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Morphological and physiological changes induced in *Olea europaea* and *Prunus dulcis* exposed to air fluoride pollution

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Abstract Air quality biomonitoring using plant leaves has been widely applied to assess the effects of atmospheric pollution. The present study was conducted to investigate the effects of gaseous hydrogen fluoride on the tree leaves of *Prunus dulcis* and *Olea europaea* around a phosphate fertilizer-producing factory constituting a major source of pollution. The photosynthesis rate, damaged leaf areas, fluoride accumulation, stomatal architecture and some biochemical responses in the leaves of *O. europaea* and *P. dulcis*, were measured. Based on visible injury, gas exchange, stomata responses and certain biochemical parameters, *P. dulcis* was a sensitive species, and *O. europaea* was tolerant. Our studies showed that fluoride had a potential effect on stomatal plasticity and confirmed the presence of different control mechanisms for pollution on each leaf surface. It was observed that evergreen tree species highly tolerated fluoride pollutants present in the air, and therefore can be effective in reducing pollutants concentration to a safer level in the environment.

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Keywords Air pollution · *Prunus dulcis* · *Olea europaea* · Fluoride accumulation · Stomatal responses · Biochemical responses

Introduction

Hydrogen fluoride is one of the most phytotoxic air pollutants (Ahmad et al. 2012). Hydrogen fluoride and other fluoride compounds in the atmosphere are deposited to vegetations surfaces either in gaseous form or in the particulates form. Fluoride released into the environment via several industrial processes especially aluminum smelters and phosphate fertilizer plants (Weinstein and Davison 2003; Ahmad et al. 2012).

Nowadays, Sfax, the second biggest city of Tunisia, accommodates important industrial complexes, among which the phosphate fertilizer factory (SIAPE) constitutes the main source of fluoride pollution in the atmosphere (Ben Abdallah et al. 2006a, b, c). Atmospheric studies performed in the industrial zone of urban Sfax (through many representative installations) showed also a high emission rate of SO_2 and dust (Gargouri et al. 2011). Mezghani et al. (2005) and Ben Abdallah et al. (2006c) reported that fluoride is the most important phytotoxic air pollutants in the vicinity of the phosphate fertilizer factory. Analysis of the air surrounding this factory showed that fluoride air contents oscillate between 3 and 12 $\mu g\ dm^{-3}\ day^{-1}$ (Mezghani et al. 2005). The fluoride pollutants emitted into the air by the phosphate fertilizer chimney are mainly HF, CaF_2 and H_2SiF_6 (Ben Abdallah et al. 2006a).

Atmospheric pollution constitutes one of the major problems in industrial environments. For several years, biomonitoring of air quality using plants has been widely applied to detect the effects of air pollution (Markert et al.

2003; Anicic et al. 2011). Lichens and mosses are recognized as most appropriate biomonitors of atmospheric contamination. However, in industrial areas, where mosses and lichens are often missing, higher plants have gained special importance and used as valuable biomonitors. The use of plants as passive sampler in biomonitoring bears the advantage of high temporal and spatial resolution due to the excellent availability of plants. Although many plant groups have been used in monitoring pollution pointing out their advantages, there has been little focus on evergreen trees (Sawidis et al. 2012). Kikuzawa and Kudo (1995) found that evergreen trees reabsorbed a higher percentage of leaf nutrients than deciduous species. Hence, evergreen plants are expected to be more convenient for biomonitoring purposes and more resistant in polluted environments (Sawidis et al. 2012).

Under the influence of air pollutants, some higher plants exhibit changes in morphology, physiology, biochemistry and growth rate (Moraes et al. 2002; Kardel et al. 2010; Kanayama et al. 2013). Criteria for the assessment of air pollution impact include analyses of visible injury (Ghosh et al. 1998; Oksanen and Holopainen 2001), accumulation of toxic substances and evaluation of biochemical and physiological pollutant-induced changes in parameters related to photosynthesis, respiration, enzyme activities, lipid synthesis, proteins and other metabolites (Herbinger et al. 2002; Bamniya et al. 2012a, b; Elloumi et al. 2014a). Air pollution can also induce qualitative and quantitative changes in the secondary metabolite composition (Kanoun et al. 2001).

It is generally believed that most airborne fluoride in polluted areas is taken up through the stomata and mainly accumulates in the tips and margins of the leaves (Braen and Weinstein 1985; Arnesen 1997). The uptake not only depends on the fluoride composition and concentration in ambient air and the exposure duration, but it also varies with the vegetation structure, plant species and growth conditions (Higueras et al. 2012).

Stomata, as regulating mechanisms for gases entering or escaping from leaves (Elagoz et al. 2006; Kardel et al. 2010), offer an excellent opportunity to study the interaction between plants and their environment, i.e., the atmosphere and its related air pollution (Robinson et al. 1998). Plants can control their stomatal characteristics, i.e., in the short term by influencing stomatal opening and closing, mainly to optimise CO_2 and water vapour exchange, and on a longer time scale (Beerling and Woodward 1997; Woodward 1998). Since stomatal characteristics are assumed to be affected by air pollution (Pal et al. 2002; Alves et al. 2008a; Kardel et al. 2010), changes in the density, distribution and morphology of stomata on a leaf surface may be considered as important traits in plants (Bettarini et al. 1998).

In the city of Sfax in Tunisia, the *Prunus dulcis* and *Olea europaea* trees are typical local trees growing over large areas; even where high levels of pollution exist, they can be seen almost everywhere in industrial and agricultural areas. The aim of this present work was to (1) survey the leaves of local fruit trees *P. dulcis* and *O. europaea* grown in industrialized areas of Sfax city for fluoride accumulation and (2) study some morphological and physiological parameters of trees exposed to industrial emissions.

Materials and methods

The study region is a low plain along the Mediterranean sea-side. It is submitted both to continental dry winds and to highly humid sea coastal winds. The prevailing winds from the southeastern sector have a frequency of 25.5 %, and those of the southwestern sector appear with a frequency of about 16.25 %. However, northwest and northeast winds occur with intermediate frequencies. The average total year rainfall is 210 mm, the average minimum air temperature of the coldest month (January) is 6.5 °C and the average maximum air temperature of the hottest month (August) is 31 °C. Most of the total annual rainfall is mostly occurring from October to December; the dry period is during June–September (data provided by the Meteorological Station of Sfax city).

The studied species were taken at distances of 1 km SW of the factory. They consist of two fruit species: *O. europaea* (cv. Chemlali), and *P. dulcis* (cv. achaak) trees planted in a loamy sand soil with a fine polyhedral structure. Another experimental station, called "control station'' (Mezghani et al. 2005; Ben Abdallah et al. 2006a), was installed at 30 km West from the factory. Air pollutants in this station are normally present in low concentrations (Mezghani et al. 2005; Ben Abdallah et al. 2006a). No fluoride emission sources are present at this location. Therefore, this area is commonly used as a reference site in biomonitoring studies (Mezghani et al. 2005; Ben Abdallah et al. 2006a, b, c). For each species three trees of approximately the same age were selected per site. Three subsamples (50–70 fully developed leaves) were randomly chosen from all sides of the crown. Leaf samples from each species were taken from several branches in different parts of the tree side exposed to the factory fume. Control samples, of the same age and same cultivars as polluted ones, were gathered by the same sampling techniques. Only leaves occupying the middle of the shoots were taken. In both sites, sampling was carried out from February to October. Leaves were thoroughly washed in distilled water to remove deposited particles from the surface (Ugolini et al. 2013). Parallel to the sampling, weekly field observations conducted regularly in the area allowed an accurate record of the necroses appearance time on both leaf species.

Leaf photosynthesis was measured using a portable infrared gas analyzer (CID 301 PS, USA) on attached leaves in the field, occupying the middle of shoots, between 9:30 and 10 am. Only the healthy leaf area was introduced in the leaf chamber. In order to avoid the effect of light intensity variation, all measurements were made on sunny days, with Photosynthetic Active Radiation being higher than 1600 µmol m^{-2} s^{-1}, by orienting the leaf chamber to obtain maximum light absorption. The average leaf temperature was 34 ± 2.55 °C.

Trichomes were removed from the lower surface of the leaves using an adhesive tape before measuring the stomatal density because the lower leaf surface was densely covered with trichomes. Observations were conducted using the Windias software and a microscope (type Leitz DIALUX 22 EB) with 250 times enlargement (Elloumi et al. 2014b).

Powdered plant samples (500 mg) were ashed at 550 °C for 1 h with 4 g of a sodium–potassium carbonate mixture, and the temperature was raised to 950 °C for an additional 30 min. The cooled ashed material was then dissolved in 20 mL of 1 M HCl, filtered into a volumetric flask, and the volume was diluted to 50 mL with demineralized water (Elloumi et al. 2005). For the potentiometric measurement of total F, the diluted solution supernatant was mixed with TISAB buffer solution (1:10) to dissociate F complexes, stabilize pH and maintain a constant ionic strength (Adriano and Doner 1982).

Proline content was measured using the methods of Bates et al. (1973). Soluble sugar and starch contents in the ground leaves were analysed according to the procedure of McCready et al. (1950) and Staub (1963).

Statistical analyses were performed with Student's *T* test. p values ≤ 0.05 were considered to indicate significant differences and are shown by * for $p \leq 0.05$, ** for $p \leq 0.01$ and by *** for $p \leq 0.001$.

Results and discussion

Regular field follow-up in the polluted area allowed us to recognize various expressions of damage caused to *P. dulcis* and *O. europaea* trees. In *P. dulcis*, damage appeared on leaf tips and margins from the beginning of the growing season. They consisted of apical brick yellow necroses extending to leaf margins (Fig. 1). Ben abdallah et al. (2006a) reported that marginal leaf slices show a high-level accumulation of fluoride suggesting, in other words, that necroses appearing in these areas probably represent fluoride-induced symptoms. In contrast, the *O. europaea* tree did not show any morphological abnormalities such as chlorosis, leaf curling, or necrosis. The under surface of *O. europaea* leaf tended to accumulate dust and appeared white. On the other hand, the leaves of the plants exposed in the control area did not show any morphological abnormalities. Mean leaf F concentrations were significantly higher in the polluted sites than in the control area. These concentrations in washed leaves collected near the fertilizer plant showed that the uptake rate differed greatly between the two species during February through October (Fig. 2). The leaf concentration of F varied from 4 µg g^{-1} dry weight in *P. dulcis* to 290 µg g^{-1} dry weight in *O. europaea* tree but remained below 10 µg g^{-1} in the plants at the control area. Since fluorine is an extraneous element to plant metabolism, its fluctuations (from 5 to 10 µg g^{-1} of dry weight), in the non-polluted area were not significant and, hence, not represented on the figures. The background level of fluoride in vegetation is generally quite low (often as low as 1 and usually less than 10 µg F/g

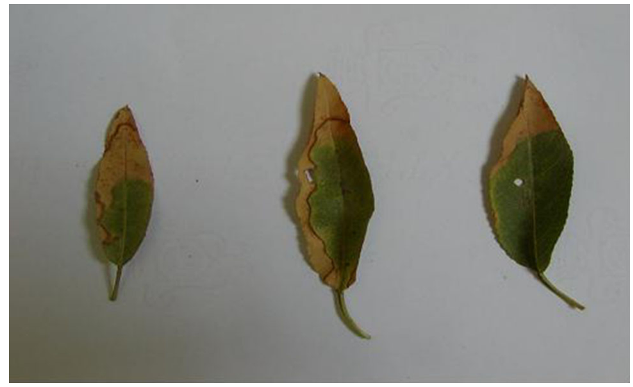

Fig. 1 Abnormal characteristic of the leaf of *Prunus dulcis* tree collected at 1 km from the phosphate fertilizer factory

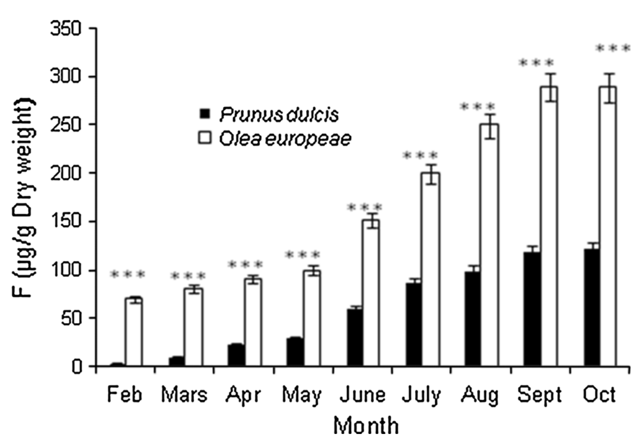

Fig. 2 Temporal variations of fluoride in *Prunus dulcis* and *Olea europaea* leaves in the polluted area. Data are mean ± SE ($n = 3$). ***$p \leq 0.001$

dry weight in most species) (Mezghani et al. 2005). Large increases in the foliar F concentration during the exposure periods were detected each month in the two species, increasing significantly from February to October. This increase coincided with a marked decrease in precipitation from February to September. Although precipitation is low during experimental period (February–September), a true dry period exists from June to September. The rainy season occurs during the autumn (October–December) and is characterized by high humidity and low temperatures. On the other hand, rain may decrease fluoride particulate concentrations in the environment, washing the material deposited on the leaves, and therefore decreasing the intensity of the pollution. The dry period is an important factor that strongly influences air pollutant concentrations. In polluted areas, the dry season accelerates pollutant deposition; therefore, pollutant availability for plant absorption is greater during drier periods. In arid regions, although high levels of solar radiation, evapotranspiration is high due to the low humidity in these areas (Allen et al. 2006). Therefore, the low relative humidity in experimental periods increases evapotranspiration, which might favour a greater accumulation of contaminants in leaf tissue.

Comparing the potential of the two studied species as pollution accumulators, it was observed that *O. europaea* showed a higher capacity to accumulate fluoride than *P. dulcis*, once the foliar F concentration on the first species was 3–3.4 times higher than in the other. Leaves of the *P. dulcis* tree, one of the most sensitive to F toxicity, may become necrotic with 65 μg F/g dry weight. *O. europaea* accumulate fluoride without exhibiting any symptoms of F toxicity with F concentrations up to 300 μg F/g dry weight. The high fluoride values obtained in sites close to the phosphate fertilizer-producing factory suggest that this factory constitutes an important source of pollution. Our results have shown that the degree of F accumulation differs substantially between both species. In our condition, *P. dulcis* and *O. europaea* leaves are affected by factory emissions, but we relate the higher values observed in *O. europaea* leaves to the longer leaf-lifespan and, therefore, to a longer duration of exposure to atmospheric pollutants. Because *O. europaea* is an evergreen species, air pollution biomonitoring can be done all year around. On the other hand, *P. dulcis* is a deciduous species. It has a leaf lifespan of only a few months, accumulating dust only during the vegetational period. All vegetables do not accumulate fluoride to the same extent and variations among them are high (Khandare and Rao 2006; Jha et al. 2008). There are different factors that can influence the leaves ability to retain atmospheric fine particles. These include exposure duration, leaf surface and texture and stomata capacity to adsorb pollutants. According to Liu et al. (2012), dust-retention amount increases with the increase of the number of stomata, suggesting that stomata and guard cells absorb dust more easily. The exposure duration seems to be one of the main factors controlling pollution retention intensity. Overall, the results of our study are consistent with previous studies comparing Mediterranean evergreen and deciduous species, which showed a much higher tolerance in evergreen species (Calatayud et al. 2010). Depending on plant species, leaf age and soil characteristics, the leaves normal fluoride content generally ranges from 1 to 10 μg g^{-1} dry weight (Mezghani et al. 2005). Many authors believe that injury to highly susceptible plants would also occur below 50 μg g^{-1} dry weight, while susceptible plants develop lesions with foliar F-amounts between 50 and 200 μg g^{-1} dry weight (Klumpp et al. 1994). Weinstein and Davison (2003) noted that highly sensitive classes included plants that showed visible injury at the furthest distance from the source, while the non sensitive (tolerant) plants showed little or no injury even when adjacent to the source.

The leaf photosynthesis rate (Fig. 3) was affected by pollution although the effect varied with species. Our results showed that pollution caused a decrease in the photosynthesis rate for both species. The reduction of the

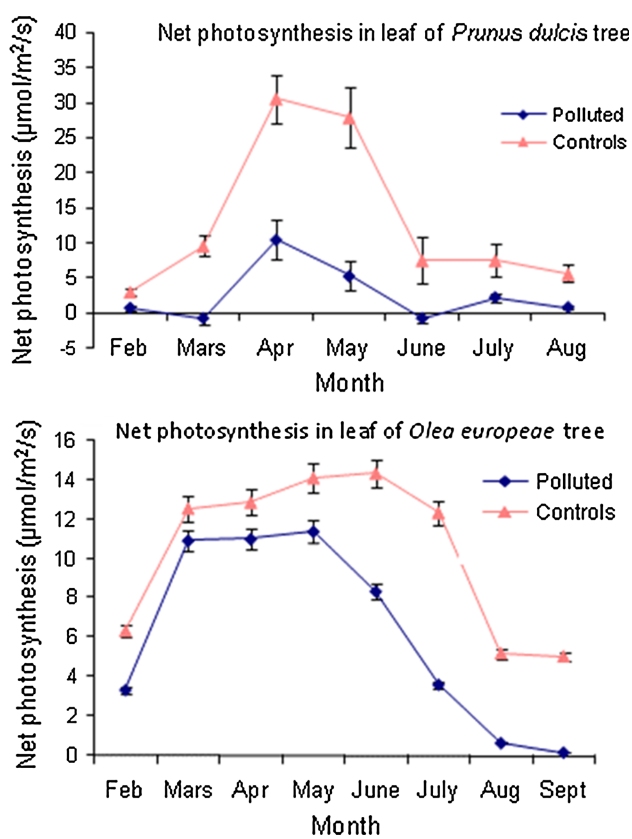

Fig. 3 Net photosynthesis of leaves of *Prunus dulcis* and *Olea europaea* trees in polluted and non-polluted area. Data are mean ± SE ($n = 20$)

photosynthesis rate uptake ranged from 18 % for *O. europaea* to 70 % for *P. dulcis*. The fluoride effects on photosynthesis were different between species. In fact, *P. dulcis* was more affected by fluoride while *O. europaea* was the most tolerant. The photosynthetic rate for the control species showed lower values in *O. europaea* compared with *P. dulcis*. In both species, photosynthesis rates were higher in spring, decreased and reached a minimum in August (Fig. 3). *P. dulcis* had significantly higher photosynthesis rates (mean maximum rate was 32 µmol). *O. europaea* had the lowest rates (mean maximum rates was 14 µmol). In April, in deciduous leaves with current season leaf expansion, the photosynthesis rate was significantly higher in *P. dulcis* leaves. Furthermore, for *O. europaea*, the photosynthesis peaks were noticed during June, coinciding with the intense vegetative growth phase of the *O. europaea* tree.

Our results showed that both species had a pronounced seasonal variation in the photosynthesis rates which were affected by date of the year, depending on ambient conditions. The high temperatures and high air vapor pressure deficit occurring during summer caused a reduction in leaf photosynthesis rates for both species. These rates were also affected by leaf age.

In summer, *P. dulcis* and *O. europaea* trees strongly reduce photosynthetic rate by limiting fluoride uptake; this would further contribute to preventing fluoride effects on these species under field conditions. The photosynthetic rate for the control species showed lower values in *O. europaea* compared with *P. dulcis*. According to Naidoo and Chirkoot (2004), the dense mat of trichomes on the lower surface would be expected to reduce light incidence on the photosynthetic tissues. Reduction of CO_2 exchange on dust covered leaves was probably due to reduction in light penetration to the photosynthetic tissues (Naidoo and Chirkoot 2004).

Observation from SEM indicate that olive leaves surface possesses stomata and is covered by trichomes (Fig. 4). The presence of large numbers of dense trichomes on the abaxial epidermis of xeromorphic *O. europaea* leaves that often overlap allowed particle trapping. Our study demonstrated that trichome density on *O. europaea* leaf surfaces was relatively greater in plants growing surrounding the phosphate fertilizer plant compared with control (Table 1). The most numerous trichomes are thus associated with a high degree of environmental pollution. These modifications seem to indicate leaf cuticular variations as an adaptation to a relatively polluted environment. On the abaxial epidermis, the dense trichomes act as a pollution screen for the small and abundant stomata. The dense trichome layer provides much protection against air pollution, keeping away air particles from the epidermal stomata.

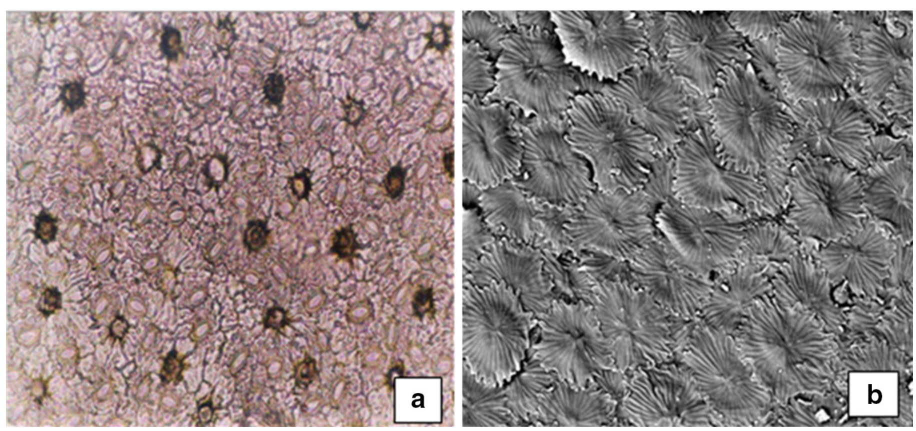

Fig. 4 Lower leaf surface of *Olea europaea* **a** Stomatal forms. **b** Dense trichome layer of abaxial surface protecting the stomata (SEM micrograph)

Table 1 Leaf properties of *Prunus dulcis* and *Olea europaea* trees from control and phosphate fertilizer industry

Leaf parameters	*Prunus dulcis* leaf		*Olea europaea* leaf	
	Control site	Industrial site	Control site	Industrial site
Stomatal density (no mm^{-2})	228.35 ± 12.80	328.83 ± 9.5***	429.17 ± 12.37	550.54 ± 10.96***
Stomatal length	23.89 ± 3.16	21.91 ± 1.57*	17.17 ± 1.96	17.90 ± 2.96
Trichomes density			197.67 ± 13.42	226.73 ± 11.62**
Trichomes length			149.06 ± 12.54	153.73 ± 17.73

Data are means per site of $n = 10$ (±SD)

Significant differences determined by Student's *t* test are indicated as follows: $p \leq 0.001$ by ***, $p \leq 0.01$ by **, and $p \leq 0.05$ by *, respectively

This may be an explanation for *O. europaea* being a pollution proof tree (Sawidis et al. 2012). No significant change in trichomes length was noticed on the lower surface of olive leaves (Table 1).

The stomatal density also seemed to indicate a significant increase in relation to polluted areas (Table 1). No apparent pattern in stomatal size in relation to habitat was discernible in *O. europaea* species (Table 1). Furthermore, stomatal lengths were lower on *P. dulcis* leaves from the industrial area than on those from the control site (Table 1). Moreover, the interaction between plants and the atmosphere occurs mainly via stomata. Therefore, stomata are ideal, integrating air quality indicators. Modification of the stomata frequency and sizes as a response to the environmental stress is an important manner for controlling pollutant absorption by plants (Gostin 2009). Gostin (2009) reported the decrease in stomatal size in *Lotus corniculatus, Trifolium montanum, Trifolium pratense,* and *Trifolium repens*. Verma et al. (2006) found a significant decrease of stomatal density and frequency in *Ipomea pes-tigridis* grown under various degrees of environmental stress. Alves et al. (2008a) reported that the stomatal density of *Eugenia uniflora* was significantly increased in an urban area in Brazil in comparison with a rural environment. An increase in stomatal density together with a reduction in stomatal size lead to an optimal control adjustment of gas exchange in general and pollutants entrance through stomata in particular (Alves et al. 2008b). Stomatal responses to air pollutants are complex and vary between species depending on leaf and plant age and are in conjunction with other environmental stressors (Robinson et al.1998; Paoletti and Grulke 2005). Furthermore, in the case of *O. europaea*, the dense trichome layer present in the lower leaf surface increases boundary layer resistance, and may eventually increase the surface of reaction with fluoride. Our study clearly demonstrated that micromorphological differences contributed to greater dust loading on the lower surfaces of *O. europaea* leaves. Specifically, leaf surfaces with trichomes have a higher capability of dust retention, while leaves with a smooth surface have a lower capacity for dust retention. This study provides further evidence of the role played by leaf characteristics in controlling dust accumulation on the leaf surface.

Soluble sugar content showed no marked differences between *O. europaea* plants in polluted and control sites (Table 2), but the amount of starch was decreased in comparison with control leaves (Table 2). For *P. dulcis*, a decrease in the soluble sugar content percentage was noted in plants situated in the polluted sites (Table 2). On the contrary, starch concentration was increased in comparison with control leaves (Table 2). These results clearly showed that the amount of starch was decreased in species that accumulated more fluoride. Singh et al. (2005) reported that starch and reducing sugar contents also decreased with increasing levels of air pollutants while soluble sugars increased. Divan Junior et al. (2007) reported that in *Chloris gayana* and *Philodendron maximum* the most notable effect of the increase in leaf fluoride content was the reduction in starch content. Hashem et al. (2013) believed that under heavy metal stress accumulation of sugar along with other compatible solutes contribute to an osmotic adjustment that allow the plants to minimize sufficient storage reserves to support basal metabolism under stressed environment. These results are in agreement with our results that were obtained for the sensitive specie "*P. dulcis*". On the other hand, the tolerant specie "*O. europaea"* showed no marked differences in soluble sugar content. This finding is also supported by Narwal and Singh (1993) who reported a decreased concentration of reducing, non-reducing and total sugars as a result of heavy metal supply in different plant species. This may be attributed to the reduced activity of net photosynthetic rates due to high external metal concentration.

This increase in starch percentage is in agreement with similar observations reported earlier on maize and barley by Bisht et al. (1976) concluding that the accumulation of starch in polluted plants was due to the inhibition of sugar and starch utilization. The above findings are also supported by various works on different plants (Bisht et al. 1976; Sanita di Toppi and Gabbrielli 1999). These effects may be due to decreased chlorophyll content, hence, a decreased photosynthesis (Alacantara et al. 1994).

Table 2 Biochemical parameters of *Prunus dulcis* and *Olea europaea* at industrial and control sites of Sfax city

Species	*Prunus dulcis* leaves		*Olea europaea* leaves	
Parameters	Control site	Industrial site	Control site	Industrial site
Proline (µmol/g fresh wt)	0.54 ± 0.05	0.83 ± 0.08**	0.46 ± 0.06	0.61 ± 0.06*
Soluble sugars (mg/g dry wt)	42.67 ± 1.29	22.24 ± 0.51***	56.76 ± 1.29	56.05 ± 2.39
Starch (mg/g dry wt)	167.2 ± 0.6	263.4 ± 0.8***	242.8 ± 0.6	125.4 ± 0.8***

Significant differences determined by Student's *t* test are indicated as follows: $p \leq 0.001$ by ***, $p \leq 0.01$ by **, and $p \leq 0.05$ by *, respectively

It has also been suggested that proline accumulation can serve as a selection criterion for the tolerance of most species to stressed conditions (Ashraf and Foolad 2007). In our case, the significant increase in proline contents (Table 2) is an important factor for providing higher tolerance to fluoride. In addition, increasing proline content is referred to as a protective mechanism due to the generation of reactive oxygen species by fluoride.

Conclusion

This study demonstrated that *O. europaea*, which is very well adapted to the climate conditions of Sfax, possesses a distinct accumulative capacity of fluoride, and additionally shows a significantly high potential of epidermis characteristics, such as stomatal density, trichome density and length, for the biomonitoring of urban habitat quality. This study favours the choice of *O. europaea* as a biomonitoring species of urban habitat quality due to its wide cosmopolitan distribution, its occurrence in a wide range of environments and its low morphological diversity (Chemlali variety). Moreover, this species is easy to sample, has a suitable height for biomonitoring and its leaves are less delicate than those of *P. dulcis*.

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