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MULBERRY LEAVES AS A BIOINDICATOR OF FLUORIDE POLLUTION IN THE VICINITY OF A PHOSPHATE FERTILIZER FACTORY LOCATED IN SFAX, TUNISIA

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ABSTRACT: Since the response of plant species to air pollutants is variable, the classification of plants into sensitive and tolerant groups is vital because the former can serve as biological indicators and the latter as sinks for the air pollutants. The morphological, biochemical, and physiological effects of fluoride stress were investigated in mulberry plants (*Morus alba* L.) grown at different distances from a phosphate fertilizer factory in Tunisia. Gas exchange (photosynthetic rate, stomatal conductance, and transpiration rate), photosynthetic pigments (chlorophyll and carotenoids) and water status (leaf relative water content) were decreased in the mulberry leaves grown near the polluted sites. However, lipid peroxidation (malondialdehyde content) and osmotic regulator (proline and soluble sugars content) levels were increased as compared to the control site. The obtained results suggest that *Morus alba* leaves could be considered as an air pollution bioindicator.

Keywords: Chlorophyll; F stress; Leaf relative water content; Mulberry plant; Net photosynthesis; Proline accumulation; Soluble sugars.

INTRODUCTION

Pollution is a major environmental concern, particularly in cities. The quality of urban soil and air is adversely affected by emissions from motor vehicles and industrial sources to the detriment of the health of people, animals, and plants alike. The response of plant species to air pollutants, such as fluoride (F) is variable, and consequently the classification of plants into sensitive and tolerant groups is vital because the former can serve as biological indicators and the latter as sinks for air pollutants.

With F air pollution, plants can uptake F directly from the air via the leaves.[1,2] The phytotoxic effects of F are probably a consequence of its interference with a number of metabolic processes.[3] According to Elloumi et al.,[1] F has adverse effects on the physiological behavior of plants, such as alterations of gas exchange parameters, inhibition of photosynthetic pigments, water status, and development. The exposure of plants to fluoride creates suboptimal conditions as the F is phytotoxic and results in the production of reactive oxygen species (ROS). These ROS react very rapidly with lipid membranes, resulting in the induction of lipid peroxidation.[4,5,6] Furthermore, plants exposed to F pollution react with physiological and biochemical adjustments which help survival under these stressful conditions.[1,3,6] In addition, a strong correlation between higher cellular levels of proline and soluble sugars, and F stress tolerance has been shown in some plants.[7]

Over the years, biomonitoring has become an important complement to the traditional techniques for measuring air quality. Bioindicator organisms are able to reveal alterations in air quality before it severely affects human health or the biotope.

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Such organisms are characterized by a low ecological tolerance to certain chemicals. Indeed, they can present a physiological, morphological, or behavioral alteration, when they are exposed to certain pollutants. Nowadays, Sfax, the second city of Tunisia, accommodates one of the most important industrial complexes, among which the (SIAPE) factory constitutes the main source of F pollution in the atmosphere. Considering the role of plants in pollution biomonitoring, the present study was conducted to clarify the morphological, physiological, and biochemical responses to fluoride pollution stress of adult mulberry (*Morus alba* L.) leaves, including lipid peroxidation, gas exchange, and the content of photosynthetic pigments, proline, and soluble sugars.

MATERIALS AND METHODS

The studied adult mulberry (*Morus alba* L.) plants used for the study were taken from three experimental sites located along the coast of Sfax: polluted sites *S1* and *S2* at distances of 1 and 3 km, respectively, from the phosphate fertilizer factory and a control site (*S3*) at a distance of 30 km west of the factory. The main pollutants emitted by this factory are fluoride compounds.[1,3] However, no pollution sources were present in the control site which is an area commonly used as an unpolluted site. The mulberry plants from the three sites (*S1*, *S2*, and *S3*) were similar in age, plant density, and training system. They are planted on a sandy loamy soil. The polluted and control sites presented very similar geochemical, ecological, and climatic conditions. The three sites are subject to an arid climate. The mean annual precipitation is 220 mm (the minimum mean precipitation [1 mm] was in July, while the maximum mean precipitation [300 mm] was in December). The mean annual temperature is 19.0ºC (the minimum mean temperature [6.5±2ºC] was in January, while the maximum mean temperature [32.5±2.5ºC] was in July). Their distances to the sea were 1 and 3 km for the polluted and control sites, respectively, and thus they were not subject to sea sprays.

Five samples (50–70 leaves) were taken from several branches chosen from all sides of the plant. The same type of leaves was used for all measured parameters. Control samples were gathered by the same sampling technique. In all the sites, sampling was carried out in May 2014. After harvesting, the leaves were washed extensively with distilled water, and then samples of leaves were collected and frozen in liquid nitrogen at –80ºC. Other samples of leaves were oven-dried at 70ºC to a constant mass and then ground.

Fluoride content: F content was determined using the potentiometric technique as previously described. [5]

Photosynthetic pigments and gas exchange measurements: Chlorophyll, carotenoids, net photosynthesis, stomatal conductance, and transpiration rate were determined as previously described. [1]

Malondialdehyde content: The levels of lipid peroxidation were estimated by the content of the malondialdehyde. Malondialdehyde was determined as previously described. [5]

Leaf relative water content, proline content, and soluble sugars content: Leaf relative water content, proline content, and soluble sugars content were determined as previously described.[7]

Statistical analysis: A one-way analysis of variance (SPSS software, 18.0) was performed. Tukey's test ($p \leq 0.05$) was used to compare the averages of all the measured parameters. At least, five replicates were performed for each measurement.

RESULTS

The results describing the F levels in the atmosphere of the different sites (*S1*, *S2*, and *S3*) are presented in Table 1. In fact, in the site closer to the phosphate fertilizer factory, the F levels in the atmosphere were higher, with the highest levels being registered in *S1* (11.35 µg/dm^3).

Table 1. Atmospheric fluoride concentrations (µg/dm^3) in the three different sites: *S1* = 1 km from the phosphate fertilizer factory, *S2* = 3 km from the factory, and *S3* = 30 km from the factory (control)

Parameter	Site		
	S1	*S2*	*S3*
Fluoride in atmosphere (µg/dm^3)	11.35±0.63[a]	5.6±0.56[b]	3.0±0.14[c]

Values are means of five samples±standard deviations.
Different letters indicate significant differences ($p \leq 0.05$, Tukey's test) between the atmospheric fluoride concentrations in the different sites.

Similarly, the results obtained in the present study showed that the leaves of the mulberry plants grown in the polluted sites (*S1* and *S2*) accumulated a higher F content compared with the plants grown in the control site (*S3*) (Figure 1). The increases were 22- and 8-fold for the mulberry plants grown in the *S1* and *S2* sites, respectively, in comparison with the F content in the leaves of the control plants.

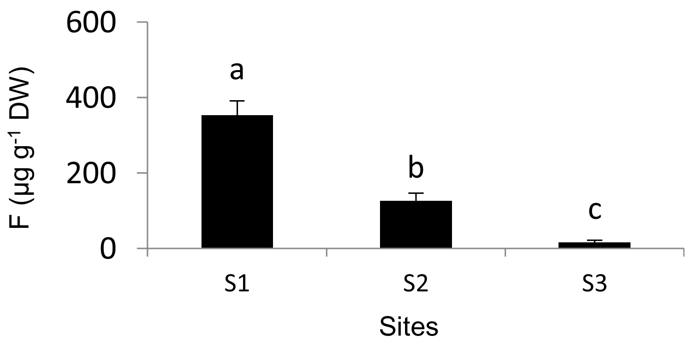

Figure 1. Fluoride content in the leaves of mulberry plants grown in the control (S3) and the polluted sites (S1 and S2). Values are the means of five samples±standard deviations. Different letters indicate significant differences ($p \leq 0.05$, Tukey's test) between the plants grown in the different sites.

The results displaying the leaf malondialdehyde (MDA) content in the leaves of mulberry plants grown in the different sites revealed that this parameter increased with increasing F stress (Figure 2). Compared with the control plants, the MDA leaf content increased about 93% and 48% in the plants grown in the *S1* and *S2* sites, respectively.

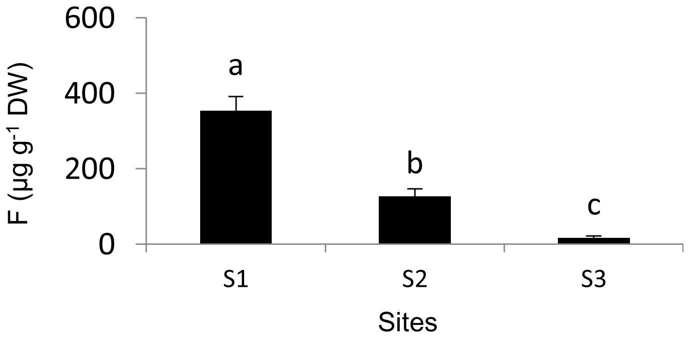

Figure 2. Malondialdehyde content in the leaves of mulberry plants grown in the control (S3) and the polluted sites (S1 and S2). Values are the means of five samples±standard deviations. Different letters indicate significant differences (p≤0.05, Tukey's test) between the plants grown in the different sites.

Concerning the water status, the results obtained showed that the relative water content in the leaves (LRWC) of the polluted mulberry plants was significantly ($p \leq 0.5$) lower than those of plants grown in the unpolluted site (Figure 3).

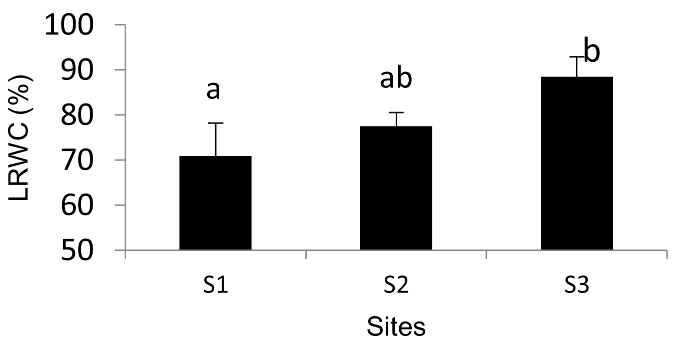

Figure 3. Leaves relative water content of mulberry plants grown in the control (S3) and the polluted sites (S1 and S2).
Values are the means of five samples±standard deviations. Different letters indicate significant differences (p≤0.05, Tukey's test) between the plants grown in the different sites.

Table 2 shows changes in the gas exchange parameters (net photosynthesis Pn, transpiration rate E, and stomatal conductance Gs) in the leaves of the mulberry plants exposed to F stress pollution. As compared to the control site *S3*, the mulberry plants grown in both the *S1* and *S2* sites showed a a significant decrease ($p \leq 0.05$) in their Pn, Gs, and E parameters, with the levels being significantly lower ($p \leq 0.05$) in the plants grown in S1 compared to those grown in S2. Moreover, the decrease of the gas exchange parameters in the fluoride-stressed mulberry plants was associated with many reductions in photosynthetic pigments (Table 2). The highest reduction of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids was recorded in plants grown in *S1*. For example, in comparison to control plants, Pn and total chlorophyll decreased about 53% and 58%, respectively, in mulberry plants grown in *S1*.

Table 2. Gas exchanges, chlorophyll content, and carotenoids content of mulberry plants growing at different sites

Parameter	Site		
	S1	*S2*	*S3*
Net photosynthesis (μmol m^{-2} s^{-1})	9.86±1.84[c]	16.59±1.90[b]	21.07±2.96[a]
Stomatal conductance (mmol m^{-2} s^{-1})	103.33±24.70[c]	166.00±18.75[b]	303.66±64.63[a]
Transpiration rate (mmol m^{-2} s^{-1})	2.27±0.44[c]	4.04±1.44[b]	7.55±2.04[a]
Chlorophyll a (mg g^{-1} FW)	1.11±0.22[c]	2.73±0.07[b]	3.75±0.15[a]
Chlorophyll b (mg g^{-1} FW)	1.26±0.27[b]	1.58±0.30[ab]	1.88±0.15[a]
Total chlorophyll (mg g^{-1} FW)	2.38±0.30[c]	4.31±0.36[b]	5.63±0.22[a]
Carotenoids (mg g^{-1} FW)	0.89±0.10[ab]	0.9±0.12[a]	1.01±0.09[a]

Values are means of five samples±standard deviations.
Different letters indicate significant differences ($p \leq 0.05$, Tukey's test) between the atmospheric fluoride concentrations in the different sites.

Furthermore, in this study the F-stressed mulberry plants showed visual leaf symptoms toxicity such as necrosis and/or chlorosis (Figure 4). Less visible leaf injury was observed at the *S2* site than at the *S1* site, while no visible injury was found in the control plants.

Under fluoride stress, the proline and soluble sugars content in the leaves of the mulberry plants increased with increasing F stress (Figures 5A and 5B). In the plants grown in *S1*, these parameters increased by 172% and 192%, respectively, in comparison to the plants grown in the control site, *S3*.

Figure 4. A control mulberry leaf from the control site (on left) and a leaf with fluoride damage from a F-polluted site (on right).

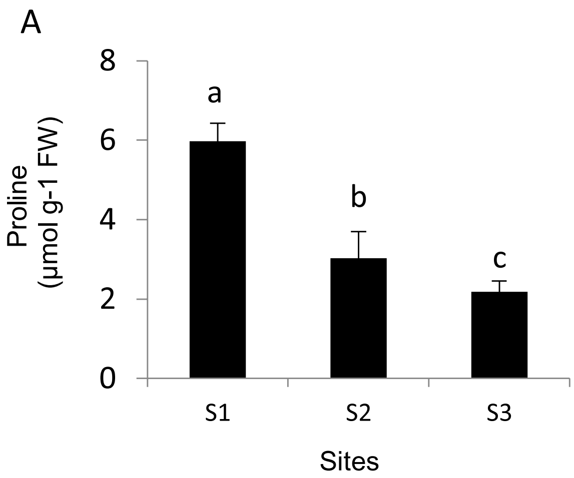

Figure 5A. Proline content in leaves of mulberry plants grown in the control (S3) and the polluted sites (S1 and S2).

Values are the means of five samples±standard deviations. Different letters indicate significant differences (p≤0.05, Tukey's test) between the plants grown in the different sites.

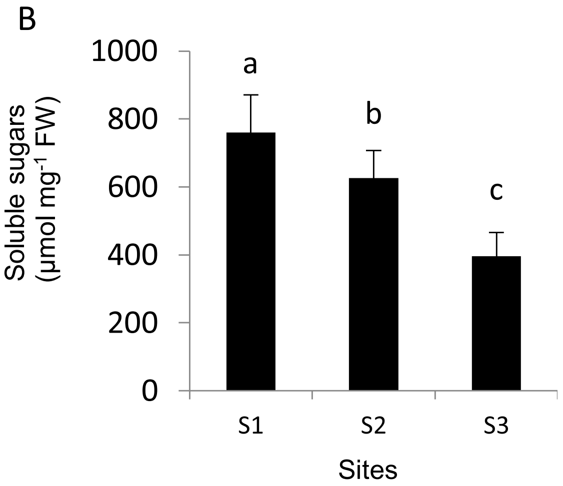

Figure 5B. Soluble sugars content in leaves of mulberry plants grown in the control (S3) and the polluted sites (S1 and S2).

Values are the means of five samples±standard deviations. Different letters indicate significant differences ($p \leq 0.05$, Tukey's test) between the plants grown in the different sites.

DISCUSSION

The obtained results demonstrated that the mulberry plants grown in the polluted sites showed high levels of F in the leaf tissues. These results confirm the presence of F stress pollution. Previous reports indicated that plants grown under F-contaminated sites efficiently accumulated F in their foliage.[1,2] Not all plants accumulate F to the same extent, with high variations between different species. Referring to Elloumi et al.,[1] several plant species showed a high accumulation of F. Furthermore, these species survive without any fluoride toxicity and are highly tolerant. However, other plant species which uptake fluoride at a lower concentration show necrotic symptoms and are F sensitive.[3] In the present study, the presence of visual leaf symptoms of toxicity, such as necrosis and/or chlorosis, in the mulberry plants grown in the polluted sites confirms the sensitivity of this species to F and indicates that the species can be used as a significant morphological biomarker of F pollution. In our present study, the increase in the MDA content in the mulberry leaves under F stress reflected the oxidative stress induced by F. The stress level in the plants increased as the F content in plant tissue increased. Similar findings were obtained by Elloumi et al.[4] in loquat trees (*Eriobotrya japonica*) grown under F stress. The significant increase of the MDA content in the F-stressed mulberry plants could be considered as an effective biomarker of the F-sensitivity of this species and as an indicator of cellular oxidative damage as suggested by Zouari et al.[5]

F accumulation seems to alter the leaf water status in stressed mulberry plants. Previous reports have also suggested that reductions in LRWC are among the earliest responses detected in plants under stressed conditions, such as water deficit, salt stress, or pollution stress.[6,7,8] This decrease in leaf water status of F-stressed mulberry plants could be explained by the high fluoride content in the leaf plant tissues, which results in osmotic stress and partial dehydration at the cellular level.

Gas exchange parameters (Pn, E, and Gs) and the chlorophyll content of the plants signifies the plant photosynthetic capacity as well as the capacity for growth and development. According to Kumar et al.[9] and Li et al.[10], the alteration of the photosynthetic machinery is widely used as an indicator of pollution stress. It is evident that the Pn and the chlorophyll content vary with the tolerance and the sensitivity of the plant species, i.e., a higher level of plant sensitivity to pollution stress is associated with a lower level of Pn and chlorophyll content.[11,12,13] Our results showed that the photosynthetic capacity was significantly reduced in parallel with an increase in the concentrations of F in the leaves of mulberry plants (Table 1). Decreases in the gas exchange parameters and the photosynthetic pigments have been reported for other plant species grown in F-polluted areas, including *Prunus dulcis* and *Vitis vinifera*.[14,15] Elloumi et al.[4] reported that the abnormality of stomata in terms of a reduction of stomata density, the closure of stomata, and a decrease of stomata pore size which can alter stomatal conductance and thereby reduce the photosynthetic rate and the photosynthetic pigment content in F-stressed mulberry plants. According to Zouari et al.,[16] the reduction of the photosynthetic capacity in the F-stressed mulberry plants could also be related to the disorganization of the thylakoid system. The obtained results are in agreement with those of Rhimi et al.[3] who reported that one of the most common impacts of air pollution is the gradual disappearance of chlorophyll and concomitant leaf chlorosis and necrosis that may be associated with a consequent decrease in photosynthetic capacity.

Despite these biochemical and physiological alterations, the F-stressed mulberry plants have an ability to grow in F-contaminated sites. Mulberry plants react to the stressful condition of F-pollution through several adjustments which assist survival.

Recent studies have shown great interest in the use of osmoticum biomarkers as a means for monitoring the environment. The present data showed a significant increase in soluble sugars and proline content in the mulberry plants grown in the F-polluted sites. Similar results were observed by Singh et al.[17] who reported an increase of proline content in the leaves of *Solanum melongena* grown under F-pollution stress. These authors have proven that an increase or inhibition of the content of certain osmotic adjustment substances is a possible response to the environment stress.

CONCLUSIONS

Different biomarkers, such as morphological symptoms, F content, MDA, LRWC, gas exchange, photosynthetic pigments, proline and soluble sugars of mulberry (*Morus alba* L.) leaves, were significantly changed in the plants grown in the F-polluted area in comparison with the control plants. The presence of these alterations in mulberry leaves due to F air pollution suggests that mulberry leaves may be a suitable bio-indicator of F air pollution.

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