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Atmospheric Absorption of Fluoride by Cultivated Species. Leaf Structural Changes and Plant Growth

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Abstract Fluoride (F) is an air pollutant that causes phytotoxicity. Besides the importance of this, losses of agricultural crops in the vicinity of F polluting industries in Brazil have been recently reported. Injuries caused to plant leaf cell structures by excess F are not well characterized. However, this may contribute to understanding the ways in which plant physiological and biochemical processes are altered. A study evaluated the effects of the atmospheric F on leaf characteristics and growth of young trees of sweet orange and coffee exposed to low (0.04 mol L^{-1}) or high (0.16 mol L^{-1}) doses of HF nebulized in closed chamber for 28 days plus a control treatment not exposed. *Gladiolus* and ryegrass were used as bioindicators in the experiment to monitor F exposure levels. Fluoride concentration and dry mass of leaves were evaluated. Leaf anatomy was observed under light and electron microscopy. High F concentrations (~180 mg kg^{-1}) were found in leaves of plants exposed at the highest dose of HF. Visual symptoms of F toxicity in leaves of citrus and coffee were observed. Analyses of plant tissue provided evidence that F caused degeneration of cell wall and cytoplasm and disorganization of bundle sheath, which were more evident in *Gladiolus* and coffee. Minor changes were observed for sweet orange and ryegrass. Increase on individual stomatal area was also marked for the *Gladiolus* and coffee, and which were characterized by occurrence of opened ostioles. The increased F absorption by leaves and changes at the structural and ultrastructural level of leaf tissues correlated with reduced plant growth.

Keywords Air pollution · Leaf analysis · Anatomy · Bioindicator · *Citrus* · *Coffea*

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1 Introduction

The rapid technological advance of the modern world resulted in increased environmental pollution and has become a global concern due to the large volume of emissions of gases and particles that are currently occurring in the atmosphere. Among the air pollu-

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tants, fluoride (F) is considered one of the most important in the atmosphere along with the sulfur dioxide (SO_2) and ozone (O_3), which can cause phytotoxicity to susceptible plant species at very low atmospheric concentrations (Weinstein 1977; Jha et al. 2008).

The presence of F in the atmosphere is related to emissions of highly reactive compounds such as hydrofluoric acid (HF) and silicon tetrafluoride (SiF_4) after heating rocks and earth materials at high temperatures, what is conducted mostly by incinerators, cast aluminum and other nonferrous metals, producers of superphosphate and other mineral fertilizers, glass and ceramics (Arndt et al. 1995; Oliva and Figueiredo 2005). Despite measures recommended by Cetesb—the state environmental protection agency—to control F emissions from industries in Brazil, based on the use of filters for solid particles and washing columns for avoiding losses of gaseous forms, reduced growth of cultivated plants and production losses were recently assessed for corn (Fortes et al. 2003), tree species (Klumpp et al. 2002; Sant'Anna-Santos and Azevedo 2007) and sugar cane (Otto et al. 2007). Often, our laboratory for plant nutrition analyses has received samples of plants of other crops with suspected toxicity caused by pollution with fluorine.

Given this context of pollution, the adverse impact on the environment must be assessed in view of the need for control measures and appropriate corrections for the maintenance of different ecosystems, including those in agricultural production. Biomonitoring has been used in recent decades to study air quality and effects of pollutants on plant species. Ryegrass (*Lolium multiflorum* Lam.) and cultivars of *Gladiolus* spp. are commonly used in monitoring studies, the first as a tolerant species and the second a susceptible one to contamination of atmospheric F (Fornasiero 2001; Weinstein and Davison 2003). Based on these characteristics, one can take advantage and use such bioindicators in studies under controlled conditions in which the development of studies detailing the response of plants to environmental contamination is more appropriate.

The atmospheric F is absorbed by the leaves of plants, mainly through the stomata and to a lesser extent, through the cuticle and lenticels (Braen and Weinstein 1985). Besides visible injuries, fluoride causes significant physiological and biochemical disorders, which consist of changes in cellular respiration (Miller and Miller 1974), reduction in photosynthetic pigment concentration (Lal and Ambasht 1981), inhibition of photosynthesis (Darral 1989), changes in composition and membrane function (Façanha and de Meis 1995), carbohydrate metabolism (Asthir et al. 1998), and anatomical and ultrastructural disorders of cells (Fornasiero 2003; Sant'Anna-Santos and Azevedo 2007). Furthermore, studies related to cell and tissue anatomy has been scarce, however, may contribute to understand the ways in which physiological and biochemical processes are altered.

The aim of this study was to evaluate the effects of the exposure of two species of economic interest to atmospheric fluoride on F accumulation in plant tissue and anatomical leaf characteristics that affect plant growth by correlating absorption levels of sensitive and tolerant bioindicator species to toxicity to the element.

2 Material and Methods

2.1 Plant Material and Experimental Design

Young trees of sweet orange [*Citrus sinensis* (L.) Osbeck] and coffee (*Coffea arabica* L. cv Obatã IAC 1669–20), ryegrass (*L. multiflorum*) and *Gladiolus* spp. var. White Goddess were placed in plastic pots with capacity of 5–10 L, with soil collected from the arable layer of a Ferralsol under grassland [MO=34 g dm^{-3}; pH ($CaCl_2$)=5.2, CEC=87.9 $mmol_c$ dm^{-3}, base saturation (V)=61%, total F=203 mg kg^{-1}, and clay=300 g kg^{-1}]. The pots were fertilized with macro and micronutrients with application of nutrient solutions to ensure adequate growth, considering the nutrient content in plant dry matter and the initial soil chemical characteristics determined according to van Raij et al. (2001). Pots were daily irrigated to replace the water lost.

The experiment was conducted in a greenhouse in a completely randomized design with treatments defined by the combination of plant species and two intensities of exposure to atmospheric F (low and high) applied by a misting system with 0.04 and 0.16 mol L^{-1} HF solutions, plus a control not exposed, beginning the summer between November and December 2009. Estimated atmospheric F con-

centrations were 0.065 and 0.260 mmol m^{-3} of F, respectively, for the low and the high exposures in the semi-open nebulization chamber used in this study and described below. Dry mass production of plants and F concentration in the leaves were subjected to analysis of variance and treatment effects were evaluated by mean comparison using the GLM mode of the statistical package SAS® (SAS Institute, Inc. 1996).

2.2 Controlled Exposure to Fluoride

The plant exposure to F was conducted in a closed chamber (2.4×1.5×1.7 m) with capacity for eight pots, built with a frame made of PVC pipes with 5 cm in diameter, and with sides, top and bottom closed with anti-UV transparent polyethylene film, 150 μm thick. In the upper chamber was attached a ultrasonic nebulizer (mod. compact Pulmosonic Star, Soniclear, São Paulo, Brazil), responsible for homogeneous nebulization of fluorine in the form of HF droplets <4 μm in diameter. At the top of the chamber and below the nebulizer, a screen of fine mesh (0.87× 0.30 mm) was installed to form a small anteroom and to prevent damages to the leaves by direct contact of any microdroplets formed during the nebulization of HF. Immediately below the screen, two small fans, 18-cm in diameter with three blades, were placed in diagonal position, to move air at low speed (800 rpm) and spread evenly the F inside the chamber. Tests were conducted with alkali collecting plates to verify the homogeneity of distribution of F in the chamber, following the procedure recommended by the Companhia Ambiental do Estado de São Paulo (Cetesb 1998).

The plants in the F treatments were exposed to the contaminated environment during three non subsequent days in a single week, for a period of 30 min after exposure and remained in the chamber for another 60 min until removal to the external environment. A volume of 10 mL of HF (0.04 or 0.16 mol L^{-1}) was placed in the plastic container of the nebulizer for every application event up to 28-days after begining the experiment. The level of exposure of studied plants was monitored by observing the appearance of visual symptoms of F toxicity on the leaves of *Gladiolus* and the maintenance of integrity of those on the ryegrass, which can accumulate F without showing any injury caused by its excess.

2.3 Light Microscopy

After 28 days of treatment, two fully developed and photosynthetically active leaves of both bioindicator and citrus and coffee plants were used for studies under light microscopy. Tissue samples were collected from the middle third of the leaf with approximate 4×5 mm between 9:00–10:00 am and fixed in Karnovsky solution (Karnovsky 1965). After 12 h fixation, dehydration of the sampled material was conducted in increasing ethanol series [30, 50, 70, 90, 100 (three times)] for 15 min in each concentration. It was then infiltrated with acrylic resin glycol methacrylate (Leica®) and 100% ethanol at a ratio of 1:1 and remained for about 5 h in refrigerator. Then, it was performed infiltration in pure resin for one night, also with refrigeration and then brought to polymerization temperature (Von Ruetze and Schmitt 1986). The blocks obtained were cut on a manual rotary microtome (Spencer Lens, USA) with C-type stainless steel knife (Leica, Wetzlar, Germany) with 5.0 μm thick. The slides were stained with toluidine blue 0.05% at pH 2.6 (Feder and O'Brien 1968) after drying, were mounted on glass slides with mounting medium as Entellan®. Analyses were performed by light microscope (mod. Zeiss Axioskop 2, Zeiss, Berlim, Germany) and images were recorded as digital files through a camera (mod. MRc, Zeiss, Berlim, Germany) coupled to the microscope.

2.4 Scanning Electron Microscopy

The samples used for this step were simultaneously collected from the same region previously determined, with dimensions of approximately 3×4 mm. After fixation, as conducted for light microscopy, it was performed post-fixation for 1 h with OsO_4 1% in cacodylate buffer 0.05 mol L^{-1}. The sample was then submitted to dehydration in increasing series of acetone [30, 50, 70, 90, 100 (three times)] and immediately taken to drying at critical point (Balzers CPD 30) with CO_2 used as a media. The dried samples were sticked on stubs, exposing them to both sides of the leaf surface and taken to metalization with gold plating (mod. MC 50, Balzers, Germany) and then analyzed under a scanning electron microscope (mod. LEO 435 VP, Cambridge, England). Obtained images were stored in digital files. A total of 15 images were taken of different leaf samples for each

plant species for measurements of the length and width of 50 stomata per treatment to determine the approximate area occupied by them. These measurements occurred at random, looking for homogeneous stomata, which was determined through a formula for the area of an ellipse (area = π*a*b, where a is the distance from the center to a vertex and b is the distance from the center to a co-vertex).

2.5 Dry Mas Determination and Fluoride Content in Plant Leaves

Immediately after sampling leaves for microscopy analyses, plants were separated into leaves (older than 4–6-months, mature 2–4-month-old, and fully expanded leaves younger than 2-months) and stems, and dried at 65°C for 48 h. Leaf samples were weighed, ground and subjected to chemical analysis for determination of total F concentration by alkaline fusion based on the method described by Frankenberger et al. (1996) and adapted in our laboratory. The modification refers to the preparation of the total ionic strength adjustment buffer solution (TISAB) and the use of a standard curve of F in place of the standard addition method for the calculation of F in the sample. Five hundred milligrams of dried and ground samples were weighted into nickel crucibles, moistened with deionized water and mixed with 6 mL of 17 mol L^{-1} NaOH solution. The crucible was placed in an oven at 150°C for 1 h, which was removed after solidification of the mixture. Then, the crucible was transferred to a mufle furnace at 300°C and the temperature was gradually increased to 600°C to allow the sample to melt for 30 min. After cooling, 10 mL of di-water was added and the fusion was re-heated until dissolution. Fifteen milliliters of 6 mol L^{-1} HCl was slowly added to the mixture, stirring to lower the pH to 8–9, and then transferred to a volumetric flask making up a final volume of 50 mL with di-water. The resulting solution was filtered through Whatman filter paper (No. 40). An alliqot of 25 mL of the filtrate was transferred into a 150 mL polyethylene flask and added 25 mL of TISAB. The pH was adjusted to 5.2. Blanks were also prepared. Samples were measured using a fluoride-sensitive electrode (mod. 60502.150, Metrohm, Switzerland) coupled to an ionmeter (mod. 692, Metrohm, Switzerland) against a stardad curve. To assure the quality of the F analyses standards of timothy grass (Ref. 2695, National Institute of Standards and Technology, NIST, Gaithersburg, US) containing 64± 8 mg kg^{-1} (low) and 277±27 mg kg^{-1} (high) of the element was used as controls.

3 Results and Discussion

3.1 Fluoride Concentration in Leaf Tissues

Control plants not exposed to HF in the nebulization chamber showed leaf F concentration of about 30 mg kg^{-1}, whereas it increased proportionally to the dose of nebulized HF for all four plant species up to 180 mg kg^{-1} (Fig. 1). Background fluoride concentrations in plant leaves are in the range of 5–15 mg kg^{-1} (Franzaring et al. 2006), slightly below the concentrations observed in the present study. According to the literature, F levels in polluted areas close fertilizer producing plants in Brazil may exceed 600 mg kg^{-1} in plant leaves (Klumpp et al. 1996; Domingos et al. 2003).

Ryegrass showed no visible symptoms of F toxicity even at levels of ~180 mg kg^{-1} leaves (Fig. 1a). For plants of *Gladiolus*, concentration less than 75 mg kg^{-1} F in the leaves, independent of age, was sufficient to cause toxicity and damage to their development (Figs. 1b and 2a–d). Studies suggest that sensitive plants may be injured when the leaf concentration exceeds 30 mg kg^{-1} F (Treshow and Anderson 1989). For coffee and citrus species, the concentration of ~100 mg kg^{-1}, observed with higher F exposure, caused toxicity to young leaves (Figs. 1c, d and 4e–j). The penetration of the anion in the apoplast of young tissues is facilitated by smaller amounts of cuticle, epicuticular wax and by the increased presence of pectins, which associated with more vigorous vegetative growth and high rates of water movement favored higher accumulation of F in the apices and margins of terminal leaves (Leece et al. 1982; Treshow and Anderson 1989). On the other hand, greater concentrations of F in old tissues would be explained by longer exposure periods they are subjected, expressing a cumulative effect (Fig 1b–d).

3.2 Symptoms of Floride Toxicity in Plant Leaves

Toxicity caused by F exposure occurred in new leaves of *Gladiolus* and coffee and in mature leaves of citrus (Fig. 2). The plants of *Gladiolus* firstly expressed

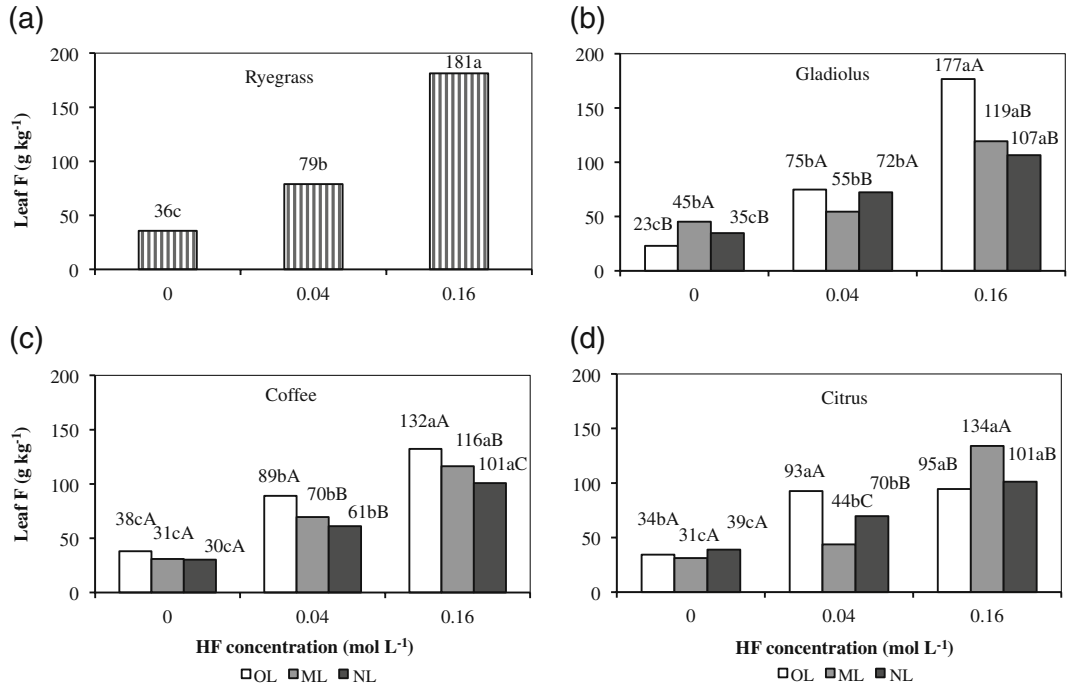


Fig. 1 Fluoride concentrations in leaves of ryegrass, and in old leaves (*OL*, >4–6-month-old), mature leaves (*ML*, 2–4-month-old), and new leaves (*NL*, fully expanded leaves younger than 2-month-old) of *Gladiolus*, coffee and citrus. Means followed by same small letters do not differ among HF doses and means followed by same capital letters do not differ within HF doses at 5% probability by the Duncan test

visual symptoms of stress to the pollutant, which appeared after the second day of exposure in the nebulization chamber with the higher dose of F and, after the fourth day, when plants were exposed to the lower one. Chlorosis started at the tip of the new leaves, and spread to the intercostal areas that became pale necrosed; lesions progressed into the leaf blade (Fig. 2a, b). Early in the second week of nebulization all leaves of *Gladiolus* plants showed evidence of F toxicity (Fig. 2c, d). Such description of symptoms was firstly made by McNulty and Newman (1957); appearance and progression of damage due to F were similar to those observed in this experiment.

In citrus, we observed the occurrence of leaf chlorosis after the third week of exposure with the highest dose of HF (0.16 mol L^{-1}) and which started only at the extremities and spread along the margins and into the leaf blade between the veins, and as the F concentration in leaves increased, the yellowing intensified and became a pale colored necrosis (Fig. 2e–g) similarly as described with *Psidium guajava* L. plants (Moraes et al. 2002). Additionally, Bryan (1957) found that the early stages of chlorosis by F in plants resemble the initial stages of boron toxicity, but when the effect is due to boron a resinous excretion may appear under the surface of leaves, which does not occur with toxicity by F. Also according to the author, the element affects leaf size, especially those new which later show tissue burning. In coffee plants, blackened spots appeared on petiole and young leaves in the same period (Fig. 2h, i), also initiated by the margins and which spread to the internal surface of the leaf blade (Fig. 2j). Fluoride injuries occur only after a certain period of accumulation in the plant tissue reaching phytotoxic levels, what was not observed with reygrass, which did not show any visual symptoms of injury.

In plants of *Hypericum perforatum* L., the total quantities of pigments were reduced leading to the emergence of necrotic tissue in some parts of the leaf (Fornasiero 2003). Therefore, phytotoxic effects on leaf development are probably due to the action of F on the biosynthesis of photosynthetic pigments, which induce disturbances in the early stages of carotene synthesis and porphyrin and, therefore, the

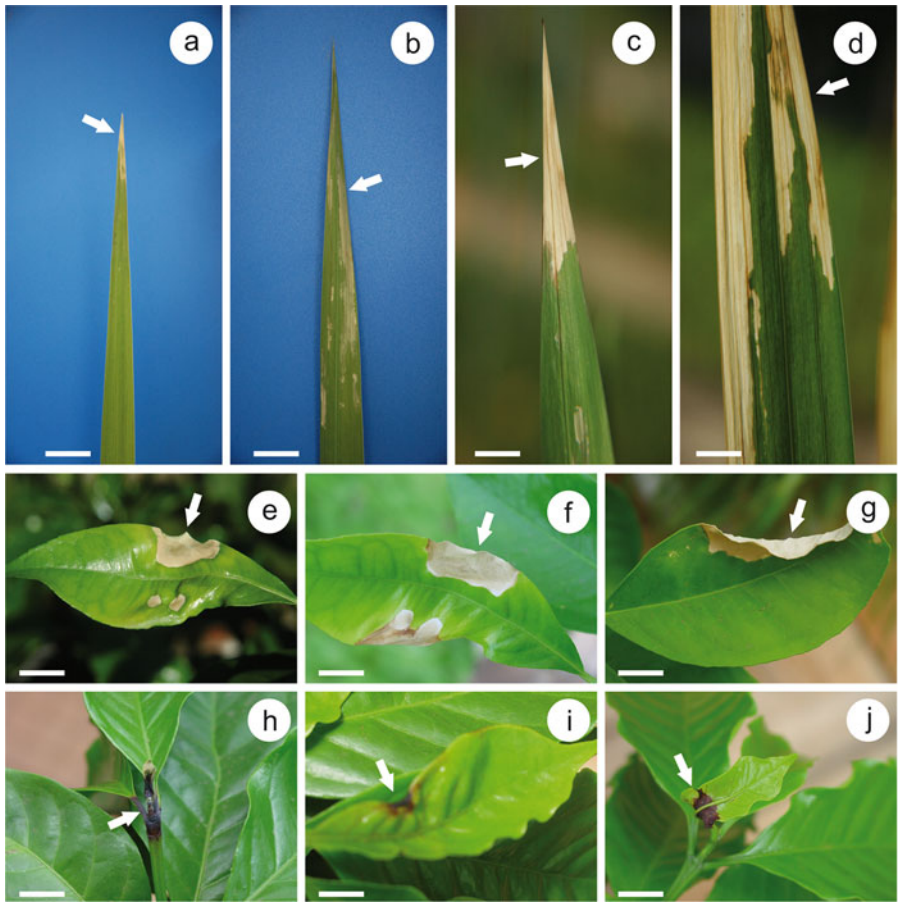


Fig. 2 Symptoms of leaf injury in plants caused by fluoride atmospheric pollution (after exposure to 0.16 mol L^{-1} HF nebulized in closed chamber); **a**–**d** (*bar*=0.2 cm): *Gladiolus*, symptoms starting at the leaf tip (*arrow*) and progressing into the leaf blade (*arrow*); **e**–**g** (bar=0.5 cm): citrus, symptoms starting from leaf margins (*arrow*) and progressing into the leaf blade (*arrow*); **h**–**j** (*bar*=0.2 cm): coffee, stem and leaves with blackened necrotic spots (*arrow*) progressing into the leaf blade

chlorophyll (Oliva and Figueiredo 2005). Also, it is likely that the leaf injury is associated to the change in lipid matrix and its relation to the soluble proteins of the membrane, which affects metabolic function and alters the activity of associated enzymes, such as H^+-ATPases (Façanha and de Meis 1995).

3.3 Light Microscopy

Leaves of ryegrass that were not exposed to the contaminated environment by F showed stratified epidermis with cells of varying sizes (Fig. 3a). Furthermore, in the adaxial side in the region of vascular tissue, larger cells were observed. In this region, the mesophyll presented parenchyma cells with laxo arrangement and concentrated in peripheral areas adjacent to the epidermis. The vascular bundle showed arrangement characteristic of grasses with cells of the xylem and phloem surrounded by a sheath extended to the epidermis through a single layer of cells (Esau 1976). In leaves of *Gladiolus*, the cells showed organization with a compact arrangement, since intercellular spaces in the mesophyll were not observed (Fig. 6c). The chlorenchyma cells showed elongated shape with larger wall parallel to the leaf surface. The vascular bundle also showed a sheath with isodiametric cells that did not bind to the epidermis and bicolateral phloem without the formation of typical design of grasses (Esau 1976).

In the cross-section of leaves of coffee plants under the control treatment, the palisade parenchyma was shown to be composed of a single layer of cells

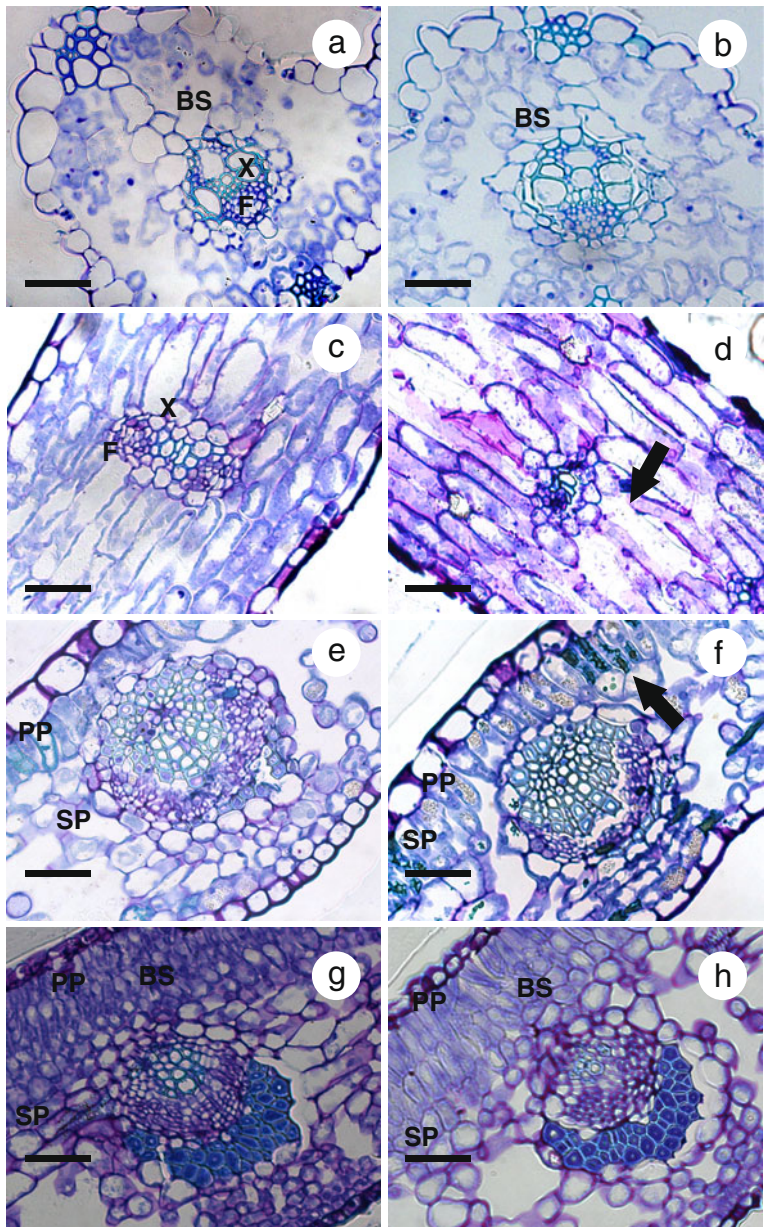


Fig. 3 Light microscopy of transverse sections of leaves from plants affected by atmospheric fluoride (not exposed or exposed to 0.16 mol L^{-1} HF nebulized in closed chamber); **a** ryegrass not exposed to F (*bar*=25 μm), chlorenchyma with laxo arrangement, and bundle sheath prolonged till the adaxial epidermis; **b** ryegrass exposed to F (*bar*=30 μm), changes in cells of palisade and spongy parenchyma, epidermis slightly damaged; **c** *Gladiolus* not exposed to F (*bar*=19 μm), chlorenchyma with compact arrangement, cells with elliptical shapes, with wide wall parallel to the epidermis; **d** *Gladiolus* exposed to F (*bar*=25 μm), cell degeneration (arrow), disorganization of the bundle sheath cells, and changes in the structure of cell wall; **e** coffee not exposed to F (*bar*=17 μm), showing layer of palisade parenchyma and chlorenchyma irregularly arranged; **f** coffee exposed to F (*bar*=25 μm), cytoplasmic degeneration and accumulation of phenolic substances; **g** citrus not exposed to F (*bar*=19 μm), palisade parenchyma consists of 8–10 layers, spongy parenchyma with larger cells, which become smaller and more compressed near the lower epidermis; **h** alteration in the palisade parenchyma and loosening of parenchyma. Legend: bundle sheath (*BS*), palisade parenchyma (*PP*), spongy parenchyma (*SP*), xylem (*X*), and phloem (*P*)

beneath the adaxial epidermis, arranged with their long axis perpendicular to the epidermis (Fig. 6e). Following the palisade it was observed the spongy parenchyma, composed of seven to eight layers of cells arranged randomly, with large intercellular spaces. Similarly, in citrus, thick leaf cuticle with thick layer of wax was present (Fig. 6g). The palisade parenchyma in two compact layers contrasted with the spongy loose one. The spongy parenchyma composed of 9–10 layers of cells contained very large intercellular spaces. The vascular bundle showed up surrounded by a sheath of parenchyma cells.

The anatomical injuries in leaves of ryegrass exposed to higher dose of HF consisted of little loss in cell shape, retraction of the spongy parenchyma, with some degeneration of the cytoplasm (Fig. 3b). The epidermis was mildly impaired, accordingly to observed irregularity of the cells. These damages were much lower compared to other species, especially the *Gladiolus* and coffee, what confirmed its tolerance to F. Furthermore, the exposure to lower dose of HF caused no apparent effect on cell structures for ryegrass.

In the same treatment with higher HF, *Gladiolus* leaves showed degenerated cells in the region with the onset of vascular collapse, with disorganization of the bundle sheath cells, showing altered shape and size (Fig. 3d). It was also found that the arrangement between the cells became loose and showed intense cytoplasmic degeneration and collapse of the wall in some cells (arrow), creating voids that were not observed in healthy plants.

Similarly, in coffee leaves was noted increased staining intensity, mainly in the cytoplasm (Fig. 3f). These results suggest that cytoplasmic degeneration occurred due to oxidation of some substances and the accumulation of phenols. The F is deposited near the xylem endings, which explains the accumulation of phenols in this region (arrow) due to the action of the ion, and is interpreted as a defense mechanism of plants (Vaughn and Duke 1984) and has been reported in other plant species also exposed to F (Chaves et al. 2002; Sant'Anna-Santos and Azevedo 2007). Furthermore, the vascular bundle showed intensely degenerated, with the phloem and xylem separated, forming spaces that were not observed in control plants. It was noted also that separation occurs between cells of the xylem, suggesting that the middle lamella has been degenerated.

In citrus leaves there were minor changes in the palisade parenchyma (Fig. 3h). There was some separation between cells likely because of damages to the middle lamella cell responsible for cementation. Similar effects on leaf structure was found by Fornasiero (2003) in plants of *H. perforatum*, placed at different distances (1, 0.2, 0.5, and 2.5 km) of an emitter source of atmospheric F.

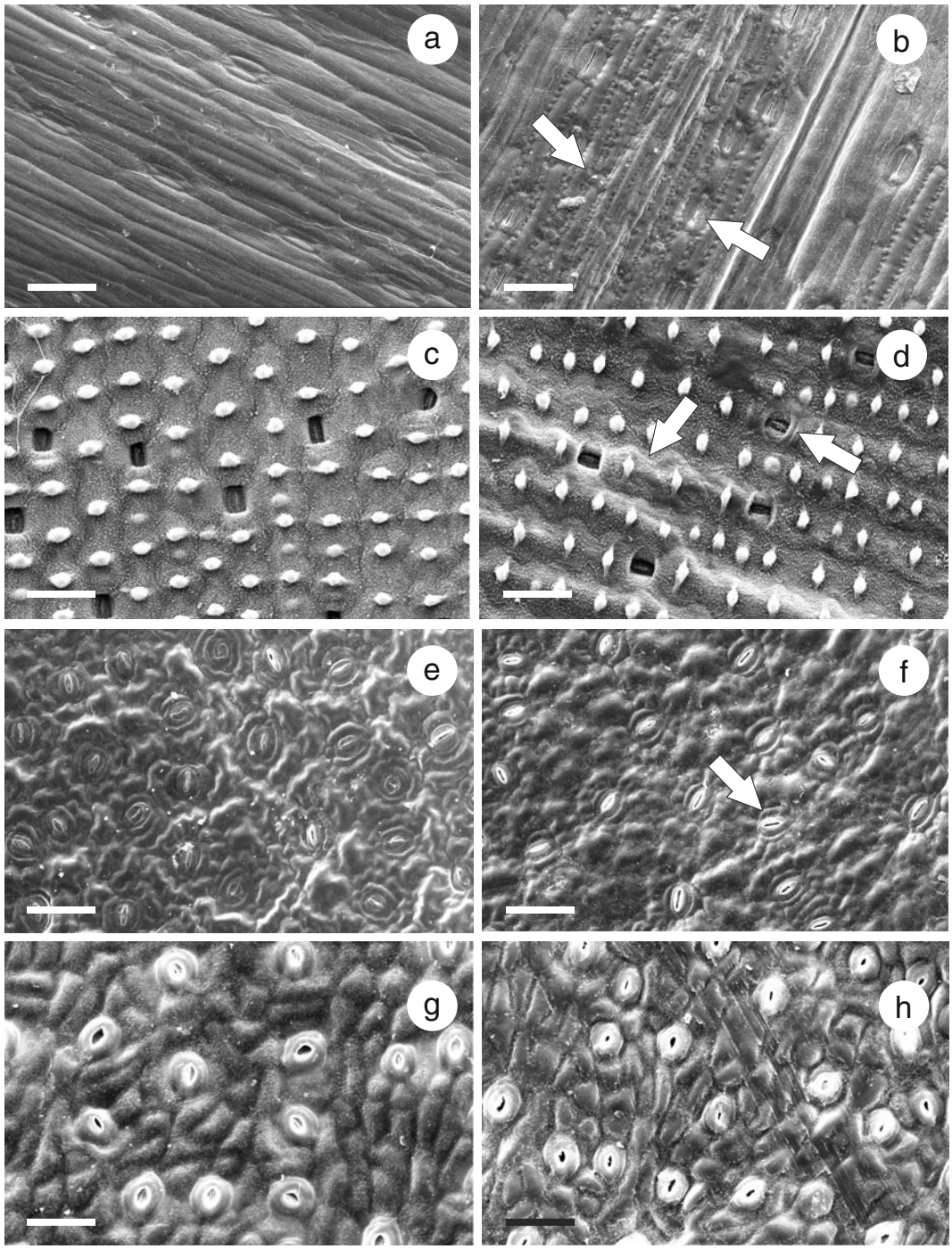
The diminution of density and loosening of the arrangement of cells evidenced by less staining of the parenchyma and observed in plants exposed to high doses of F are possibly associated with the disorder caused on the Ca in the plant (Allmendinger et al. 1950; Ben Abdallah et al. 2006). Garrec et al. (1974) demonstrated that the normal gradient of Ca^{+2} in plants is interrupted when they are exposed to environmental pollution with F, which according to Weinstein and Davison (2003) is related to the ability of F to complex Ca^{+2} cell wall middle lamella, since the F absorbed by the leaf reacts Ca in the plant to form CaF_2, which is relatively insoluble.

Furthermore, calcium is essential in physiological processes such as growth of meristematic tissues, light perception, action of growth regulators and stomatal regulation (Sanders et al. 2002), which possibly were affected indirectly by plant exposure to atmospheric F.

Fig. 4 SEM of leaves from plants affected by fluoride atmospheric pollution (not exposed or exposed to 0.16 mol L^{-1} HF nebulized in closed chamber); **a** ryegrass not exposed to F (*bar*=64 μm), guard cells and stomata closed and intact epidermis; **b** ryegrass exposed to F (*bar*=39 μm), damaged guard cells, increased deposition of wax in the epidermis, and stomata closed; **c** *Gladiolus* not exposed to F (*bar*=58 μm), abundant trichomes located on the lower epidermal cells, and stomata closed; **d** *Gladiolus* exposed to F (*bar*=10 μm), sinuosity of epidermal cells, increase in stomata, which remained open; **e** coffee not exposed to F (*bar*=37 μm), epidermal cells with intact and closed stomata; **f** coffee exposed to F (*bar*=35 μm), deformation of epidermal cells and stomata opened and damaged; **g** citrus not exposed to F (*bar*=35 μm), epidermis with wax ornamentation, stomata located on the rise and expansion ostiole protected by the wall and cuticle; **h** citrus exposed to F (*bar*=47 μm), higher sinuosity of epidermal cuticular strengthening of ostiole opened and damaged

3.4 Scanning Electron Microscopy

With the scanning electron microscopy (SEM) it was observed that control plants of ryegrass showed leaf epidermis formed by elongated cells of variable sizes (Figs. 4a and 5a). Stomata generally formed a linear



arrangement parallel to the epidermal cells. Cells remained intact and without ornamentation with wax. The stomata were located on the same level of the epidermis. In the case of the *Gladiolus*, leaf epidermis was different from that of ryegrass (Figs. 4c and 5c), because the presence of tector trichomes shaped as a pedunculated sphere and stomata inserted in cavities with rectangular outline at a level below the epidermis. The cells showed various shapes, some elliptical and others tending to rectangular. The distribution of epicuticular wax was abundant in epidermal cells, which was presented in

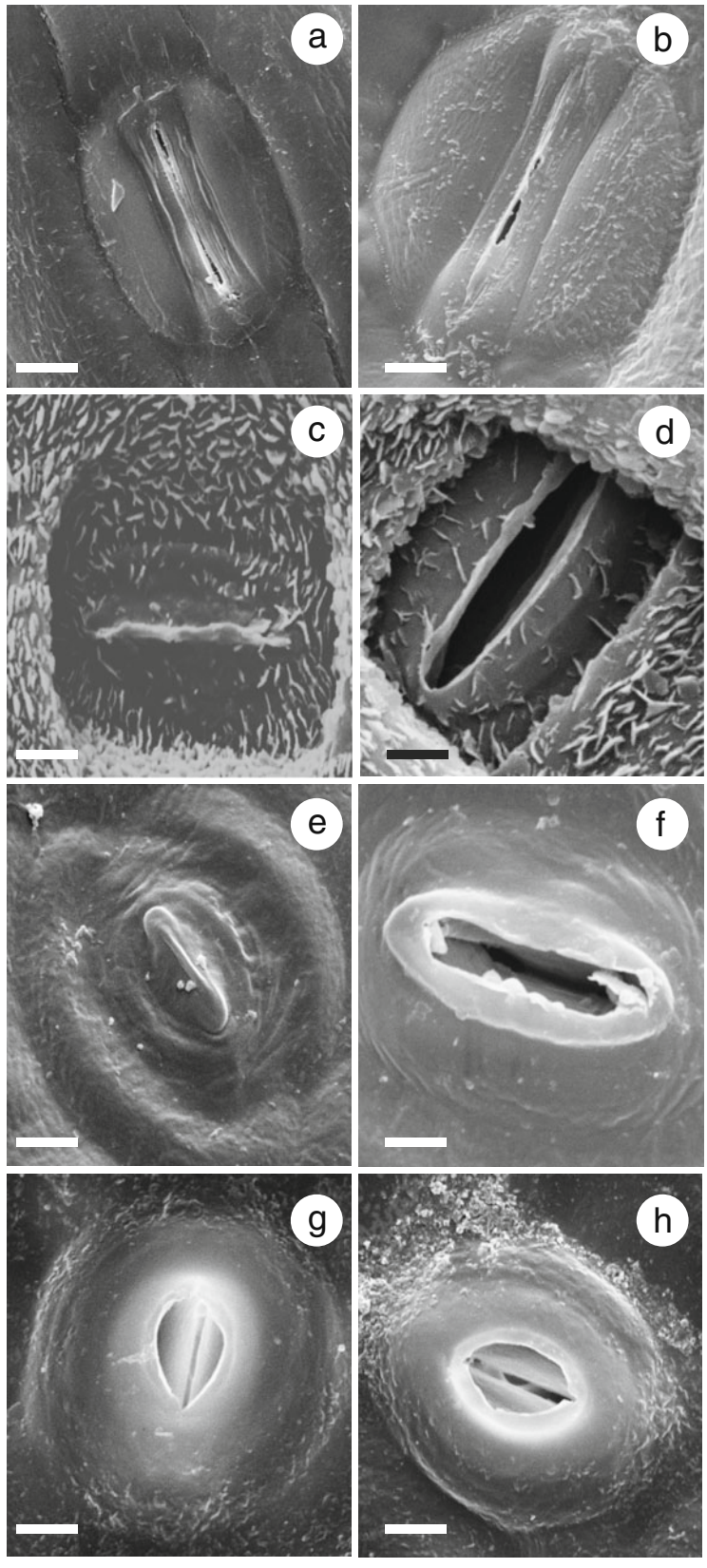


Fig. 5 SEM of leaves from plants affected by fluoride atmospheric pollution (not exposed or exposed to 0.16 mol L^{-1} HF nebulized in closed chamber); **a** ryegrass not exposed to F (*bar*= 2 μm), closed ostiole; **b** ryegrass exposed to F (*bar*= 3.14 μm), ostiole remained closed; **c** *Gladiolus* not exposed to F (*bar*=1.3 μm), deposition of wax on small plates; **d** *Gladiolus* exposed to F (*bar*=0.94 μm), severe increase in the distribution of epicuticular wax on vertical plates; **e** coffee, not exposed to F (*bar*=7 μm), cuticular strengthening suberized on ostiole; **f** coffee exposed to F (*bar*=1 μm), cuticular strengthening suberized on opened and damaged ostiole; **g** citrus not exposed to F (*bar*=2.1 μm), cuticular strengthening suberized on closed ostiole; and **h** citrus exposed to F (*bar*=1.8 μm), cuticular strengthening suberized on opened ostiole

the form of plates. The distribution of stomata in the epidermis was randomized.

On the leaf epidermis of control plants of coffee, cells were irregularly shaped with sinuous outline (Figs. 4e and 5e). The junction region of the cells formed furrows that highlight the cell contour. Stomata appeared randomly distributed and at the same level of the epidermal cells.

The epidermis of citrus leaf also showed cells with varying shape and regular contour (Figs. 4g and 5g). The central portion of the cells was raised in relation to cell junction region. The distribution of epicuticular wax was presented with ripples in the cell wall. The stomata were also raised above the epidermis.

Observations made in plants of ryegrass treated with the higher dose of HF demonstrated that probably the amount of wax on the leaf surface become more prominent due to cell wall degeneration, which caused deformation of the surface (Fig. 4b; arrow) with little deformation of the guard cells (Fig. 5b), which kept the ostiole closed.

For the *Gladiolus*, the degeneration of epidermal cells formed depressed regions where it was difficult to visualize the boundary region between them (Fig. 4d; arrows). This also caused deformation of cells around the stomatal cavity. The stomata were open suggesting a possible impairment on the opening and closing regulation mechanism as observed in *Gallesia integrifolia* (Spreng.) Harms, a tropical species subjected to simulated acid rain (1.0 mol L^{-1} H_2SO_4; pH=3.0) (Sant'Anna-Santos et al. 2006). The authors pointed out that such impairment exposes the more internal plant tissues to the direct effects of pollution. In those plants, photosynthesis decrease is expected after damages related to stomatal movement, light interception and CO_2 fixation (Moraes et al. 2002), what explain reduced plant growth, as measured by leaf dry mass of *Gladiolus* and coffee, in the present study.

Similar damages were reported for leaf samples of *Spondias dulcis* Sol. ex Parkinson—a sensitive species to F pollution—under SEM, which were exposed either to simulated acid rain (Sant'Anna-Santos et al. 2006) or rain fluoride (2.5 10^{-4} mol L^{-1} KF) (Sant'Anna-Santos and Azevedo 2007). According to these authors, the effect of F on leaf surface caused stomatal outer ledge rupture. Reduction of leaf thickness was also associated with impairment of the mesophyll, which in the control treatment (no F) presented five to six cell layers of spongy parenchyma contrasting with only three to four layers of F treated plants. On the contrary, tolerant plants may present increased specific leaf area in more pollutted areas (Kardel et al. 2010).

In coffee, it was noted the same characteristic of degeneration of the cell wall, leading to the formation of depressed regions, as in Sant'Anna-Santos and Azevedo (2007), which were not observed in control plants (Fig. 4f; arrow). The ostiole of the stomata was also opened (Fig. 5f).

Peeling of epicuticular wax also occurred in citrus probably because of losses of integrity of the leaf tissue (Fig. 4h). This might explain the fact that leaves of plants under the highest dose of HF were less coriaceous to the touch (Fig. 5h).

The area of the stomata of *Gladiolus* and coffee was affected by F exposure in the nebulization chamber with 54% and 25% increase, respectively, when plants were nebulized with 0.16 mol L^{-1} HF compared to healthy control ones (Table 1). These later correlated with higher stomatal conductance of same plants (data not shown). Increase on stomatal pore surface appear as a morphometric characteristic that also turns plants prone to pollution stress by causing low adjustment of gas exchange and consequent favoring entrance of pollutants through stomata (Alves et al. 2008; Kardel et al. 2010).

3.5 Dry Mass Production

The production of total leaf dry weight of ryegrass did not significantly change between control plants and those exposed to HF (Fig. 6a). On the contrary, the effect of atmospheric F caused reduction of about 25% in the production of leaf dry matter of *Gladiolus* even at the lowest F dose compared to the control ones not exposed to F (Fig. 6b). For coffee, the reduction in leaf dry weight was 36% at the highest F dose (Fig. 6c). Probably significant amount of carbon was not used to the normal processes of growth leading to a reduction in leaf dry mass production. These were in line with increased changes observed at the structural and ultrastructural level of leaf tissues of these two species as described earlier. It is assumed that carbon was diverted to production of phenolic compounds produced by plants, as anthocyanins, causing to coincide with the appearance of reddish leaves of *H. perforatum* plants (Fornasiero 2003). The citrus showed no change in dry mass of leaves in the presence of F (Fig. 6d). Furthermore, according to Brewer et al. (1969), the

Table 1 Effect of atmospheric fluoride on leaf stomatal characteristic of plants exposed or not to HF nebulization in closed chamber

HF concentration (mol L^{-1})	Ryegrass	*Gladiolus*	Coffee	*Citrus*
	Stomata area (μm^2)			
0	810a	138b	888b	376a
0.16	856a	301a	1,187a	345a
Mean	833	220	1,038	361
F test	0.19ns	17.42[a]	5.28[b]	0.67ns
CV, %	17.5	25.1	17.6	15.0

Means followed by same letter in columns for each species are not statistically different at 5% probability by the Duncan test

ns not significant

[a] Significant at 1%

[b] Significant at 5%

effect of the gradual accumulation of F in citrus leaves is more harmful in the flowering and fruit production stages. Therefore, it could explain the limited injury caused by F exposure on the later.

4 Conclusion

The results of this study confirmed that plants exposed to HF nebulized in a closed chamber showed increased F concentrations in leaf tissue. Overall, high F concentrations (~180 mg kg^{-1}) were found in leaves of plants exposed to the highest dose of HF. Control of exposure of plants under contaminated environment was adequate for study purposes as revealed by visual toxicity symptoms observed in leaves of the sensitive *Gladiolus* at 0.04 mol L^{-1} of HF and, by absence of symptoms in the tolerant ryegrass bioindicator at 0.16 mol L^{-1} of HF. Light microscopy analyses of plant tissue provided evidence that F

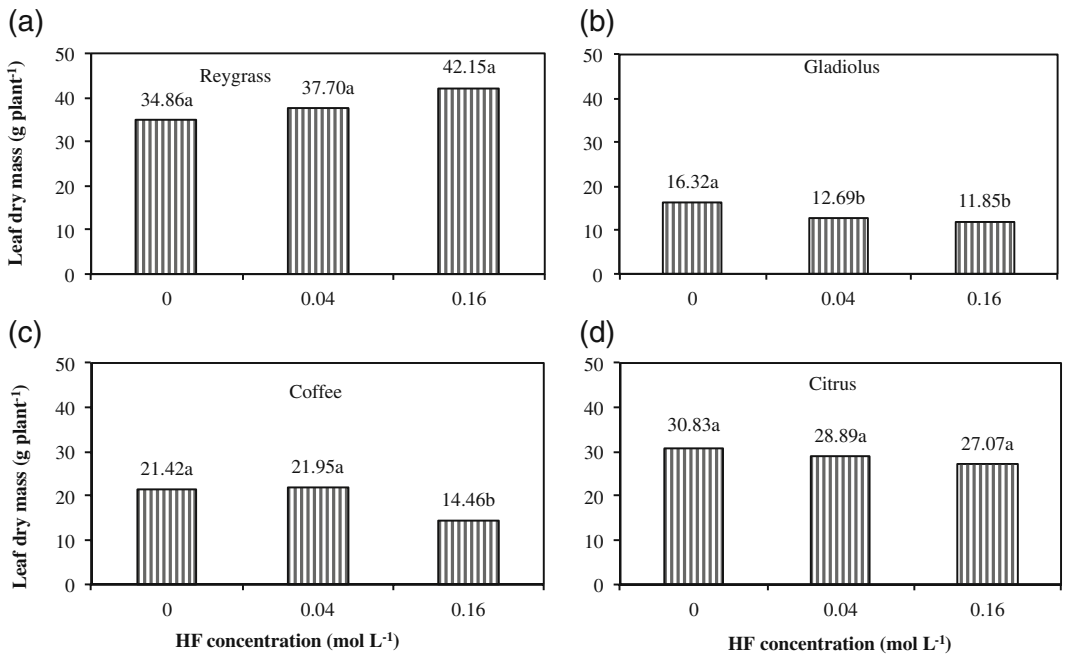


Fig. 6 Effect of doses of fluoride on the dry matter production of leaves of ryegrass, *Gladiolus*, coffee, and citrus after exposure to HF in a nebulization chamber. Means followed by same letter in columns for each species are not statistically different at 5% probability by the Duncan test

caused direct injuries on leaf cells characterized by degeneration of cell wall and cytoplasm and disorganization of bundle sheath, which were more evident in the *Gladiolus* and coffee. Minor changes were observed for the sweet orange and the ryegrass. Electron microscopy pointed out to damages to epicuticular wax and also to epidermal cells. Deformation of stomatal cells, with significant increase on stomatal area, was marked for the *Gladiolus* and coffee. Interestingly, damages to stomata were also characterized by opened ostioles when plants were exposed to high HF dose, what suggested a possible impairment on the opening and closing stomatal regulation mechanism. This later did not occur in ryegrass plants. The increased F absorption by leaves and changes at the structural and ultrastructural level of leaf tissues correlated with reduced plant growth.

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