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ADAPTIVE BIOCHEMICAL RESPONSES OF *PUNICA GRANATUM* TO ATMOSPHERIC FLUORIDE POLLUTION

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ABSTRACT: Monthly observations on the southwest side of a phosphate fertilizer plant located in the coastal zone of the Gabes region of Tunisia showed that pomegranate trees (*Punica granatum*) close to the factory accumulated large quantities of the fluoride ion (F) with variable specific symptoms of toxicity. We focused on the impact of F accumulation on the photosynthetic pigment content, cell membrane, and selected osmoprotectants (proline and soluble sugars). Samples were collected at various distances from the phosphate fertilizer factory: study sites at 0.5, 2.5, and 3.5 km and a control site at 35 km. The *Punica granatum* trees accumulated significant amounts of F in the leaves, showed a marked reduction in the photosynthetic pigment content, and had damage to the cell membranes, as indicated by an increased malondialdehyde (MDA) content. The significant increases in the proline and soluble sugars content in response to fluoride accumulation may be defense mechanisms induced in response to fluoride stress.

Keywords: Airborne fluoride; Biochemical responses; Fluoride pollution; Pomegranate; *Punica granatum.*

INTRODUCTION

Fluorides are released to the environment by several industrial processes like aluminum smelters and phosphate fertilizer factories. Fluoride constitutes one of the most important phytotoxic air pollutants.[1] Atmospheric pollution constitutes one of the major problems in industrial environments. The fluoride concentration in the air varies according to industrial emissions, topography, and weather factors. Fluoride compounds in the atmosphere are deposited on vegetated surfaces in gaseous or particulate forms[2]. The leaves of higher plants intercept pollutants from wet and dry atmospheric deposition and accumulate aerial pollutants from the atmosphere. Morphological and physiological differences may also explain why different species from the same location may have varying accumulation potential of airborne pollutants and different responsiveness.[3] Weinstein and Davison[1] reported the existence of a relationship between the fluoride ion (F) concentration or dose and damage in tree leaves. Mezghani et al.[4] showed large differences in F concentrations among different plant species and large variations in the degree of plant tolerance to F pollution. Fluoride is absorbed through leaf stomata and moved by transpiration into the principal sites of accumulation at the tip and leaf margins, where it may reach toxic concentrations which result in typical necrotic

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lesions.[5,6] Fluoride concentrations in the range 1–10 µg F/g dry weight (dw) are considered as the normal background values in plant leaves.[7,8] Different plants species have been suggested for active and passive biomonitoring of airborne fluoride effects. [9,10]

Nowadays, Gabes city in Tunisia accommodates important industrial complexes, among which the phosphate fertilizer factory constitutes the main source of fluoride pollution in the atmosphere. In the city of Gabes, the pomegranate (*Punica granatum*) trees are typical local trees growing over large areas. Even where high levels of pollution exist, they can be seen almost everywhere in the industrial and agricultural areas. The aims of the present work were (i) to survey the leaves of local fruit trees *Punica granatum* grown in industrialized areas of Gabes city for fluoride accumulation and (ii) to study some morphological and biochemical parameters of trees exposed to industrial emissions.

MATERIALS AND METHODS

The study was carried at Gabes city, located 376 km south-east of Tunis on the southern side of the Gulf of Gabes (Mediterranean Sea Gabes city), which has an arid climate with a low average rainfall (from 167 to 176 mm average annual pluviometry) and an average annual temperature from 18.8 to 19.3ºC.

In this study, we selected three oases located relatively close to the factory complex (site 1 [S1] 0.5 km, site 2 [S2] 2.5 km, and site 3 [S3] 3.5 km from the factory), and, for a control oasis with less exposure, another more distant oasis (35 km from the factory).

Three trees of approximately the same age were selected at each site. Three subsamples (50–70 fully developed leaves) were randomly chosen from all sides of the crown.[3] Leaf samples from each species were taken from several branches in different parts of the tree side exposed to the factory fume. Only leaves occupying the middle of the shoots were taken. In the different sites, sampling was carried out from April to June.

Powdered plant samples (500 mg) were ashed at 550ºC for 1 hr with 4 g of a sodium-potassium carbonate mixture, and the temperature was raised to 950ºC 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.[11] 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.[12]

Chlorophyll a (chl a), chlorophyll b (chl b), and total chlorophyll (chl a+b) determinations were taken from fully expanded leaves of plants. 0.1 g of leaves were weighed and ground in 5 mL of 80% acetone. After filtration, the extraction was adjusted to 10 mL with 80% acetone, and the content of photosynthetics pigments was determined spectrophotometrically according to the method of Arnon.[13]

The level of lipid peroxidation in the leaf tissues was measured in terms of malondialdehyde content (MDA, a product of lipid peroxidation) determined by the thiobarbituric acid (TBA) reaction using the method of Heath and Packer,[14]

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with minor modifications as described by Zhang and Kirham.[15] A 0.25 g leaf sample was homogenized in 5 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 g for 5 min. We added 4 mL of 20% TCA containing 0.5% TBA to a 1 mL aliquot of the supernatant. The mixture was heated at 95ºC for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000 g for 10 min, the absorbance of the supernatant was read at 532 nm and the value of the nonspecific absorption at 600 nm was subtracted. Proline content was analyzed according to Bates et al.[16] Soluble sugars were analyzed according to Robyt and White.[17] 0.3 g of leaves was mixed with a 5.0 mL of methanol (80%) and boiled at 70ºC for 30 min. After the mixture had cooled, 1 mL of the extract was mixed with 1 mL of phenol and 5 mL of concentrated sulfuric acid. The soluble sugars concentration was calculated using glucose solutions to develop a standard curve.

All statistical analyses were performed with SPSS version 17 software. Duncan's multiple range test was used to determine the significance of differences between treatments, calculated at 5% level.

RESULTS

Compared to the leaves of the *Punica granatum* trees at the control site, the F concentration in the leaves were significantly increased, at all three sampling times in April, May, and June, at S1 (maximum 150 µg/g dw) and S2 (maximum 90 µg/g dw), and in April and May at S3 (60 µg/g dw, Figure 1).
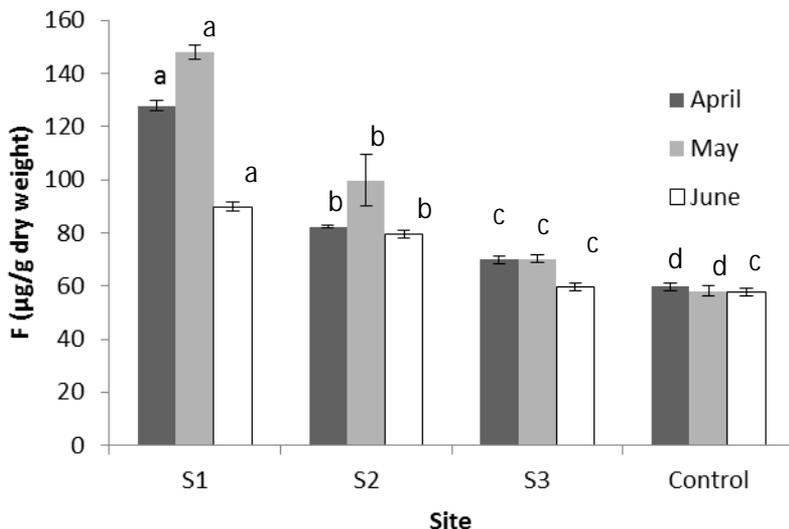

Figure 1. Temporal variation of fluoride (µg/g dry weight) in *Punica granatum* leaves at the polluted sites at increasing distances from the phosphate fertilizer factory (S1 at 0.5 km, S2 at 2.5 km, S3 at 3.5 km, and the control site at 35 km from the factory). For each month, means with different letters (a, b, c, and d) indicate a significant difference between the sites at p≤0.05 using the Duncan multiple range test.

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Additionally, the regular field follow-ups in the polluted area allowed us to recognize various expressions of damage caused to the pomegranate trees. The damage appeared on the pomegranate leaf tips and margins from the beginning of the growing season. They appeared as apical brick yellow necroses that extended to the leaf margins (Figure 2).

The pomegranate trees began to show signs of intoxication, with the characteristic signs of F phytotoxicity, at the end of May with a F content of 145 µg/g dw (Figure 1). The signs of intoxication were limited to S1. At S2, S3, and the control site, the pomegranate trees tolerated the F accumulation without exhibiting any symptoms of F toxicity.

The chlorophyll content significantly decreased maximally at S1 (depicting highest F contamination) followed by S2 (depicting moderate F contamination) and S3 (depicting lowest F contamination, Figure 3).

Figure 2. Pomegranate tree leaf showing fluoride phytotoxicity. The leaf was collected at site 1, 0.5 km from the Gabes phosphate fertilizer factory.
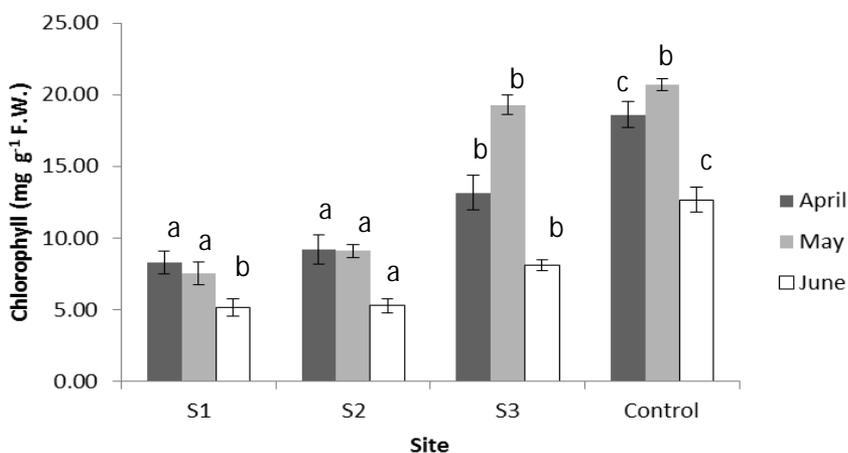

Figure 3. Total chlorophyll contents (mg/g fresh weight) in the leaves of *Punica granatum* trees at increasing distances from the phosphate fertilizer factory (S1 at 0.5 km, S2 at 2.5 km, S3 at 3.5 km, and the control site at 35 km from the factory). For each month, means with different letters (a, b, and c) indicate a significant difference between the sites at p≤0.05 using the Duncan multiple range test.

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Chl a showed no significant changes in response to fluoride accumulation. However, total chlorophylls and chl b were more sensitive to F stress. Chl b content decreased about 60% after three months of exposure to fluoride (Figure 4).
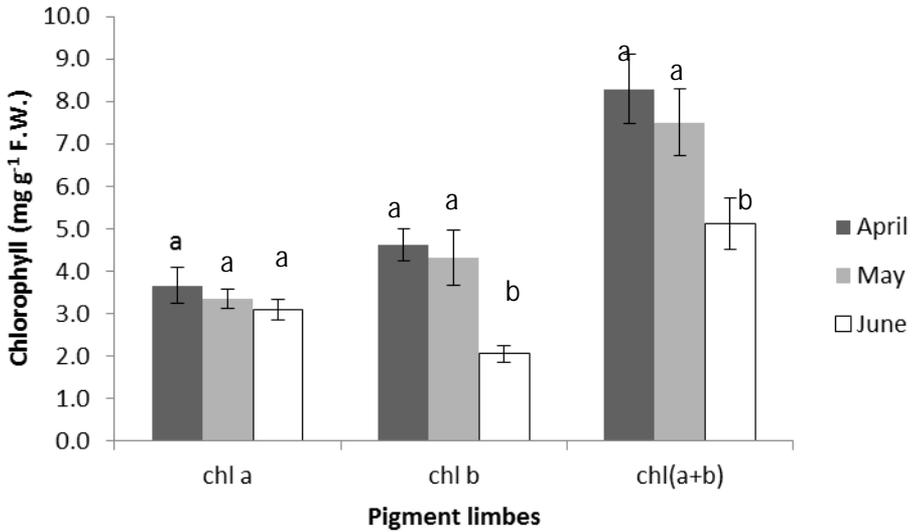

Figure 4. Chlorophyll a (chl a), chlorophyll b (chl b) and total chlorophyll (chl a+b) contents (mg/g fresh weight) in the leaves of *Punica granatum* trees at a distance of 0.5 km from the phosphate fertilizer factory. For each pigment, means with different letters (a and b) indicate a significant difference between months at $p \leq 0.05$ using Duncan multiple range test.

As an indicator of oxidative stress due to fluoride toxicity, an enhanced formation of malondialdehyde (MDA) could be demonstrated (Figure 5).
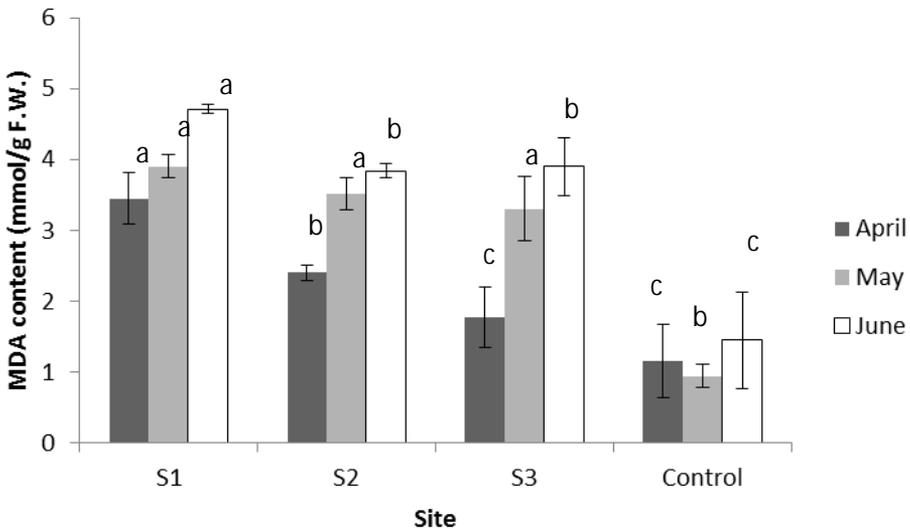

Figure 5. MDA content (mmol/g fresh weight) in the leaves of *Punica granatum* trees at increasing distances from the phosphate fertilizer factory (S1 at 0.5 km, S2 at 2.5 km, S3 at 3.5 km, and the control site at 35 km from the factory). For each month, means with different letters (a, b, and c) indicate a significant difference between the sites at $p \leq 0.05$ using the Duncan multiple range test.

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The MDA content rose markedly at the polluted sites indicating enhanced lipid peroxidation. The maximum increase was found in June. Large increases in the foliar MDA accumulation during the exposure periods were detected each month at all the sites, increasing significantly from April through June.

The impact of fluoride accumulation on some selected osmoprotectants (proline and soluble sugars) was determined. Compared to the control site, the proline content at S1 was significantly increased in May and June but no significant increases were found at S2 and S3 (Figure 6).
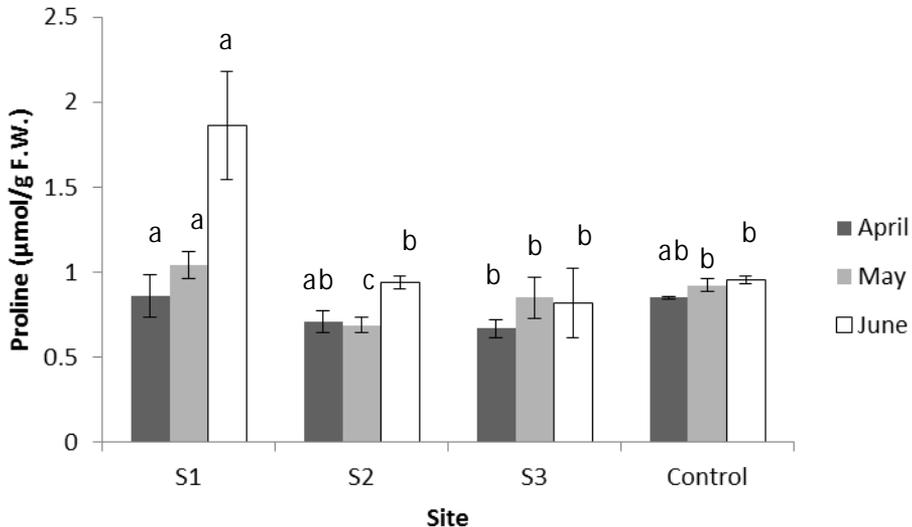

Figure 6. Proline content (µmol/g fresh weight) in the leaves of *Punica granatum* trees at increasing distances from the phosphate fertilizer factory (S1 at 0.5 km, S2 at 2.5 km, S3 at 3.5 km, and the control site at 35 km from the factory). For each month, means with different letters (a, b, and c) indicate a significant difference between the sites at $p \leq 0.05$ using the Duncan multiple range test.

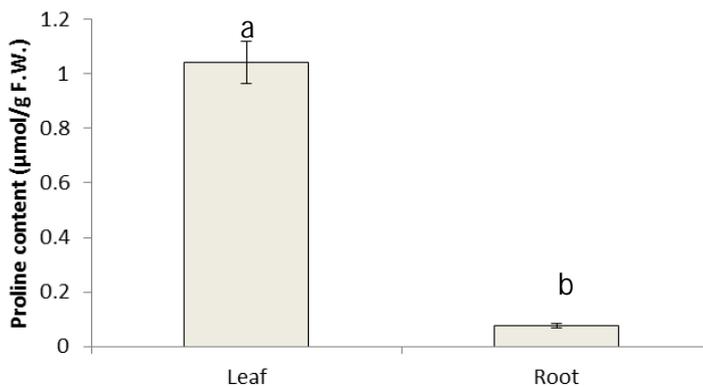At S1, the leaves showed a higher proline content than the roots (Figure 7).

Figure 7. Proline content (µmol/g fresh weight) in the leaves and roots of *Punica granatum* trees at S1, 0.5 km from the phosphate fertilizer factory. Means with different letters (a and b) indicate a significant difference between the organs at $p \leq 0.05$ using the Duncan multiple range test.

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The fluoride content also had a great influence on the leaf soluble sugars concentration (Figure 8). The lowest (S3)and the highest (S1) F concentrations induced an increase by 46 and 80%, respectively, of the leaf soluble sugars concentrations in June.
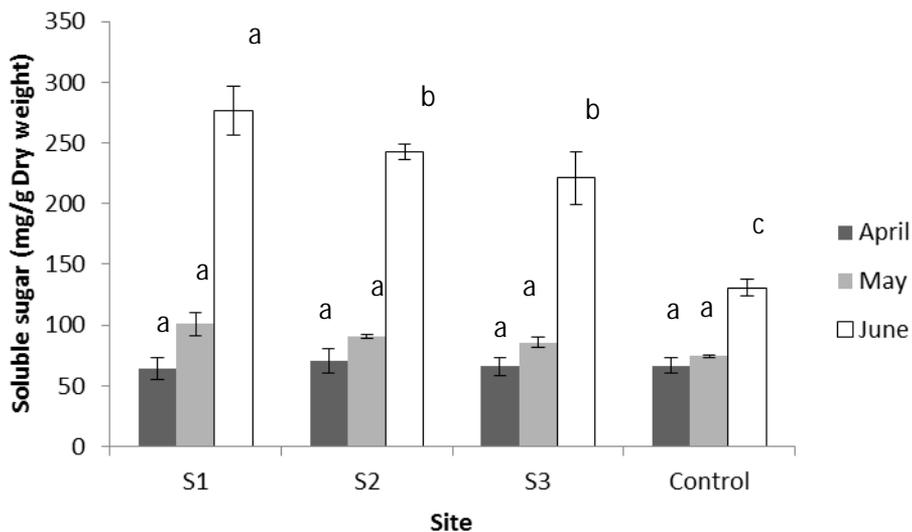

Figure 8. Soluble sugars content (mg/g dry weight) in the leaves of *Punica granatum* trees at increasing distances from the phosphate fertilizer factory (S1 at 0.5 km, S2 at 2.5 km, S3 at 3.5 km, and the control site at 35 km from the factory). For each month, means with different letters (a, b, and c) indicate a significant difference between the sites at p≤0.05 using the Duncan multiple range test.

DISCUSSION

The accumulation of fluoride in the leaves of the pomegranate trees differed significantly at the four sites and at the different sampling times. When comparisons were made for the three months at the same site, significantly higher concentrations of F were found in the May samples. The maximum F concentration was found at S1 where the plant F accumulation was two times higher than at S3 and at the control site. These results clearly showed a gradient of fluoride contamination. Large increases in the foliar F concentration, compared to the control site, were detected from April to May. A smaller significant increase, compared to the control site, was still present in June in S1 and S2 but not at S3. This decrease in the F content in June, compared to the values in April and May, coincided with a marked precipitation registered for June (117 mm). With the high fluoride stress at S1, the leaves became narrowed and suffered chlorosis and necrosis. The symptoms observed on this species at S1 are consistent with those reported in the literature.[18,19] These symptoms were restricted to the trees at the site (S1) closest (0.5 km) to the phosphate fertilizer factory. In agreement with the visual perturbations, the chlorophyll content decreased with the elevations of the F concentration. Ben Abdallah et al.[19] showed that necrotic tissue accumulates

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greater amounts of F than adjacent, healthy tissue. This reduction in the chlorophyll content of the sampled leaves, as compared to the control leaves, is attributed to the high emission and deposition of dust on the leaves, which adversely affects the metabolic activity of the plant. A significant pigment loss was noted in total chlorophyll and chlorophyll b following the exposure to fluoride air pollution. Fluoride-induced chlorosis and symptoms seem to be due to an inhibition of chlorophyll synthesis.[20] In our study, we detected the maximum decrease in chlorophyll content for site S1. The reason for this decrease could be due to disturbances of the pigment synthesis mechanism due to fluoride effects. A decreased chlorophyll content causes an inhibition of photosynthetic activity of plants. Some organic solutes in plants (such as proline and soluble sugars) act as osmoprotectants in adaptation to environmental stress such as with drought, heavy metals and increased salinity.[21,22] Sugar metabolism is adversely affected in plants growing under stressful conditions.[23] In many plant species, the accumulation of soluble sugars has been observed in response to various environmental stresses. [24,25]

CONCLUSION

The results of the present work showed that exposure of *Punica granatum* to fluoride air pollution increased proline and sugar contents. The significant increase in proline contents is an important factor for providing higher tolerance to fluoride. The increased proline content is referred to as a protective mechanism due to the generation of reactive oxygen species by fluoride. The accumulation of proline under the effect of stress provides energy for the growth and survival of the plant and helps it to tolerate stress.

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