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# Traits Driving Tolerance to Atmospheric Fluoride Pollution in Tree Crops

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**Abstract** Increased emissions of fluoride into the atmosphere contribute to reducing the sustainability of agricultural systems worldwide. In order to improve the understanding of the factors behind such phenomenon, varieties of citrus (*Citrus* spp.), Valencia sweet-orange, Ponkan mandarin, and Lisbon lemon and coffee (*Coffea* spp.), Obatã, Catuai, and Apoatã, were treated with fluoride nebulization. The trees were exposed to nebulization for 60 min inside a chamber by using medium (0.04 mol $L^{-1}$) and high (0.16 mol $L^{-1}$) doses of fluoridic acid (HF) during three nonconsecutive days in a single week, for a total of 26 days of exposure during the experiment. Sixty days after beginning nebulization, we evaluated leaf gas exchange, (ultra)structural organization, tree growth, and fluoride and nutrient concentrations in plant tissue. Photosynthesis and leaf dry mass of citrus and coffee varieties were affected differently by fluoride toxicity, and based on the tolerance index (relative leaf dry mass of control versus leaf dry mass of trees treated with 0.16 mol $L^{-1}$ HF), the order of sensitivity for the varieties of each species was as follows: for citrus, lemon > mandarin > sweet-orange; and for coffee, Apoatã > Catuaí > Obatã. The ability of the trees to control fluoride absorption most likely explained this contrast in sensitivity among varieties because both photosynthesis and leaf growth were negatively correlated with leaf fluoride concentration. Although disorganization of the thylakoids, degeneration of vascular cells, and disruption of the middle lamella occurred in leaves of all varieties exposed to fluoride, the more severe damage was observed in those with greater sensitivity to the pollutant (i.e., lemon and Apoatã coffee). Taken together, these results provided insights into the factors that explain poor performance of citrus and coffee trees under fluoride pollution and also revealed the traits driving the tolerance of these crops such a limiting condition, which included a combination of the following: (i) reduced fluoride absorption, (ii) increased photosynthesis, and (iii) improved maintenance of the ultrastructural organization of leaves.

**Keywords** Air pollution · *Citrus* spp. · *Coffea* spp. · Electronic microscopy · Photosynthesis

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## 1 Introduction

Increasing air pollution is a public concern worldwide because of its contribution to the decline of forests and

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the reduction in agricultural sustainability (Alloway 1998; Cronin et al. 2001; Ozsvath 2009). Among pollutants, fluoride is a unique, highly phytotoxic potential air contaminant because it is the most electronegative and chemically active of the nonmetals (Supharungsun and Wainwright 1982), which might explain the pronounced damage caused by fluoride to plants grown in contaminated areas (Jha et al. 2008; Sant'Anna-Santos et al. 2014). Damage includes, for example, disarranged chloroplasts and reductions in chlorophyll content (Singh and Verma 2013), with subsequent reduced leaf photosynthesis (Mesquita et al. 2013). Additionally, in response to fluoride, exposed plants show changes in the composition and function of cell membranes and cell walls (Façanha and Meis 1995) and (ultra)structural organization (Fornasiero 2003; Mesquita et al. 2011; Sant'Anna-Santos et al. 2014).

Based on observations in a field survey, in major agricultural areas of Brazil, citrus and coffee are crops frequently found near sources of fluoride emission, i.e., phosphate fertilizer and ceramic factories. In this scenario, a better understanding of the mechanisms by which air contaminated with fluoride affects the performance of these species in the field is required, since this might be useful, for example, in predicting the potential damage originating from fluoride stress events on crop yield stability. In order to achieve this, we argue that the evaluation of varieties with contrasting responses to fluoride toxicity would be a good approach and merits further investigation. Indeed, from a practical point of view, such an evaluation also increases the possibility of using more suitable varieties in areas that are prone to fluoride contamination. Although the selection of more tolerant varieties to fluoride toxicity may have beneficial effects on crop performance in the field, attempts should continue to develop techniques that reduce fluoride pollution in the environment (Jadhav et al. 2015).

The differential tolerance among citrus varieties to atmospheric contamination with fluoride has been established, with lemon and tangerine very sensitive, Valencia sweet-orange moderately sensitive, and Hamlin and pineapple orange the most tolerant (Woltz et al. 1971; Saborit 2009). However, the mechanisms underlying these distinct adaptations to fluoride stress have not been elucidated, and detailed studies are lacking that focus on the roles of the interactions among plant nutritional status, fluoride absorption, ultrastructural organization of leaf tissue, and photosynthesis characteristics in determining the differential tolerance among citrus varieties to fluoride stress. Furthermore, the genotypic response of other perennial crops of economic importance, such as coffee, to fluoride toxicity has not been determined. The view that integrated evaluation of different plant traits is critical to improve current understanding about adaptation to contaminants in the environment is supported by works dealing with multiple responses of citrus to other pollutants, i.e., ozone (Fares et al. 2010), copper (Zambrosi et al. 2013; Hippler et al. 2016), and cadmium (Lopez-Climent et al. 2014).

Based on the above discussion, in this study, citrus and coffee were selected to provide insights into the mechanisms that influence tree tolerance to fluoride toxicity. These crops were used to test the hypothesis that the differential tolerance among varieties of each species to fluoride pollution would be associated with the ability of each variety to reduce fluoride absorption through the leaves and also to sustain the organization of leaf (ultra)structure and the characteristics of photosynthesis. Therefore, the aim of this research was to evaluate the effects of fluoride nebulization on growth and nutritional status, leaf gas exchange, and (ultra)structural organization of leaf tissue in different varieties of citrus and coffee.

## 2 Materials and Methods

### 2.1 Plant Material and Growth Conditions

Young trees (approximately 1 year old) of varieties of citrus, Valencia sweet-orange [*Citrus sinensis* (L.) Osbeck], Ponkan mandarin (*Citrus reticulata* Blanco), and Lisbon lemon [*Citrus limon* (L.) Burm. f.], and coffee, Obatã (*Coffea arabica* L., Obatã IAC 1669-20), Catuai (*Coffea arabica* L. red Catuaí IAC 99), and Apoatã (*Coffea canephora* Pierre ex A. Froehner Apoatã IAC 3597-8), were used in this study. The trees were grown in 10 L plastic pots containing soil collected from the surface layer (0–0.25 m) of a Ferralsol under grassland. The soil was chemically and physically characterized according to Camargo et al. (1986) and Raij (2001) as follows: organic matter = 34 g $dm^{-3}$; pH ($CaCl_2$ 0.01 mol $L^{-1}$) = 5.2, CEC = 87.9 $mmol_c\ dm^{-3}$, base saturation = 61 %, clay = 300 g $kg^{-1}$. The pots were fertilized with nutrient solutions to ensure adequate growth, based on the nutrient content in tree dry mass and the initial soil chemical characteristics. The soil moisture was maintained at 80 % of water field capacity,

and the frequency of irrigation was determined by weighing the pots.

The experiment was conducted in an unshaded greenhouse in which the minimum and maximum average temperatures were 14 and 32 °C, respectively, and the air relative humidity ranged from 35 % to 90 %. The treatments were defined by the citrus and coffee varieties exposed to two intensities of fluoride applied inside a chamber: 0.04 and 0.16 mol $L^{-1}$ of fluoridic acid (HF) solution. An additional control was not exposed to fluoride. Each species was examined in a separate experiment, which was a completely randomized design with four replicates in each treatment. The estimated atmospheric F concentrations inside the nebulization chamber were 0.065 and 0.260 mmol $m^{-3}$ of fluoride for 0.04 and 0.16 mol $L^{-1}$, respectively, as described in Mesquita et al. (2011).

The trees in the fluoride treatments were exposed to the contaminated environment during three nonconsecutive days in a single week, totaling 26 days of exposure over the total experimental period of 60 days. The trees were exposed for 30 min and remained in the chamber for another 60 min until removal to the external environment. A total volume of 10 mL of HF for each tested concentration of the element was nebulized after being placed in the plastic container of the nebulizer, as presented in detail by Mesquita et al. (2011). The free fluoride content according to Frankenberger et al. (1996) in the soil after the experimental period was $0.532 \pm 0.09$ and $0.540 \pm 0.08$ mg $kg^{-1}$ in the control and nebulization treatment with 0.16 mol $L^{-1}$ HF, respectively.

## 2.2 Leaf Gas Exchange and Chlorophyll Fluorescence

Leaf $CO_2$ assimilation ($A$, μmol $m^{-2}$ $s^{-1}$), transpiration ($E$, mmol $m^{-2}$ $s^{-1}$), and stomatal conductance ($g_s$, mol $m^{-2}$ $s^{-1}$) were measured between 9:00 am and 11:00 am with an infrared gas analyzer (LI-6400; Li-Cor Biosciences, Lincoln, NE, USA) 60 days after the start of fluoride treatments. Measurements were taken in fully expanded mature leaves developed during fluoride nebulization, and this was performed under a photosynthetic photon flux density of 800 μmol $m^{-2}$ $s^{-1}$ and natural variations in air temperature and humidity. Data were recorded when the coefficient of variation of measurements was less than 1 %. The temperature of the leaves varied between 30 and 35 °C. The average solar radiation was $1200 \pm 2$ μmol $m^{-2}$ $s^{-1}$; the relative humidity ranged between 25 and 45 %, and the atmospheric $CO_2$ concentration was $370 \pm 3$ μmol $mol^{-1}$.

Chlorophyll fluorescence was measured simultaneously with gas exchange in light-exposed leaves in a fluorometer chamber (LCF-6400-40; Li-Cor, Lincoln, NE, USA) attached to a Li6400. The effective quantum yield of photosystem II ($\Delta F/F_m'$) was measured as $\Delta F/F_m' = (F_m' - F')/F_m'$, where $F_m'$ and $F'$ are the maximal and steady-state fluorescence yields in light-adapted leaves, respectively. In dark-adapted leaves (30 min), the maximum quantum efficiency of photosystem II ($F_v/F_m$) was determined as $F_v/F_m = (F_m - F_o)/F_m$, where $F_m$ and $F_o$ are the maximum and minimum fluorescence of dark-adapted leaves, respectively (Roháček 2002).

## 2.3 Light Microscopy and Scanning Electron Microscopy

At the end of the nebulization period, the anatomical characteristics of leaves were evaluated in samples obtained from the identical leaves that were used for measurements of leaf gas exchange (i.e., mature leaves developed after the beginning of the fluoride treatments). Tissue samples (approximately 20 $mm^2$) were removed from leaves without visual damage of four different plants per treatment. In lemon trees, the portion of the leaves removed for analysis was located immediately below the chlorotic area of the leaf. Samples were prepared according to the procedure described previously (Mesquita et al. 2011) and then analyzed by light and scanning electron microscopy. Four blocks were prepared from each treatment, and the individual areas of 100 randomly selected stomata (25 stomata and chloroplasts per block) were estimated. The area calculation was based on 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).

## 2.4 Transmission Electron Microscopy

Samples were prepared according to Mesquita et al. (2016). Briefly, the fixed leaf samples were post-fixed with 1 % osmium tetroxide, dehydrated in an increasing acetone concentration series, and infiltrated and polymerized into Spurr low viscosity epoxy resin. The blocks (four per treatment) were prepared by using a trimmer (EM Trim; Leica Microsystems Inc., USA).

Sections (70 nm thick) were obtained by using an ultramicrotome (Leica UC6; Leica Microsystems Inc., USA) and contrasted against uranyl acetate and lead citrate (Reynolds 1963). The analyses were performed by using a transmission electron microscope (EM900; Zeiss, Germany) fitted with a digital camera at 80 kV. The individual areas of 100 randomly selected chloroplasts (25 chloroplasts per block) were estimated as previously described for the stomata.

### 2.5 Tolerance Index to Fluoride Toxicity and Mineral Composition of Leaves

After sampling leaves for microscopic analyses, all the leaves of each tree were harvested and then dried at 65 °C for 48 h to estimate total leaf dry mass (DM) production per tree. Furthermore, the tolerance index of the varieties of citrus and coffee to fluoride toxicity was estimated as the ratio between the leaf DM produced at 0.16 mol $L^{-1}$ of HF and the DM produced in the control.

Leaf samples (approximately 2-month-old leaves that developed during fluoride nebulization) were ground and chemically analyzed to determine total F concentration by using the alkaline fusion based on the method described by Frankenberger et al. (1996) and adapted according to the procedure described by Mesquita et al. (2011). The analysis of the mineral composition of leaf tissue was performed according to Bataglia et al. (1983).

### 2.6 Data Analyses

The data were subjected to two-way analysis of variance (ANOVA) in a complete factorial design for citrus or coffee varieties versus fluoride treatments. When a significant ($p < 0.05$) interaction between the factors (citrus or coffee variety × fluoride concentration) was detected, varieties of each species were compared by using Tukey's tests ($p < 0.05$) for a given fluoride treatment, and the fluoride treatments were compared by using Tukey's tests ($p < 0.05$) within the same variety. When no significant interactions were detected, Tukey's tests were used to compare the mean values of the main factors. Light, scanning, and transmission electron microscopy images of citrus and coffee leaves were interpreted according to a comparative evaluation of the treatments.

## 3 Results

### 3.1 Visual Symptoms of Fluoride Toxicity

The visual symptoms of fluoride toxicity were detected in lemon leaves at the highest dose of fluoride after 3 weeks of nebulization (Fig. 1a–d). We observed chlorosis on the borders of the leaves, which spread further along the margins and into the leaf blade between the veins. Over the experimental period, the chlorosis became necrosis (Fig. 1a–c), ending with leaf abscission (Fig. 1d). By contrast, no visible injury was observed for other citrus varieties and any variety of coffee.

### 3.2 Leaf Dry Mass Production and Tolerance Index to Fluoride Toxicity

The leaf DM production of citrus and coffee varieties responded differently to the doses of fluoride, as indicated by the significant ($p < 0.05$) interaction between these factors within each species. Leaf growth of sweet-orange in the control did not differ between the two treatments with fluoride, whereas leaf DM of lemon and mandarin was reduced by 38 % and 20 %, respectively, in the treatment with the highest dose of fluoride (Fig. 2a). Furthermore, the growth of sweet-orange was more vigorous under such stress, and the leaf DM of this variety was 30 % and 20 % greater than that of lemon and mandarin, respectively.

For the coffee varieties, the leaf DM of Obatã was not influenced by the fluoride treatments, whereas leaf DM was reduced under the highest fluoride dose for Catuaí and Apoatã by 21 % and 35 % compared with the control, respectively (Fig. 2b). The leaf DM of Obatã was also higher than that of Catuaí and Apoatã in all treatments; however, the differences were much more pronounced under the application of the highest concentration of fluoride (35 % to 49 %) compared with that in the control (27 % to 29 %).

The tolerance index of citrus and coffee varieties to fluoride toxicity was also estimated, and the results indicated that the tolerance to fluoride stress was unique among the varieties. Accordingly, the following ranking was obtained for citrus and coffee trees, respectively: sweet-orange > mandarin > lemon (Fig. 3a) and Obatã > Catuaí > Apoatã (Fig. 3b).

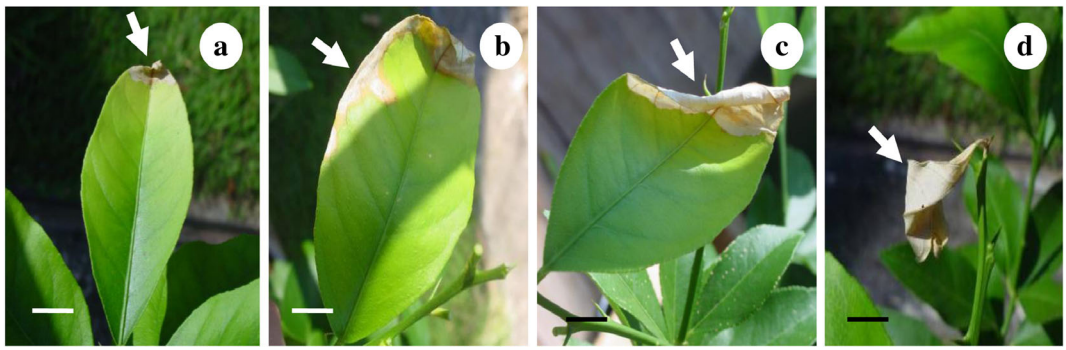


**Fig. 1** Symptoms of leaf injury in lemon plants caused by fluoride after exposure to 0.16 mol $L^{-1}$ fluoridic acid nebulized in closed chamber: **a**–**c** symptoms starting from leaf margins (*arrow*) and progressing into the leaf blade (*arrows*) and **d** leaf abscission (*bar* = 0.2 cm)

### 3.3 Fluoride and Mineral Nutrient Concentrations in Leaf Tissue

For the concentration of fluoride in the leaves, a significant ($p < 0.05$) interaction was observed between citrus and coffee varieties with fluoride doses. In citrus varieties, the increase in leaf fluoride concentration across fluoride treatments ranged from 10-fold to 15-fold (Fig. 4a). Furthermore, when the citrus varieties were compared, sweet-orange had the lowest concentrations of leaf fluoride under HF nebulization, whereas the highest concentration was in lemon.

Leaf fluoride concentration also increased in all coffee varieties following fluoride application. However, the response was different depending on the genotype, with the largest increment being found in Apoatã (Fig. 4b). For the comparison among the varieties, Apoatã had the highest concentrations of fluoride in leaves in both pollutant treatments.

The concentrations of nutrients in leaf tissue of citrus and coffee trees, with the exceptions of Ca and Mg, were not influenced by the fluoride treatments. The application of fluoride increased the concentration of Ca and did not affect that of Mg in the leaves of both sweet-

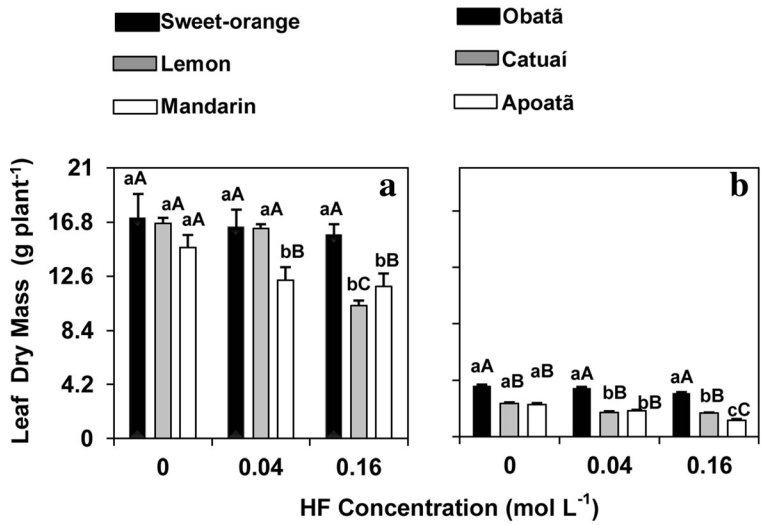


**Fig. 2** Effect of atmospheric fluoride on the dry mass production of leaves of citrus (**a**) and coffee trees (**b**), after 60 days of exposure to fluoridic acid (HF) in a semi-open nebulization chamber. HF concentration comparison: *columns followed by different lowercase letters* are significantly different by the Tukey test ($p < 0.05$). Comparison of varieties: *columns followed by different uppercase letters* within the same HF concentration are significantly different by the Tukey test ($p < 0.05$). Each histogram is the mean ± standard error ($n = 4$)

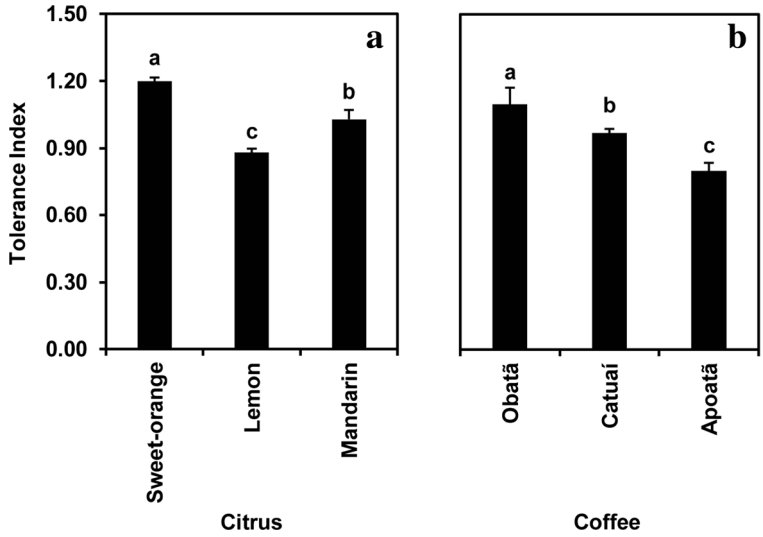


**Fig. 3** Tolerance index of citrus (**a**) and coffee varieties (**b**) to fluoride toxicity. Each histogram is the mean value ($n = 4$) and was calculated as the relative value of leaf dry mass production at 0.16 mol $L^{-1}$ fluoridic acid versus control plants. *Different letters* represent significant difference by Tukey test ($p < 0.05$)

orange and mandarin trees (Fig. 5a, c). However, in the high-dose fluoride treatment, the concentrations of Ca and Mg in the leaves of lemon were reduced by 24 % and 30 %, respectively, compared with those in the control. Furthermore, lemon had lower concentrations of Ca and Mg in leaf tissue for both fluoride treatments than those of sweet-orange and mandarin.

In coffee trees, the concentrations of leaf Ca and Mg were reduced in all varieties under the highest dose of fluoride compared with those in the control (Fig. 5b, d). Leaf Ca concentration was reduced by 12 % in Apoatã, and leaf Mg concentration was reduced by 18 and 21 % in Catuaí and Apoatã, respectively.

### 3.4 Leaf Gas Exchange and Chlorophyll Fluorescence

For gas exchange parameters, significant ($p < 0.05$) interactions were detected between citrus and coffee varieties with the fluoride treatments. In the absence of fluoride application, the values of *A* were similar for lemon and mandarin but higher than those observed for sweet-orange (Fig. 6a). At the highest dose of HF,

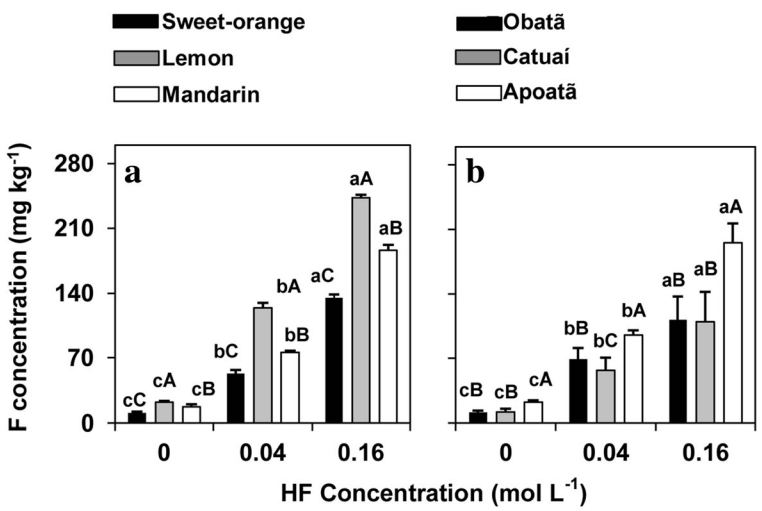


**Fig. 4** Fluoride concentrations in leaves of citrus (**a**) and coffee trees (**b**), after 60 days of exposure to fluoridic acid (HF) in a semi-open nebulization chamber. Comparison of HF concentrations: *columns followed by different lowercase letters* are significantly different by the Tukey test ($p < 0.05$). Comparison of varieties: *columns followed by different uppercase letters* within the same HF concentration are significantly different by the Tukey test ($p < 0.05$). Each histogram is the mean ± standard error ($n = 4$)

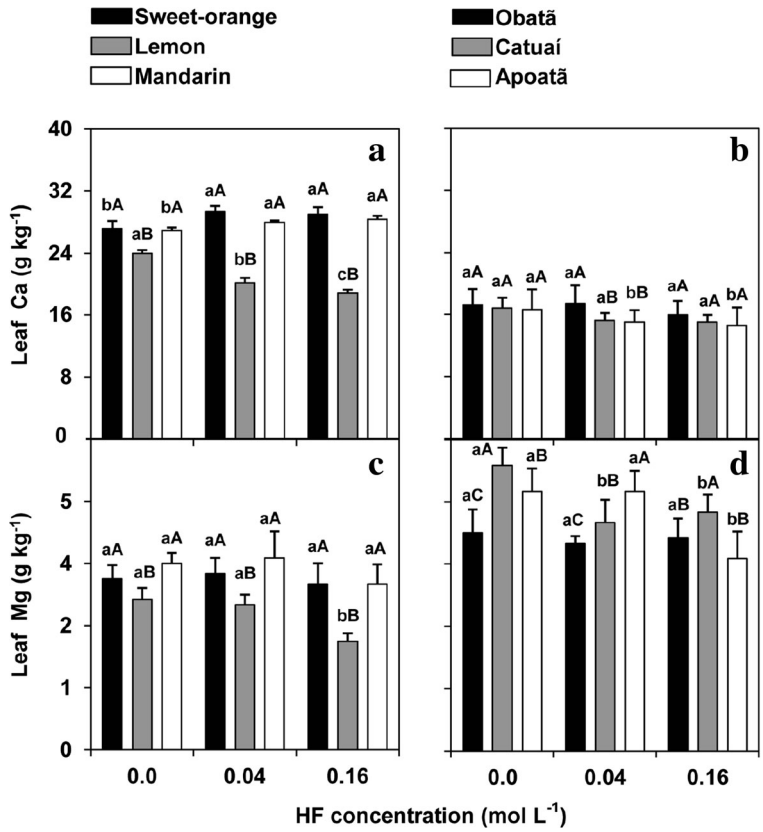


**Fig. 5** Effect of atmospheric fluoride on calcium (Ca) and magnesium (Mg) concentrations in leaves of citrus (**a**, **c**) and coffee varieties (**b**, **d**), after 60 days of exposure to fluoridic acid (HF) in a semi-open nebulization chamber. Comparison of HF concentrations: *columns followed by different lowercase letters* are significantly different by the Tukey test ($p < 0.05$). Comparison of varieties: *columns followed by different uppercase letters* within the same HF concentration are significantly different by the Tukey test ($p < 0.05$). Each histogram is the mean ± standard error ($n = 4$)

values of *A* for lemon (−30 %) and mandarin (−21 %) were reduced compared with those of the control, whereas no effect was observed on *A* in sweet-orange. Nebulization with fluoride increased the values of *E* in all citrus varieties, with lower values being observed for lemon than those for sweet-orange and mandarin under fluoride application (Fig. 6c). Stomatal conductance was not influenced by fluoride treatment in sweet-orange but increased by 40 % and was reduced by 23 % in lemon and mandarin, respectively, at the highest dose (Fig. 6e).

All coffee varieties showed significant reductions in *A* when exposed to fluoride nebulization. For example, at the highest dose of the pollutant, the reduction in *A* was 42 %, 31 % and 46 % for Obatã, Catuaí, and Apoatã, respectively (Fig. 6b). Moreover, also in the high-dose fluoride treatment, Apoatã had the lowest values of *A*. The values of *E* were also affected with the application of fluoride, and the reductions ranged from 24 % to 40 % among the different coffee varieties (Fig. 6d). Furthermore, $g_s$ was reduced by 75 % for Obatã when the plants were exposed to the highest dose of fluoride (Fig. 6f). On the other hand, Apoatã exhibited an increase in $g_s$ when exposed to fluoride toxicity, and no $g_s$ variation was detected for Catuaí.

For citrus and coffee trees, the maximum and effective quantum efficiencies of photosystem II were not significantly ($p > 0.05$) different among the varieties or between the fluoride nebulization treatments. Thus, the values of maximum and effective quantum efficiencies of photosystem II averaged across treatments were 0.866 and 0.899 for citrus and 0.694 and 0.702 for coffee trees, respectively.

### 3.5 Anatomical Changes in Leaf Tissue

The stomatal area of the control lemon trees was 21 % larger than that of trees nebulized with 0.16 mol $L^{-1}$ fluoride, whereas no effect was observed for the other citrus species (Fig. 7a). Scanning electron microscope images revealed that the stomata of lemon trees under exposure to the pollutant were completely opened with injuries at the epidermis (Fig. 8k). However, in the leaves of sweet-orange and mandarin exposed to fluoride, the stomata were not fully opened and no marked evidence of epidermal alteration was found (Fig. 8j, l).

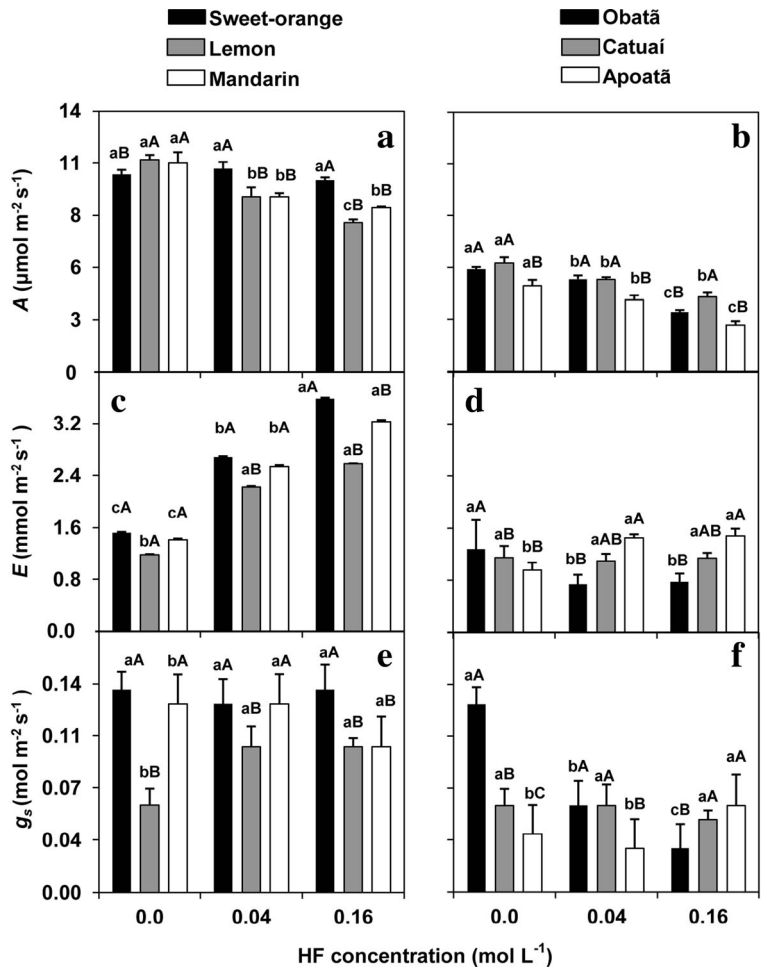


**Fig. 6** Effect of atmospheric fluoride on leaf $CO_2$ assimilation (*A*), transpiration (*E*), and stomatal conductance ($g_s$), of citrus (**a**, **c**, **e**) and coffee varieties (**b**, **d**, **f**), after 60 days of exposure to fluoridic acid (HF) in a semi-open nebulization chamber. Comparison of HF concentrations: *columns followed by different lowercase letters* are significantly different by the Tukey test ($p < 0.05$). Comparison of varieties: *columns followed by different uppercase letters* within the same HF concentration are significantly different by the Tukey test ($p < 0.05$). Each histogram is the mean ± standard error ($n = 4$)

Moreover, at this high dose of the pollutant, no changes in cell structure were observed in the leaves of sweet-orange compared with leaves of the same under the control treatment (Fig. 8d). By contrast, the leaves of lemon trees exposed to the highest dose of fluoride showed a marked disorganization of vascular cells at the bundle sheath (Fig. 8e). Damage to the xylem and phloem vessels, indicated by packed cells that lost their normal circular format, was also found in mandarin leaves under fluoride stress (Fig. 8f).

For coffee trees, the stomatal area of Apoatã was reduced by 56 % when exposed to the highest dose of fluoride, whereas the other varieties showed no variation in this measurement (Fig. 7b). The structural changes in leaf tissue were distinct among the tested coffee varieties when exposed to fluoride toxicity. For example, in Obatã, no evident alteration in the leaf structure was observed in the fluoride treatment (Fig. 9d), whereas in Catuaí, damages to the cell wall and middle lamella were observed, as revealed by the spaces between the palisade parenchyma cells and disruption of vascular bundle (Fig. 9e). For Apoatã, the shape of the phloem and xylem cells was altered and palisade parenchyma cells appeared to exhibit the accumulation of phenolic compounds (arrow, Fig. 9f). Additionally, the leaves of all coffee varieties showed disintegration of the epidermal cells surrounding the stomata, which were also wrinkled and deformed (arrows, Fig. 9j, k, l).

### 3.6 Ultrastructural Changes in Leaf Tissue

The chloroplast area of lemon leaves was reduced by 75 % when exposed to 0.16 mol $L^{-1}$ of fluoride, compared

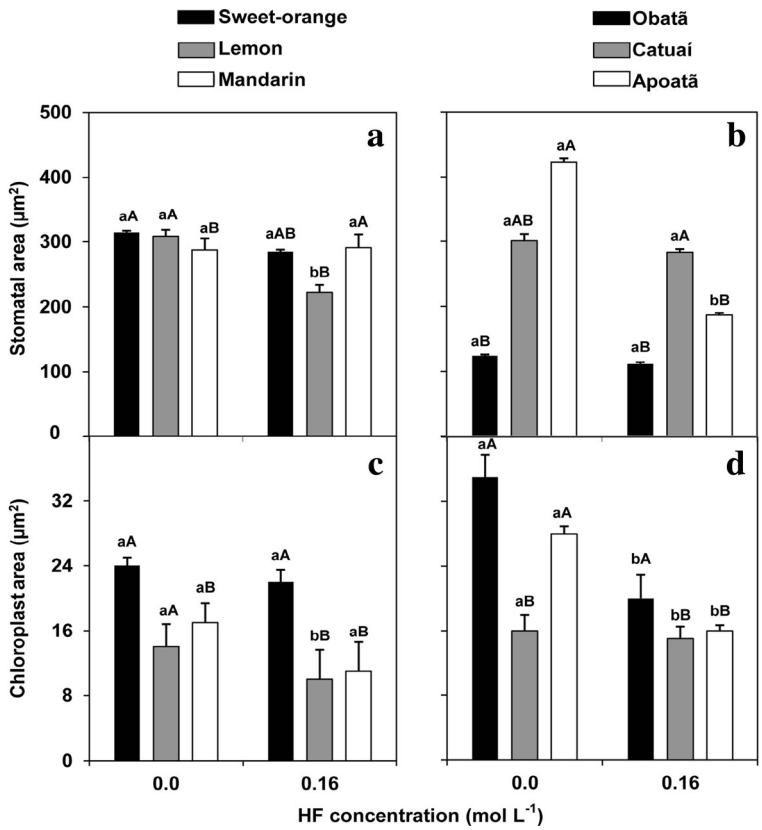


**Fig. 7** Effect of atmospheric fluoride on leaf stomatal characteristics of citrus (**a**, **c**) and coffee varieties (**b**, **d**), after 60 days of exposure to fluoridic acid (HF) in a semi-open nebulization chamber. Comparison of HF concentrations: *columns followed by different lowercase letters* are significantly different by the Tukey test ($p < 0.05$). Comparison of varieties: *columns followed by different uppercase letters* within the same HF concentration are significantly different by the Tukey test ($p < 0.05$). Each histogram is the mean ± standard error ($n = 4$)

with that of the control trees, whereas no effect was observed on the other citrus trees (Fig. 7c). Indeed, the most pronounced damage occurred in the chloroplasts of lemon trees, which showed thylakoid disorganization and accumulation of starch grains under fluoride nebulization (arrows, Fig. 10e). Moreover, lemon and mandarin showed also a thin cell wall and sectioned middle lamella (arrows, Fig. 10f). However, only minor changes in the cell walls (i.e., sinuosity scarcely visible) were observed in the leaves of sweet-orange (arrows, Fig. 10d).

In coffee trees, the chloroplast area was reduced by 43 % in Apoatã and Obatã and by 31 % in Catuai, when exposed to the highest fluoride dose (Fig. 7d). Regardless of the variety, when exposed to the pollutant, the leaves of coffee trees had cells with thin and deformed walls and with apparent fragmentation of the middle lamella (Fig. 11d, e, f). We also observed darkening of and accumulation of starch grains in the chloroplasts across all varieties. Moreover, the number of chloroplasts in the leaves of Apoatã trees was apparently reduced.

## 4 Discussion

Our results consistently revealed differential tolerance among both citrus and coffee varieties to fluoride toxicity in the environment (Fig. 3), which confirmed the genotype-dependent response observed in a number of plant species to this type of stress (Doley 1988; Weinstein and Davison 2004; Sant'Anna-Santos et al. 2006; Domingues et al. 2011). Herein, we could primarily argue that the contrast in tolerance to fluoride toxicity was related to the ability of the most tolerant varieties (i.e., sweet-orange and Obatã) to maintain a lower concentration of leaf fluoride than that of the most sensitive ones (i.e., lemon and Apoatã) (Fig. 4). The absorption of fluoride through the leaves depends on the characteristics of the leaf surface, i.e., pubescence and waxy coatings, and also on stomatal conductance (Wei and Miller 1972; Coulter et al. 1985; Vike 1999). In the present research, stomatal conductance might have been a critical component regulating the differential fluoride absorption between sensitive and tolerant varieties of citrus and coffee under the highest dose of the pollutant, as

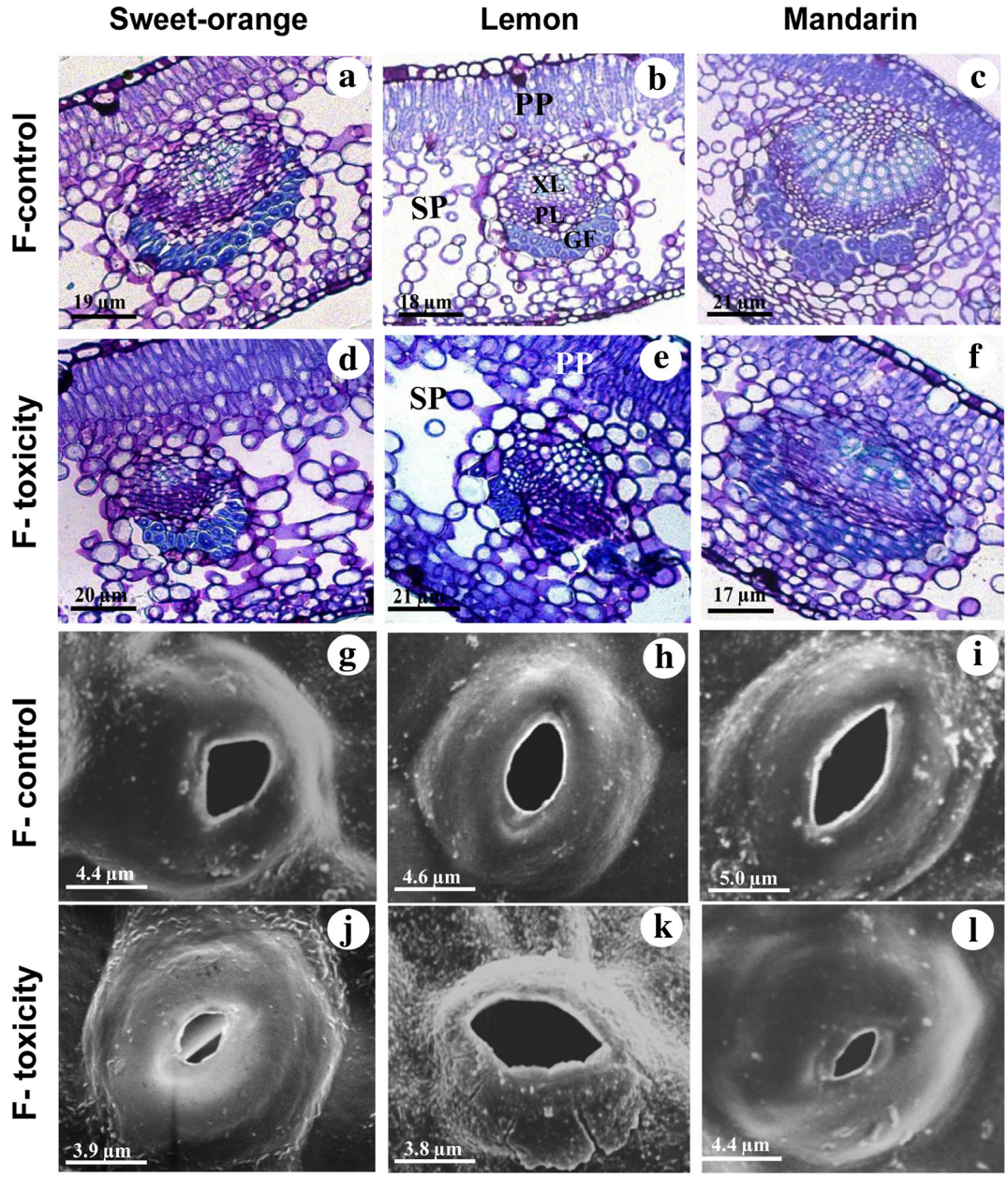


**Fig. 8** Cross sections of leaves of citrus trees under light (LM) and scanning electron microscopy (SEM): sweet-orange (**a**, **d** LM; **g**, **j** SEM), lemon (**b**, **e** LM; **h**, **k** SEM), and mandarin (**c**, **f** LM; **i**, **l** SEM). Leaves were exposed or not to atmospheric fluoride at 0.16 mol $L^{-1}$ of fluoridic acid (HF) in a closed chamber. *PP* palisade parenchyma, *SP* spongy parenchyma, *XL* xylem, *PL* phloem, *GF* gelatinous fibers. Ostiole (*arrowhead*)

suggested by the following: (i) The $g_s$ of lemon and Apoatã increased at the highest fluoride dose compared with that of the control (Fig. 6e, f), and (ii) more severe damage, and therefore, compromised function, was found in the structure of the stomata of the leaves of lemon and Apoatã than in that of stomata of sweet-orange and Obatã leaves (Figs. 8 and 9). After absorption by the stomata, fluoride is transported inside the leaves and accumulates in the tip and leaf margins (Mesquita et al. 2011), which is consistent with the visual symptoms of fluoride toxicity found in lemon leaves (Fig. 1).

The highest fluoride concentration in the leaves of the lemon and Apoatã resulted also in the most

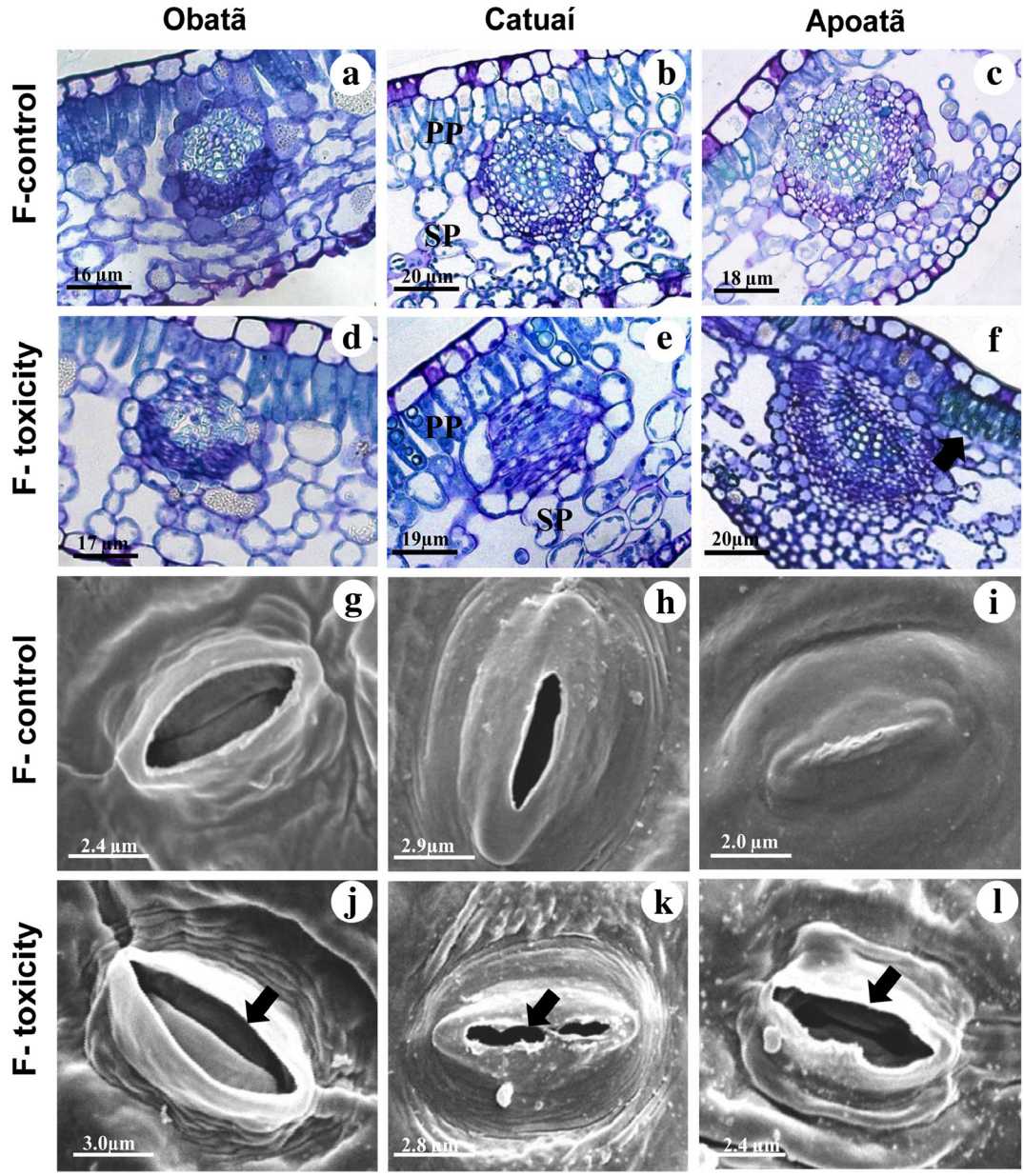


**Fig. 9** Cross sections of leaves of coffee trees under light (LM) and scanning electron microscopy (SEM): Obatã (**a**, **d** LM—oxidative stress (*arrow*)); **g**, **j** SEM), Catuaí (**b**, **e** LM; **h**, **k** SEM), and Apoatã (**c**, **f** LM; **i**, **l** SEM). Leaves were exposed or not to atmospheric fluoride at 0.16 mol $L^{-1}$ of fluoridic acid (HF) in a closed chamber. *PP* palisade parenchyma, *SP* spongy parenchyma, *XL* xylem, *PL* phloem, *GF* gelatinous fibers. Ostiole (*arrowhead*). *Arrows* in each micrograph are described in the text

pronounced decline of *A* observed for these plants, as indicated by the negative correlation between *A* and leaf fluoride concentration across both citrus ($r = -0.84$; $p < 0.001$; $n = 36$) and coffee varieties ($r = -0.79$; $p < 0.001$; $n = 36$). This type of negative relationship might be explained by the reactions of fluoride with metals, such as Mg, leading to the reduction in the activity of ribulose-1,5-biphosphate carboxylase/oxygenase and also in the biosynthesis of photosynthetic pigments (Doley 1988; Parry et al. 1984; Nilsson and Braden 1983; Doley and Rossato 2012; Singh and Verma 2013). In addition to such effects, the impaired photosynthetic activity of lemon and Apoatã trees was associated to the pronounced damage observed to the

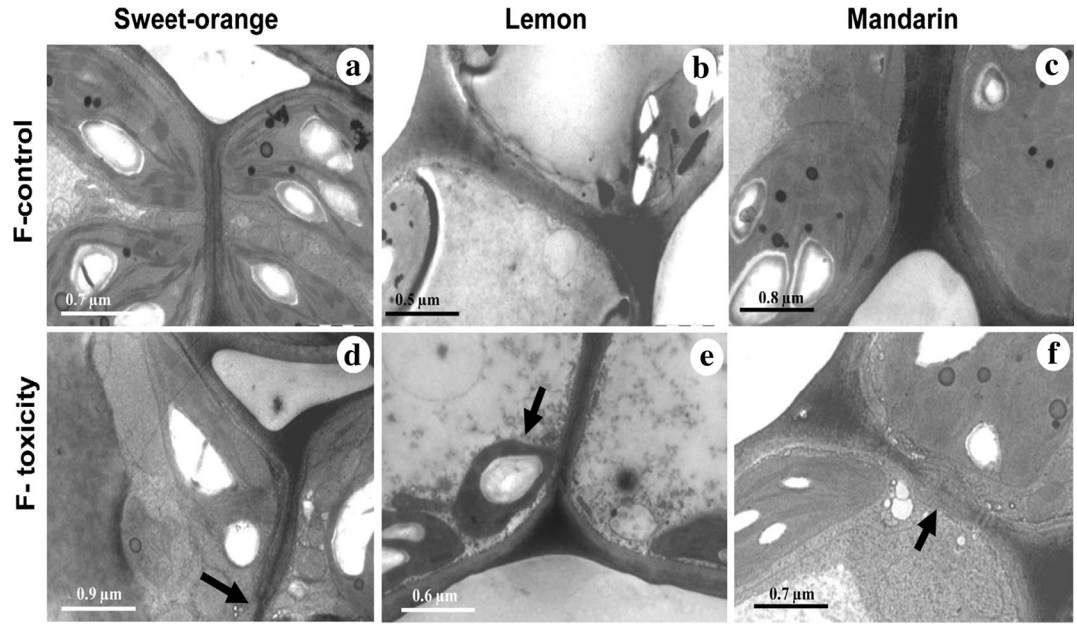


**Fig. 10** Transmission electron microscopy of leaves of citrus plants affected by atmospheric fluoride (not exposed or exposed 0.16 mol $L^{-1}$ fluoridic acid (HF) in closed chamber). Sweet-orange (**a**, **d**), lemon (**b**, **e**), and mandarim (**c**, **f**). *Arrows* in each micrograph are described in the text

ultrastructure of their chloroplasts (i.e., reduction in size and distortion of shape) and an apparent decrease in the abundance of this organelle in the mesophyll cells (Figs. 7c, d, 10e, and 11f). Indeed, chloroplast is an organelle severely affected by fluoride pollution (Fornasiero 2003) because of large accumulations of this element (Miller 1993; Bustamante et al. 1993). The impaired photosynthetic efficiency of lemon might also be related to the accumulation of starch grains (Fig. 10e) (Rennenberg et al. 1996; Soda et al. 2000; Fornasiero 2003; Sant'Anna-Santos et al. 2014), which was most likely due to an inhibitory effect of the

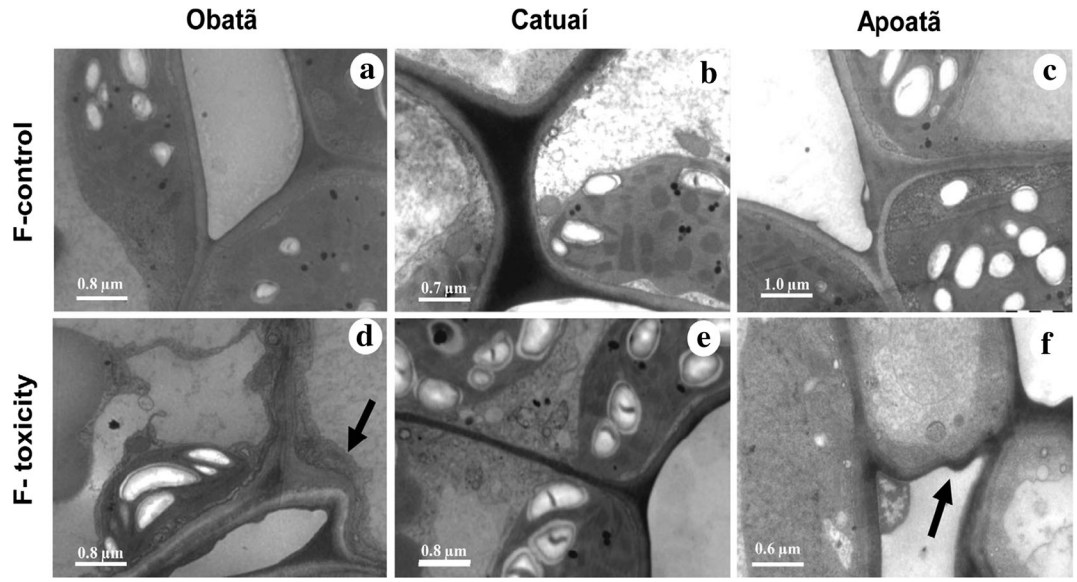


**Fig. 11** Transmission electron microscopy of leaves of coffee varieties affected by atmospheric fluoride (not exposed or exposed 0.16 mol $L^{-1}$ fluoridic acid (HF) in closed chamber): Obatã (**a**, **d**), Catuaí (**b**, **e**), and Apoatã (**c**, **f**). *Arrows* in each micrograph are described in the text

pollutant on carbohydrate transport to sink tissues (Momen et al. 2002) as a result of severe structural damage in the vascular bundle (Fig. 8e). The diminished demand for carbohydrate due to inhibition of growth by fluoride toxicity in lemon might have also contributed to starch accumulation and reduced photosynthesis (Ribeiro et al. 2012).

The disturbance in the organization of the cell wall (i.e., disruption of the middle lamella and the occurrence of a thin cell wall with sinuosity) is an additional type of damage that occurs with fluoride toxicity in plants, and the damage level is an indication of the susceptibility to the pollutant (Mesquita et al. 2011). Consistent with this relation, the damages to the leaf cell wall were more pronounced in lemon than in sweet-orange (Fig. 10) and Apoatã than in Obatã trees (Fig. 11). In the disruption of cell wall stability, the removal of Ca from cell wall components to form insoluble $CaF_2$ is the primary effect of fluoride (Ben Abdallah et al. 2006). The formation of these compounds releases Ca from fixation sites, and the cell wall is more prone to collapse with subsequent impairment of leaf physiological activity (Garrec and Chopin 1982). Accordingly, plants that have high absorption capacity and ability to transport Ca to leaves are more tolerant to contamination with fluoride because the integrity of the cell wall is maintained (Ruan et al. 2004; Ben Abdallah et al. 2006). Therefore, the difference in tolerance between sweet-orange and lemon might also be related, at least in part, to the higher leaf Ca concentration in sweet-orange than that in lemon (Fig. 5a). However, for the coffee varieties, differences in leaf Ca concentration likely did not contribute to the difference in fluoride tolerance between Obatã and Apoatã because the concentrations of Ca in the leaf tissues of the two varieties were similar (Fig. 5b). Therefore, complementary mechanisms that regulate the tolerance of a plant to abiotic stresses, such as antioxidant activity of the leaves (Li et al. 2011), might be critical components in determining the distinct performances of coffee varieties exposed to fluoride toxicity, although the details of these mechanisms require further investigation.

In conclusion, based on our results, the mechanisms controlling the tolerance of tree crops to fluoride pollution were clearly identified, which involved the following: (i) maintaining a low concentration of leaf fluoride, (ii) increasing photosynthesis, and (iii) maintaining and improving (ultra)structural organization in cells of leaf tissues. Therefore, these findings might contribute to the selection of traits to increase tree resistance to fluoride toxicity and provide insight into the factors leading to the impaired performance of agricultural crops exposed to fluoride stress in the environment.

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