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Structural changes in *Psidium guajava* 'Paluma' leaves exposed to tropospheric ozone[1]

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RESUMO

(Alterações anatômicas em folhas de *Psidium guajava* 'Paluma' expostas ao ozônio troposférico). *Psidium guajava* 'Paluma' vem sendo testada como bioindicadora de ozônio (O_3) e responde com pigmentações, entre as nervuras, na superfície adaxial, decorrente do acúmulo de compostos fenólicos. Tais compostos atuam como antioxidantes não enzimáticos que neutralizam espécies reativas de oxigênio (ROS), formadas a partir do O_3. O estudo objetivou comparar a estrutura foliar de plantas com e sem sintomas visíveis para estabelecer como esses sintomas se apresentam em nível celular. Para tanto, avaliaram-se acúmulo de substâncias fenólicas, densidade estomática e alterações estruturais nos tecidos foliares de *P. guajava* 'Paluma', exposta durante as quatro estações do ano, no Parque Estadual das Fontes do Ipiranga (PEFI), área verde da cidade de São Paulo, com altos índices de O_3. Folhas com sintomas apresentaram, na superfície adaxial, antocianina nos vacúolos das células da epiderme, da hipoderme e, quando o sintoma foi mais intenso, em até três primeiras camadas do parênquima paliçádico, além de maior acúmulo de outros compostos fenólicos, em comparação com folhas assintomáticas. Algumas células parenquimáticas contíguas à câmara subestomática mostraram crescimento intrusivo em sua direção, promovendo a oclusão da câmara, o que pode contribuir para reduzir a entrada de O_3 na folha. O acúmulo de antocianina e outros compostos fenólicos, além da oclusão da câmara subestomática são características que podem conferir proteção à planta contra efeitos do O_3. Tais características, somadas à compactação do mesofilo, contribuem para que *Psidium guajava* 'Paluma' não apresente morte celular, sintoma frequente em espécies sensíveis ao O_3.

Palavras-chave: Anatomia foliar, bioindicador, compostos fenólicos, densidade estomática, estresse oxidativo

ABSTRACT

(Structural changes in *Psidium guajava* 'Paluma' leaves exposed to tropospheric ozone). *Psidium guajava* 'Paluma' has being tested as an ozone (O_3) bioindicator and responds with pigmentation between the veins on the adaxial surface, due to the accumulation of phenolic compounds. These compounds act as non-enzymatic antioxidants that neutralize reactive oxygen species (ROS), formed from O_3. This study aimed to evaluate the leaf structure of plants with and without visible symptoms and to establish these symptoms at the cellular level. Beside this we also aimed to detect structural changes that can minimize the effects of the O_3 on the plant. The accumulation of phenolic substances, stomatal density and structural changes in *P. guajava* 'Paluma' leaf tissues exposed during the four seasons of the year were evaluated. The study was conducted at the Parque Estadual das Fontes do Ipiranga (PEFI), which is a park in the city of São Paulo that has high levels of O_3. Leaves with symptoms showed, on the adaxial surface, anthocyanin accumulation in the vacuoles of epidermal cells and hypodermis. When the symptoms were more intense this accumulation was observed even in the first three layers of palisade parenchyma. Comparing symptomatic and asymptomatic leaves, there was higher accumulation of phenolic compounds in the symptomatic leaves. Some parenchyma cells adjacent to substomatal chambers showed intrusive growth towards the stomatal pore, promoting its occlusion, which could reduce the entry of O_3 in the leaf. The accumulation of anthocyanins and other phenolic compounds, in addition to the occlusion of the chamber, protect the plant against O_3 effects. These features and the compact arrangement of the mesophyll contribute to why *Psidium guajava* 'Paluma' does not present cell death, a symptom usually observed in species sensitive to O_3.

Key words: Bioindicator, phenols, leaf anatomy, oxidative stress, stomatal density

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Introduction

Great urban centers worldwide have air pollution as a common characteristic. This is mostly due to an increase in the number of cars and industrialization, which are essential to support the population growth in these regions. São Paulo's metropolitan region, with a population of more than 17 million inhabitants, faces this problem. This city has a great amount and diversity of air pollutants as tropospheric ozone (O_3) and its precursors, and concentrations of these gases reach high levels in different locations (Cetesb 2009).

Some plants can be used as O_3 bioindicators because they show characteristic responses when exposed to this pollutant (De Temmerman *et al.* 2004; Klumpp *et al.* 2001). These responses are initiated when the O_3 enters the leaf through stomata and reacts with the intercellular water forming reactive oxygen species (ROS). These ROS initially act on the plasma membrane and, depending on the capacity of the plant to neutralize them, can cause oxidative stress (Iriti & Faoro 2009; Roschina & Roschina 2003; Bray *et al.* 2000). The ROS neutralization is done by enzymatic and non-enzymatic antioxidants, such as phenolic compounds (Soares & Machado 2007).

Some macroscopic leaf symptoms are described for O_3 bioindicator plants, such as chlorosis, necrosis, and leaf pigmentation. These symptoms must be present between the veins, and only on the adaxial surface of the leaf; besides this, microscopic analysis must assure the absence of pathogens. However, for each species, symptoms observed in the field must be compared to those exhibited by the plant when exposed to known O_3 concentrations inside a fumigation chamber (Günthardt-Goerg & Vollenweider 2007; Novak *et al.* 2003).

Psidium guajava 'Paluma' has been studied as an O_3 bioindicator plant (Furlan *et al.* 2007; Pina *et al.* 2007; Pina & Moares 2007; Dias *et al.* 2007). Controlled studies inside fumigation chambers were done by Furlan *et al.* (2007) that described phenolic compound accumulation as a symptom resulting from O_3, which causes a reddish pigmentation.

Considering that *P. guajava* 'Paluma' shows characteristic macroscopic symptoms when exposed to O_3, this study aimed to evaluate the leaf structure of plants with and without visible symptoms to establish how these symptoms are expressed at the cellular level.

Methods

Plant exposure and environmental characterization

About 110 cloned *Psidium guajava* 'Paluma' seedlings, obtained from a specialized producer, were exposed in 2008 at the Instituto de Botânica located in Parque Estadual das Fontes do Ipiranga (PEFI) (23°38'08"S, 46°38'00"W). As a control group, ten seedlings were kept, for each season of the year, inside a greenhouse equipped with a Purafil® air filter (for particles and gases) and temperature control, which was next to the location of the plants exposition.

The plants were kept on holders with plastic boxes with water inside, covered by galvanized wire grilles. The plants were continuously watered by capillarity using nylon strings with one of the ends inserted in the pot near the roots and the other in contact with the water inside the box (VDI 2003). The seedlings were fertilized weekly with Peters (10:10:10 N:P:K) and were exposed side by side, under the same conditions of temperature, precipitation, wind speed, and irradiance. The plants kept in the greenhouse received the same irrigation and fertilization of those kept outside, and they were used as a control in relation to the appearance of visible damage and stomata density.

Continuous measurements of ozone were taken by HORIBA monitors (APOA-360CE and APNA-360CE) and IDA-2000 software, and the data were used to calculate AOT40 (Accumulated Ozone Exposure over a threshold of 40 ppb) and AOTzero, which refers to the total value of accumulated O_3 during the exposure of the plants. Meteorological data were provided by the Instituto Astronômico e Geofísico da USP (IAG) localized inside PEFI.

Anatomy and Histochemical tests

To analyze the phenolic compounds, plant tissue was collected by the end of each season of the year for each of the six plants sampled, with and without visible symptoms, from leaves of the first and second nodes (from base to apex).

Transversal sections of fresh material were used to localize phenolic compounds and condensed tannins, using ferric chloride (Johansen 1940) and acid vanillin (Gardner 1975), respectively, which was seen as intense colored agglomerates. Anthocyanin detection in mesophyll cells was observed in transversal sections of fresh material, without using dyes or reagents. For those analyses, about twelve samples from each season of the year were mounted in 50% glycerin and were observed using light microscopy.

Leaf fragments up to 1 cm^2 taken from the middle of leaves on the third node, of three plants, were fortnightly collected during each season. These fragments were fixed in Karnovsky solution (Karnovsky 1965), remaining under vacuum for about thirty minutes, in fixer for forty-eight hours, and then the samples were washed with phosphate buffer and stored in 70% alcohol. The samples were embedded in glycol methacrylate (GMA) (Leica historesin), according to the manufacturer recommendations, and were transversally sectioned with a rotary microtome (Olympus Cut 4055). Sections about 5 µm thick were dyed with 0.05 toluidine blue in 0.1M, acetate buffer at pH 4.7 (O'Brien & McCully 1965), and analyzed using a Olympus BX41 light microscope.

To determinate stomatal density, samples of three plants kept inside and outside the greenhouse (with the air filtered), were analyzed at the beginning, middle and end of each

season. For this study, leaf fragments with 1cm from leaves of the fifth and sixth nodes (from base to apex), were fixed in Karnovsky solution (Karnovsky 1965), diaphanized according to Arnott (1959) *apud* Kraus & Arduin (1997), dyed with astra blue and safranin (9:1), submitted to a crescent alcohol series, and mounted in synthetic Permount™ resin.

Stomatal density was determined by observing a 200 μm^2 area of the leaf surface at 400 times magnification (n=30 per season) using a microscope equipped with an image capture system, and a semi-automatic measurement system (Pro-Express 4.0.1, Media Cybernetics). This quantification was not done for plants kept inside the greenhouse during the autumn once this material was lost.

For the statistical analysis that correlated stomatal density with O_3 concentration, the Pearson correlation method was used. The comparison between stomatal density of plants kept inside the greenhouse with those outside the greenhouse was done through variance analysis (Anova on Ranks); when these analysis showed significant differences with $p \leq 0,05$, a multiple comparison analysis was done (Kruskal-Wallis).

Results

The highest AOTzero values occurred during the winter, followed by autumn and spring, while in the summer the concentration was the lowest. Average temperature values were higher during the summer and equal during the autumn and winter. Temperature data during the spring were not provided by IAG-USP. Average air humidity values did not conspicuously vary during spring, autumn and winter, but the average was comparatively lower in summer (Tab. 1).

Table 1. Data related to average temperature (T - ºC), relative air humidity (RAH - %), ozone concentrations values above 40ppb to the end of seasons (AOT40 – ppb).

	T average	RAH average	AOT40
Spring		80.95	575
Winter	17.22	77.63	661.3
Summer	20.32	16.32	360
Autumn	17.65	80.15	658

In relation to the location of phenolic compound accumulation, leaves without visible symptoms, here called asymptomatics (Fig. 1A), showed differences in cellular content when compared to symptomatic leaves (Fig. 1B). The presence of anthocyanins in the vacuole of the leaves was registered in epidermal cells on the adaxial surface and hypodermis. When symptoms were more intense, a very intense accumulation of anthocyanin in the upper layers of the palisade cells was registered.

Histochemical analysis using acid vanillin (Fig. 1C and D) and ferric chloride (Fig. 1E and F) on fresh material of asymptomatic (Fig. 1C and E) and symptomatic (Fig. 1D and F) leaves showed the presence of phenolic compounds in both types; however, when symptoms appeared, the occurrence of phenolic compounds, highlighted by intense colored agglomerates, visibly increased.

Stomatal density on leaves from the second node, that had expanded during exposure, in plants kept in and outside the greenhouse during different seasons of the year are indicated in figure 2.

Results obtained were compared through analysis of variance (Anova on Ranks - Kruskal Wallis), and showed that the plants that remained outside the greenhouse during the winter showed the highest stomatal density values. These values were higher than those from plants kept inside the greenhouse during the winter. Also, they were higher than those from plants kept outside the greenhouse during other seasons of the year that did not differ between each other. In the spring, the plants exposed to the environment did not statistically differ from those kept inside the greenhouse, while in those exposed during the summer a variation was observed, and the stomatal density of the plants outside the greenhouse was higher. The stomatal density of the plants exposed during the autumn that remained inside the greenhouse could not be analyzed after they were contaminated by fungi.

For the plants exposed to the environment, we correlated stomatal density with O_3 concentration, obtaining through the Pearson method a regular positive correlation with $r = 0.579$ and significant $p = 0.00279$ in all seasons of the year.

With the progression of visible symptoms, a conspicuous increase in the phenolic content in the epidermal cells on both surfaces was observed (Fig. 3A and 3B). In the hypodermis, besides this increase, it was possible to observe the membrane cell invagination in symptomatic leaves (Fig. 3C).

In the palisade cells there was a conspicuous increase in the content of phenolic compounds, in all layers of this tissue (Fig. 3D-F). A visible reduction in chloroplasts was also observed in symptomatic leaves in comparison to the asymptomatic leaves (Fig. 3A and 3D). Parenchyma cells showed changes, especially those closer to the abaxial surface of the leaf, which were smaller when compared to those from the upper layers (Fig. 3 A-F). Some parenchyma cells contiguous to the substomatal chamber showed an intrusive growth in the direction of the substomatal chamber, promoting its occlusion (Fig. 3F).

Discussion

Asymptomatic leaves of *P. guajava* 'Paluma' showed phenolic compounds in all mesophyll layers and in epidermal cells. However, in symptomatic leaves, besides these phenolic compounds being more apparent, the presence of anthocyanin was observed, which was accumulated in the upper mesophyll layers. This indicates that anthocyanin is mainly responsible for the visible symptoms that this plant

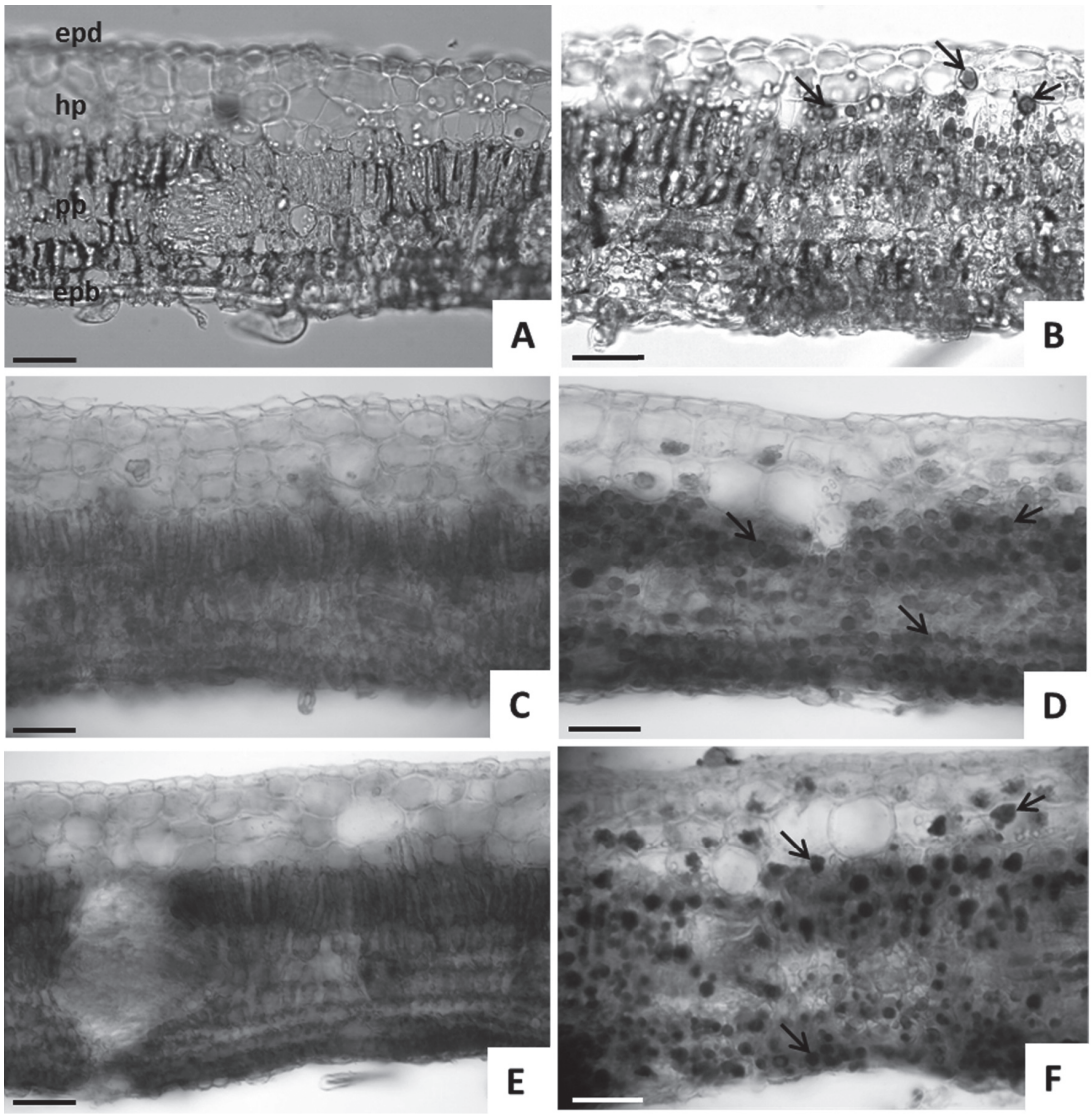

Figure 1. Transversal sections of *Psidium guajava* 'Paluma' leaves. A, C and E. Asymptomatic leaves. A. Control from histochemical analyses (material without dye or reagents). B, D and F. Symptomatic leaves. B. Anthocyanin (arrows). D. Condensed tannins (arrows). F. Total phenolic compounds (arrows). epd = adaxial epidermis; hp = hypodermis; pp = palisade parenchyma; epb = abaxial epidermis. Bars = 50 µm.

shows when exposed to O_3; these symptoms were characterized by reddish pigmentation between the veins, only on the upper surface of older leaves as described by Furlan *et al.* (2007) and Pina & Moraes (2007) as stippling. Indeed, the *P. guajava* 'Paluma' symptom is reddening since there is no cell death. According to Vollenweider & Günthardt-Goerg (2003), the term reddening is the most correct in this case, when there is an increase in vacuolar phenolics associated with the absence of cell death.

In general, phenolic compounds and especially anthocyanins act as antioxidants, neutralizing the ROS and minimizing its effects on cell membranes (Gould *et al.* 2002). Besides the direct action of the antioxidant, anthocyanins protect the chloroplasts from excess excitation by activating a controlled energy dissipation process and, hence, the potential for oxidative damage (Gravano *et al.* 2004).

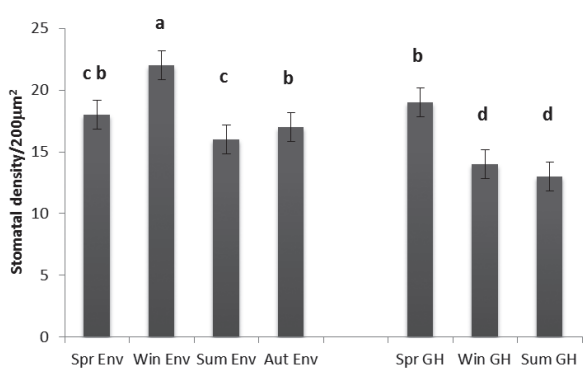

Figure 2. Comparative values of stomata density between plants of *Psidium guajava* 'Paluma' exposed to the environment and those kept inside the greenhouse with filtered air. Spr Env, Win Env, Sum Env and Aut Env respectively represents plants exposed to the environment during spring, winter, summer and autumn, while Spr GH, Win GH and Sum GH, are the plants inside the greenhouse during the same seasons of the year. Data from greenhouse plants exposed in the autumn were not obtained.

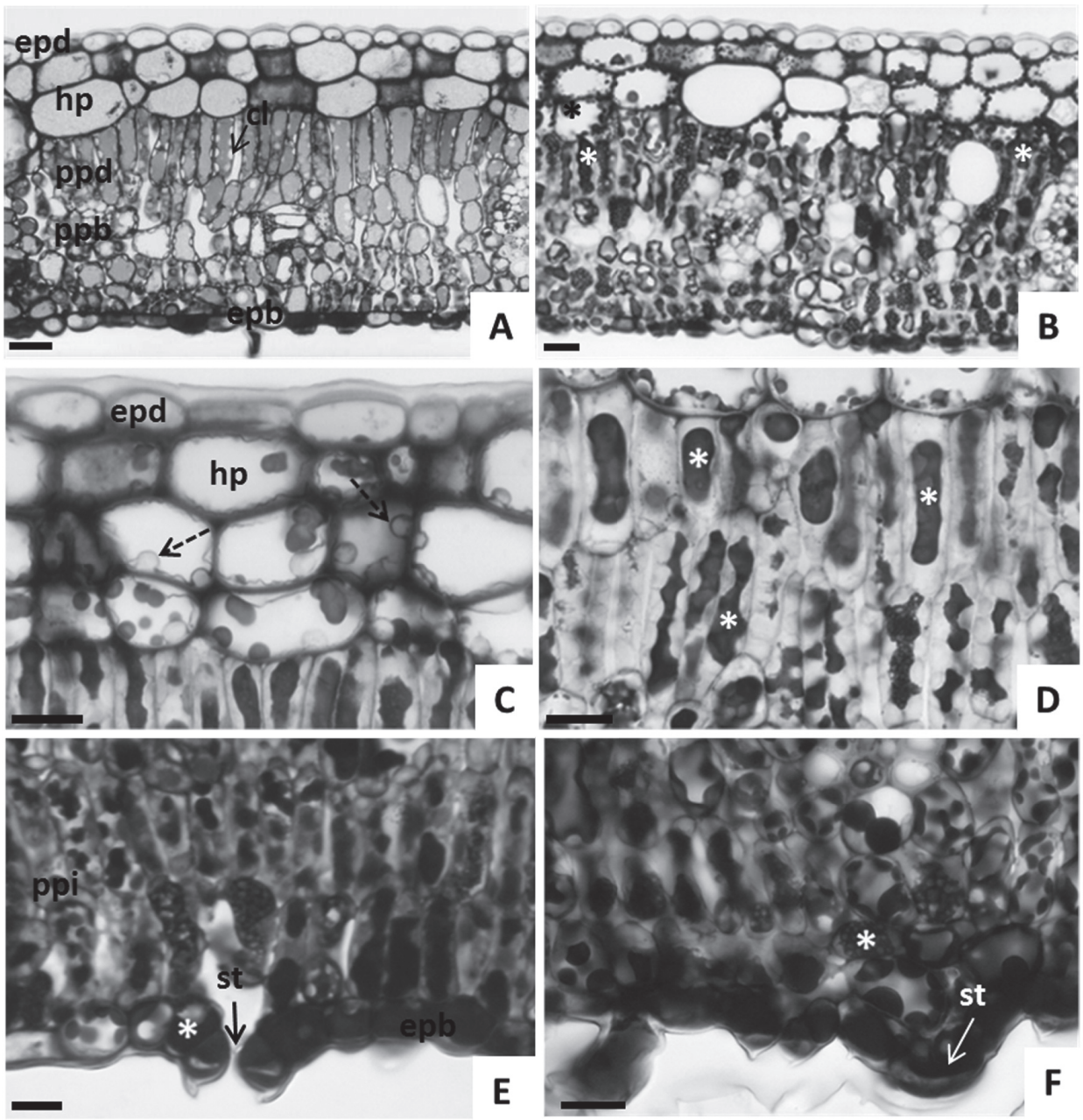

Figure 3. Transversal sections of *Psidium guajava* 'Paluma' leaves. A. Asymptomatic leaves of plants from the greenhouse. B – F. Symptomatic leaves of exposed plants (from outside the greenhouse). C. Hypodermis detail, showing the invagination of the plasma membrane (dashed arrow). D. Detail from upper layers of palisade parenchyma. E. Detail of inferior layers of palisade parenchyma, and sinuosity from cell wall (white arrow) and stomata (arrow). F. Parenchymatic cell growth causing the substomatal chamber occlusion (white arrow). Asterisks indicate the conspicuous accumulation of phenolic compounds. epd = adaxial epidermis; hp = hypodermis; ppd = adaxial palisade cells; ppb = abaxial palisade cells; epb = abaxial epidermal cells; st = stomata. A-B. Bars = 50 µm. C-F. Bars = 20 µm.

It is known that the over absorbed light can cause ROS production, that will damage nucleic acids, proteins, and membrane lipids (Foyer *et al.* 1994). This way, the photoprotector factor, plus the direct antioxidant action, indicates that anthocyanins protect the plant from different stress-causing agents (Neill & Gould 2003).

Unlike other O_3 bioindicator species, such as *Nicotiana tabacum* 'Bel-W3', a sensitive cultivar that responds to the appearance of leaf necrosis due to cell death (Vergé *et al.* 2002), *Psidium guajava* 'Paluma' exhibits pigmentations instead of cell death, so it is more O_3 tolerant than sensitive species like *N. tabacum*, for example. This tolerance can also be associated to the compact arrangement of the leaf tissues of *P. guajava*. Compact arranged mesophyll, a xeromorphic feature (Fahn 1990), makes ROS diffusion into intercellular spaces more difficult and can help avoid large amounts of damage to cells further from the substomatal chamber. The relationship between compactly arranged mesophyll and O_3 sensitivity in plants has already been confirmed by some authors. Ferdinand *et al.* (2000), analyzing two *Prunus*

serotina genotypes, found a higher compact arrangement of the parenchyma cells within the tolerant genotype. The same was verified by Pedroso & Alves (2008) in two tobacco cultivars, one sensitive (*Nicotiana tabacum* 'Bel-W3') and the other tolerant (*N. tabacum* 'Bel-B'), the last with mesophyll comparatively more compact.

Besides the compact arrangement of the tissues, stomata characteristics can limit air pollutant entrance into the leaf. In *P. guajava* 'Paluma' substomatal chamber occlusion was observed due to the growth of parenchyma cells. This fact indicates that the species has mechanisms to efficiently control gas entrance, and the loss of water by transpiration as well. This change was observed in symptomatic and asymptomatic plants kept outside the greenhouse.

In *P. guajava* 'Paluma' seedlings exposed to the environment the tendency for an increase in stomatal density was observed when compared to plants kept away from pollutants (inside the greenhouse with an air filter). Plants exposed during the winter, a period with higher values of AOT, showed a higher stomatal density, while those from the summer (lower AOT) showed a lower density. On plants exposed during spring and autumn, a positive correlation between stomatal density and AOT was verified as well.

On the studied species, the increase of stomatal density can be related to a higher efficiency on the stomata opening-closure mechanism. Relations between O_3 sensitivity, stomata aspects, and stomatal conductance were pointed out by some authors, sometimes, however, with contradictory results. Alves *et al.* (2008) observed an increase of stomatal density in *Eugenia uniflora* exposed to the same local of our study, in comparison to control plants kept in a place little affected by air pollutants. Paoletti *et al.* (2009) evaluating physiological and structural parameters in symptomatic leaves of *Fraxinus ornus,* observed a stomatal conductance reduction due to the changes in stomata or in other epidermal cells. Saitanis & Karandinos (2002) comparing the most sensitive cultivar of *Nicotiana tabacum* 'Bel-W3' with the most tolerant (K63) did not observe significant differences in stomatal density. While Pedroso & Alves (2008), also comparing tobacco cultivars, 'Bel-W3' (O_3 sensitive) and 'Bel-B' (O_3 tolerant), registered a higher stomatal density in the tolerant cultivar.

Therefore we conclude that the anatomical changes found in *P. guajava* 'Paluma' seedlings exposed to an O_3 contaminated environment were more intense in symptomatic leaves, which indicates that these will reflect in visible symptoms. The most evident changes were an increase in phenolic compound content, ROS neutralizers, and the occlusion of the substomatal chamber, characteristics that protect the plant against the effects of O_3. These characteristics and the compactly arranged mesophyll, contribute to why *Psidium guajava* 'Paluma' did not present cell death, a symptom that is usually observed in species sensitive to O_3.

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