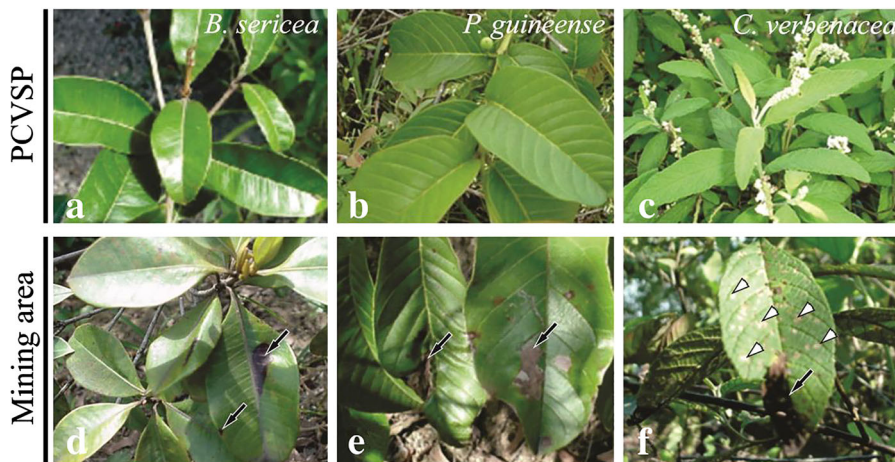
epicuticular wax flaking; and breakage, especially near stomata (Fig. 2d), which obliterated them.

At PCVSP, leaves of *P. guineense* showed epidermal common cells with conspicuous anticlinal cell walls and the smooth layer type of epicuticular wax deposition pattern (Fig. 2e). Stomata and turgid non-glandular trichomes occurred on the abaxial leaf surface (Fig. 2f, g). On the other hand, in *P. guineense* individuals growing near the pelletizing plant, epidermal cells of the adaxial surface showed turgor loss (Fig. 2h), while on the abaxial leaf surface, we found plasmolyzed non-glandular trichomes (Fig. 2i) and flaccid stomatal ledges (Fig. 2j).

*C. verbenacea* individuals growing at PCVSP showed high density of both glandular and non-glandular trichomes on the adaxial leaf surface (Fig. 3a, b). Epidermal common cells were isodiametric and had sinuous walls (Fig. 3a, b). Stomata and non-glandular trichomes were present on the abaxial leaf surface (Fig. 3c, d). However, asymptomatic leaves of *C. verbenacea* showed the highest amount of micromorphological injuries among the studied species. In individuals growing near the pelletizing plant, we found cuticle erosion, epidermal rupture, and exposure of internal tissues in leaf regions with more conspicuous damage (Fig. 3e). We also found turgor loss of the following: glandular and non-glandular trichomes and epidermal common cells (Fig. 3f, g). Stomata were frequently obliterated, some of which had flaccid, non-functional stomatal ledges (Fig. 3h).

#### 3.3 Anatomical Characterization of Unexposed Plants

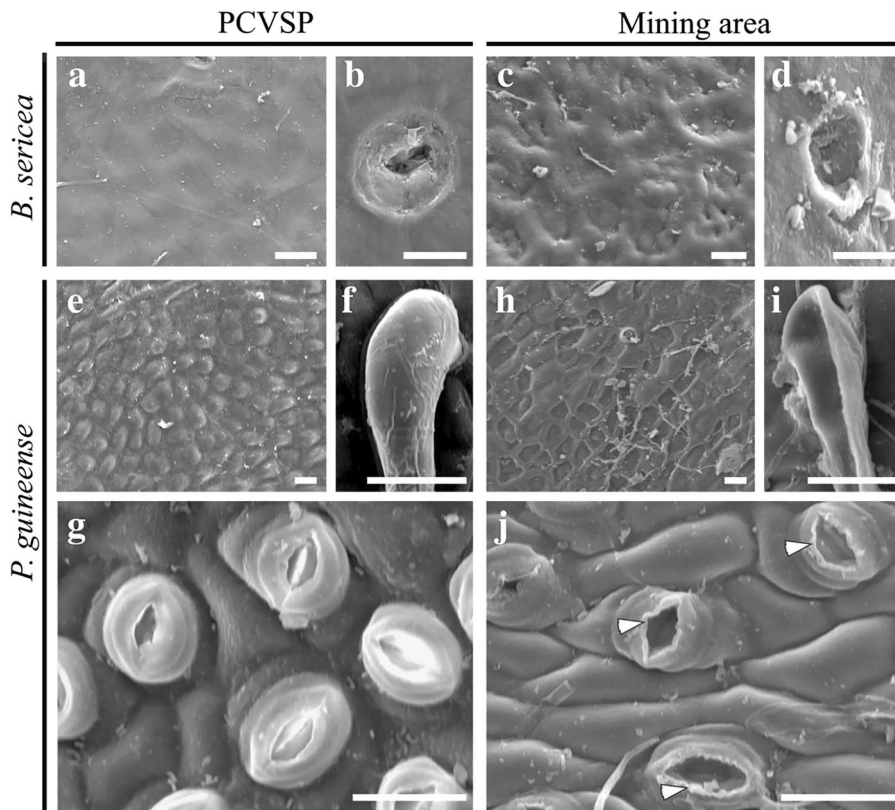
Leaves of *B. sericea*, *C. verbenacea*, and *P. guineense* are hypostomatic and dorsiventral. The mesophyll has one cell layer of palisade parenchyma in *C. verbenacea* (Fig. 5a) and 2–3 cell layers in *B. sericea* and *P. guineense* (Fig. 4a, c) and 3–7 cell layers of spongy parenchyma in all species (Figs. 4a, c and 5a). Epidermis is uniseriate in the three species. Leaves of *P. guineense* and *B. sericea* have non-glandular trichomes (Fig. 4a) while leaves of *C. verbenacea* have both glandular and non-glandular trichomes (Fig. 5c, i). A hypodermis composed of 1 and 3 cell layers is present on leaves of *B. sericea* and *P. guineense*, respectively (Fig. 4a, c). Collenchyma cells occur immediately



**Fig. 1** Individuals of *Byrsonima sericea*, *Psidium guineense* and *Cordia verbenacea* growing at the PCVSP conservation unit (a–c) and in a forest remnant near an iron ore pelletizing plant (d–f). Black arrows: necrosis on the leaf blade. White arrowhead: chlorosis

below the epidermis on the midrib of *P. guineense* and *C. verbenacea* leaves (Figs. 4d and 5d). Vascular bundles in leaves of *P. guineense* are

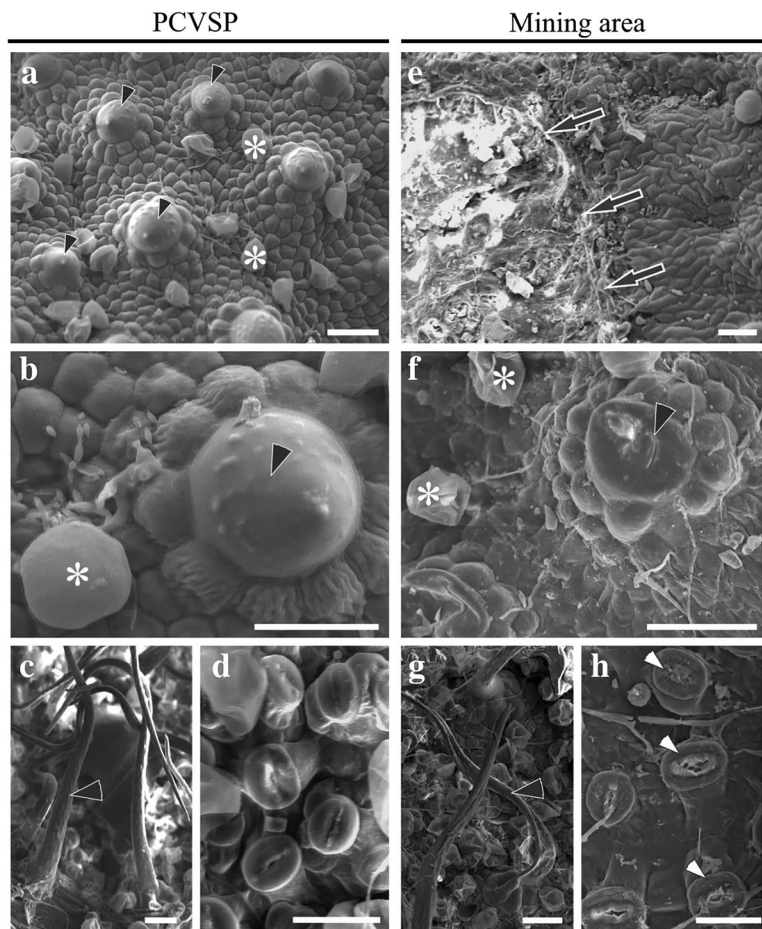
bicollateral and surrounded by parenchyma (Fig. 4d, e). *P. guineense* leaves have secretory cavities in the mesophyll (Fig. 4c).



**Fig. 2** Leaves of *Byrsonima sericea* (a, b) and *Psidium guineense* (e–g) collected from individuals growing at the PCVSP conservation unit and near an iron ore pelletizing plant (c, d, h–j), observed

in scanning electron microscopy. Adaxial surface (a, c, e, h). Abaxial surface (b, d, f, g, i, j). White arrowhead: flaccid stomatal ledges. Bars = 20 μm

**Fig. 3** Leaves of *Cordia verbenacea* collected from individuals growing at the PCVSP conservation unit (**a–d**) and near an iron ore pelletizing plant (**e–h**), observed in scanning electron microscopy. Adaxial surface (**a, b, e, f**). Abaxial surface (**c, d, g, h**). Black arrowhead—non-glandular trichomes, white asterisks—glandular trichomes, black arrow—epidermal erosion, white arrowhead—obliterated stomata with flaccid ledges. Bars = **a–c, e–g** 50  $\mu\text{m}$ ; **d, h** 20  $\mu\text{m}$



### 3.4 Anatomical Alterations of Exposed Plants

We found anatomical alterations in leaves of all species. In *B. sericea*, we found invaginations on the cell wall (Fig. 4b). In *P. guineense*, there were turgor loss and cell collapse of epidermal cells on the adaxial leaf surface (Fig. 4f). In the hypodermis of *P. guineense*, we found protoplast retraction; cell plasmolysis; cell hypertrophy; and accumulation of substances, probably phenolic compounds, in a few cells (Fig. 4f). Secretory cavities in that species were deformed, showing collapsed epithelial cells (Fig. 4f). In vascular bundles, phloem cells were collapsed as well (Fig. 4g, h).

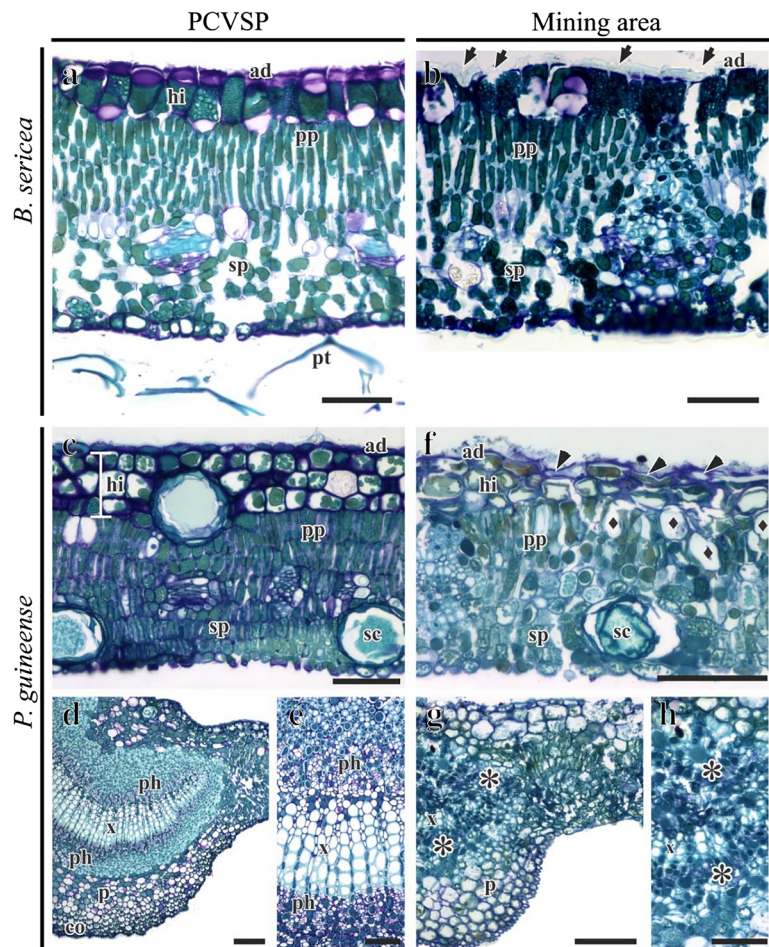
Epidermal cells on the adaxial leaf surface of *C. verbenacea* were also collapsed, which resulted in degradation of the epidermis and ultimate exposure of the mesophyll (Fig. 5e, f). Such collapse occasionally compromised trichomes (Fig. 5g). In the palisade parenchyma, we found not only collapsed cells but also

hypertrophied ones (Fig. 5e). In the spongy parenchyma and midrib, cell collapse resulted in formation of large *intercellular spaces* (Fig. 5f, i). Near necroses, we found hypertrophied cells and formation of a wound tissue (Fig. 5h).

## 4 Discussion

We characterized the morpho-anatomical alterations that occur in leaves of *B. sericea*, *C. verbenacea*, and *P. guineense* individuals growing near an iron ore pelletizing plant. The visual symptoms we found reveal that pelletizing plant emissions can affect nearby vegetation, thereby damaging plant vegetative organization at different levels (Silva et al. 2017; Arrivabene et al. 2015) and decreasing biodiversity and ecosystem resilience (Grantz et al. 2003). Visual injuries such as chlorosis and necrosis of leaves are commonly reported to

**Fig. 4** Leaves of *Byrsonima sericea* (a) and *Psidium guineense* (c–e) collected from individuals growing at the PCVSP conservation unit and near an iron ore pelletizing plant (b, f–h), observed in light microscopy (cross sections). Black arrow—cuticle rupture, black arrowhead—loss of cell turgidity, diamond—cell hypertrophy, asterisks—collapse of phloem cells. p parenchyma, pt peltate trichome, ph phloem, x xylem, co collenchyma, ad adaxial epidermis, pp palisade parenchyma, sp spongy parenchyma, hi hypodermis, sc secretory cavities. Bars = a–d, f, g 50  $\mu$ m; e, h 25  $\mu$ m



plant species that are sensitive to particulate matter emissions (Lopes et al. 2002; Silva et al. 2006, 2017).

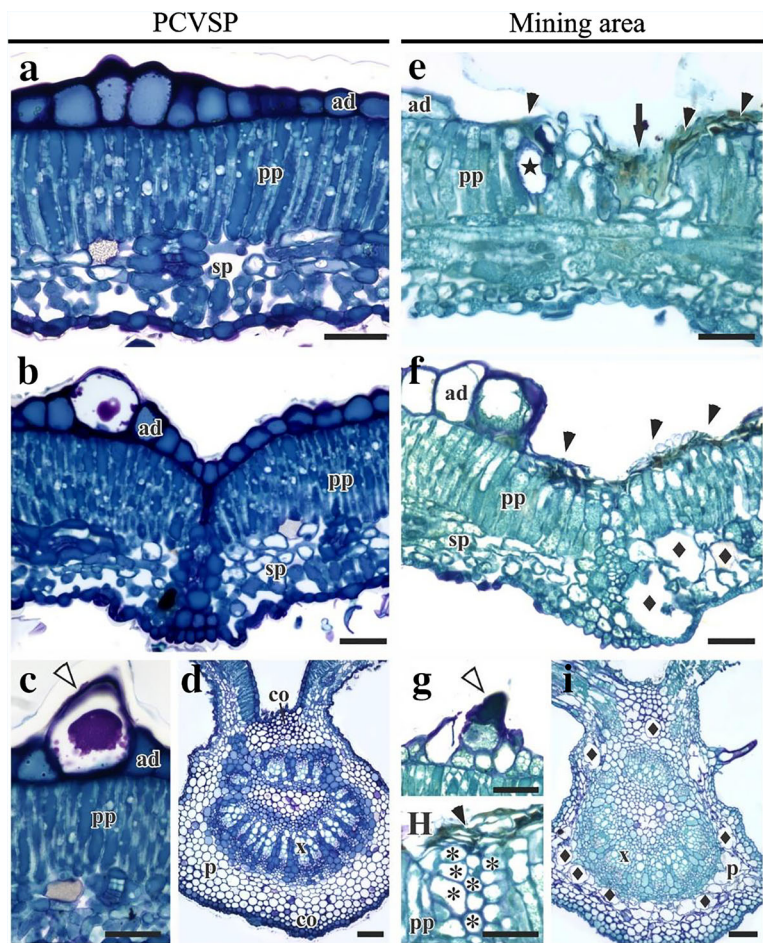
We found micromorphological alterations even in asymptomatic leaves of individuals growing near the pelletizing plant. Alterations in leaf structure have already been shown to precede the onset of visual symptoms, which thus demonstrates the prognostic value of anatomical analyses (Silva et al. 2005; Sant'Anna-Santos et al. 2012; Rocha et al. 2014).

Micromorphological and anatomical leaf features, including the epicuticular wax deposition pattern, may influence the sensitivity of plant species to airborne pollutants (Andrade and Silva 2016). Thus, leaf anatomical structure is directly related to the sensitivity or resistance of different plant species to pollutants (Rocha et al. 2014). In *B. sericea*, for instance, the glabrous leaves and waxy cuticle might have determined the lower deposition of  $SPM_{Fe}$  onto the organ, which justifies the lower amount of leaf damage found

in this species, as the effects of particulate matter also depend on the extent of exposure to it (Grantz et al. 2003).

The invaginations among epidermal cells and trichomes of *P. guineense* and the high amount of trichomes in *C. verbenacea* may have determined a greater particle adhesion to leaves of those species, probably enhancing the resulting harmful effects. These findings are in agreement with those of other authors, who also attributed the degree of particle adhesion to epidermal and cuticular features (Prusty et al. 2005; Kuki et al. 2008a; Arrivabene et al. 2015; Silva et al. 2017). Trichome basal cells are more permeable to pollutants (Azevedo 1995; Chaves et al. 2002), therefore representing a particular site of pollutant uptake through the leaf. The loss of turgidity in trichomes of *C. verbenacea* and *P. guineense* suggests that these structures were impacted by the exposure, thus corroborating the abovementioned hypothesis.

**Fig. 5** Leaves of *Cordia verbenacea* collected from individuals growing at the PCVSP conservation unit (**a–d**) and near an iron ore pelletizing plant (**e–i**), observed in light microscopy (cross sections). Black arrowhead—epidermal collapse, black arrow—mesophyll exposure, white arrowhead—non-glandular trichomes, star—cell hypertrophy, asterisks—wound tissue, diamond—intercellular spaces. Ad adaxial epidermis, sp spongy parenchyma, pp palisade parenchyma, x xylem, p parenchyma, co collenchyma. Bars = **a–c, e–h** 50  $\mu$ m; **d, i** 100  $\mu$ m



Cuticle and epicuticular wax erosions were common micromorphological injuries in *C. verbenacea*, which may be related to the abrasive effect of particulate matter. Due to the species micromorphological features,  $SPM_{Fe}$  might well have been deposited for a longer period on the species leaf surface (Rai et al. 2010; Rocha et al. 2014; Silva et al. 2017). The cuticle and epicuticular wax are the first protective barriers of the leaf (Pal et al. 2002). Their erosion therefore favors the entry of pollutants into the organ. Moreover, cuticle and epicuticular wax erosion can cause stomata obliteration, as we found in *C. verbenacea* and *P. guineense* and as other authors have reported as responses to particulate iron deposition (Rocha et al. 2014; Silva et al. 2017). Furthermore, the ultrastructural alterations found in the stomata of all species may compromise their opening and closing, making them non-functional and then facilitating the entry of air pollutants, such as  $SO_2$ ,

pollutants emitted by the iron ore pelletization factory (Manninen and Huttunen 2000).

In a research conducted under the same conditions as those of our study, Silva et al. (2006) have chemically and histochemically detected the presence of iron in leaf tissues of exposed individuals. Although iron is an essential element, high concentrations of it in plant tissues tend to generate oxidative stress due to formation of reactive oxygen species (ROS) (Kobayashi and Nishizawa 2012; Rout et al. 2015; Neves et al. 2009). Excess ROS react with polyunsaturated fatty acids, causing lipid peroxidation and loss of membrane permeability (Becana et al. 1998; Gill and Tuteja 2010). Such imbalance in cellular biochemistry is reflected in the anatomy of leaf tissues, in the form of alterations such as invaginations, in *B. sericea* leaves; turgor loss, protoplast retraction, cell plasmolysis, deformation of secretory cavities, and collapse of phloem cells, in *P. guineense*; and cell collapse progressing to necrosis,

in *C. verbenacea*. Anatomical alterations have been reported by several studies on the effects of excess iron (Arrivabene et al. 2015; Silva et al. 2017; Araújo et al. 2014; Santana et al. 2014).

The anatomical alterations found on the leaf blade of *C. verbenacea* were drastic and as such could significantly compromise photosynthesis in the species and consequently interfere with plant development. In *C. verbenacea*, a wound tissue was formed near necrotic regions. This effect represents a defense strategy, insofar as such tissue acts as a barrier that prevents progress of the necrosis to other healthy leaf regions, as already observed with other types of pollutants (Silva et al. 2005; Sant'Anna-Santos et al. 2006; Siqueira-Silva et al. 2012; Araújo et al. 2014). *C. verbenacea* showed more damage in its leaf structure compared with *B. sericea* and *P. guineense*. The damage found in *C. verbenacea* was more severe, which enabled us to classify this species as the most sensitive one among all three evaluated in our study.

## 5 Conclusions

We found visual, micromorphological, and anatomical alterations on leaves of individuals occurring near an iron ore pelletizing plant, which thus reveals that the studied species are impacted by pollutants emitted thereat. The three species showed differential sensitivity to the pollutants, *C. verbenacea* being the most sensitive one. We believe that anatomical features such as higher leaf roughness and abundant glandular trichomes may have contributed to the onset of more severe damage in *C. verbenacea*. On the other hand, the presence of a hypodermis may have prevented the occurrence of more extensive micromorphological and anatomical damage in leaves of *B. sericea*. Leaf morphoanatomy might well be a key factor in determining the sensitivity or resistance of native species to airborne pollutants. In that sense, anatomical and micromorphological studies are therefore of major importance to properly assess plant sensitivity to polluting agents.

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