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Research Article

SOME INDICATORS OF DAMAGES AND RESPONSES OF *XANTHORIA PARIETINA* (L.) Th. Fr. TO FLUORIDE AND LEAD INDUCED-STRESS

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ABSTRACT. One of the major problems of current time is air pollution, the assessment of air quality through the use of bioindicators is a major concern at the moment. Our work aims to study the response of *Xanthoria parietina* (L.) Th. Fr. to fluoride- and lead-induced stress as an example of the phytotoxic air pollutants. For this purpose, lichen thalli have been treated with sodium fluoride (NaF) and lead nitrates ($Pb(NO_3)_2$) solutions at 0, 0.5, 1.0, 5.0 and 10 mM, for time scale of 0, 24, 48 and 96 h. Lipid peroxidation measured by Malondialdehyde (MDA) and chlorophyll degradation measured by optical density OD_{435}/OD_{415} ratio are used as results of stress induced by fluoride and lead, and the accumulation of proline and soluble sugars are measured as indicators of responses used by *X. parietina.* Based on the obtained results, it was noted that lipid peroxidation increased correlating with increasing concentrations of NaF and $Pb(NO_3)_2$ (r=0.773, p=0.000712*** and r 0.865, p=0.000031***, respectively), however, chlorosis and proline increased correlating with increasing exposure time of NaF (r=- 0.737, p=0.0011** and r=0.783, p=0.00032***, respectively) and $Pb(NO_3)_2$ (r=-0.926, p<0.0001*** and r=0.811, p=0.00013***, respectively), whereas soluble sugar contents increased according to increasing concentrations of NaF (r=0.678, p=0.0010***) and according to increasing exposure time of $Pb(NO_3)_2$ (r=0.780, p=0.00036***). Although lead was significantly more toxic than fluoride (p=0.02*), *X. parietina* offers a very high sensitivity to fluoride, which allowed us to conclude that the toxicity of fluorine is comparable to that of lead.

Keywords: *Chlorophyll, fluoride, lead, MDA, proline, soluble sugars.*

INTRODUCTION

Lichens present a very important model of symbiotic organisms associating a mushroom called mycobiont and green algae and/or cyanobacteria called photobiont [1], one of its main uses is the use as bioindicators of air quality [2, 3, 4]. Lichens have the ability to absorb significant amounts of trace elements from the atmosphere [5, 6]. Lichens are also used as biomonitors [7, 8, 9] and bioaccumulators of heavy metals [10, 11, 12, 13]. They are capable of growing on difficult supports such as coastal rocks [14], and differed from most other eukaryotic organisms in their physiology, morphology and their ability to tolerate extreme stresses [15]. Despite their distribution and diversity are influenced by climate, soil chemistry and geography [16], lichens can resist various stressful conditions such as drought and temperature extremes [17], salinity [18], heat [19] heavy metals [12] and nutrient deficiency [20]. These stresses are the source of reactive oxygen species (ROS), the most important adaptation mechanism used by lichens for tolerance to stressful conditions is the scavenging of reactive oxygen species (ROS) [21]. Lichens can also respond to stressful situations by displaying conventional stress-tolerant traits including reduced growth rates, considerable longevity, low nutrient demand and the presence of particular morphological and physiological adaptation and changes in ecological behaviour for surviving in the world most hostile situations [22].

Stressful conditions caused several biochemical and physiological changes in the plants. The most commonly used parameters to study the toxicity of atmospheric pollutants on lichens are chlorophyll degradation [23, 24] and lipid peroxidation [25, 26].

Exposure to abiotic stress triggers the accumulation of amino acids and amines in different plant species. Soluble sugars play a crucial role in a variety of metabolic processes, acting as a signal to control gene expression in photosynthesis, osmolyte production, and sucrose metabolism [27]. Proline, on the other hand, is crucial for plants; it protects plants from various stresses and helps in their faster recovery from stress [28].

Our work which relates to the study of the toxic effect induced by fluoride and lead on *X. parietina*, is the first to be carried out in our region.

The aim of this study is to investigate the toxic effect of fluoride and lead on lipid peroxidation and chlorophyll degradation in *X. parietina* (L.) Th. Fr. lichen, and to check for the accumulation of proline and soluble sugars as indicators of responses used for adaptation to induced stress.

MATERIALS AND METHODS

Lichen material

Lichen thalli samples of *X. parietina* were gathered in the Beni Metrane and Djimar region, south of Jijel (Algeria) in February-March 2018. After collection, samples were transferred to the laboratory in clean closed boxes. Impurities were removed, and samples were washed with distilled water to remove superficial dust and adherent particles. In each experimental vessel, fresh weights of thalli were isolated and acclimatized to laboratory conditions until analysis.

Fluoride and lead treatment

In comparison to distilled water, the lichen thalli of *X. parietina* were incubated in solutions of NaF and $Pb(NO_3)_2$ at 0, 0.5, 1.0, 5.0 and 10.0 mM concentrations at room temperature. H_2SO_4 or HNO_3 were added to the solutions immediately before treatment to adjust pH to 3.5. These solutions were then stored in the dark for 0, 24, 48, and 96 h at room temperature. The samples were rinsed three times with distilled water after treatment and before each assay [29].

MDA assay

For the MDA assay, the Health and Packer [30] method was used. 200 mg of the lichen thalli were homogenized in 2 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 10000 g for 20 min. To 1 mL of supernatant, 1 mL of 20% TCA containing 0.5 mL of thiobarbituric acid and 0.001 mL of butylated hydroxyl-toluene BHT at 4% solution in ethanol were added. The mixture was heated at 95 °C for 30 min and centrifuged at 10000 g for 15 min, the supernatant absorbance was read at the wavelength 532 nm and corrected at 600 nm. MDA was calculated by multiplying by the coefficient of 155 $mM^{-1}cm^{-1}$; the results were expressed in µmol g^{-1} FW.

Integrity of chlorophyll assay

According to Ronen and Galun [31], the integrity of chlorophyll was calculated. About 20 mg of lichen thalli were extracted in 3 mL of dimethylsulfoxide (DMSO) in dark at 65 °C for 40 min. The optical densities at 435 nm and 415 nm for the extract were read, and the ratio OD_{435}/OD_{415} was calculated to assess the degree of chlorophyll degradation. Ronen and Galun (1984) estimate that a ratio between 1.4 and 1.45 was calculated in lichens in the case of minimal chlorophyll degradation into phaeopigments.

Proline assay

The method used for the determination of proline is that of Troll and Linsley [32], 100 mg of fresh lichen thalli were extracted in 2 mL of 40 % methanol at 85 °C for 60 min. After cooling, 1 mL of acetic acid and 1 mL of a mixture containing (120 mL of distilled water, 300 mL of acetic acid and 80 mL of acid orthophosphoric acid and 25 mg of ninhydrin) were added to 1 mL of the extract. The solution was brought to the boil for 30 min, it gradually turns red. After cooling, 5 mL of toluene was added; the upper phase which contains the proline was recovered and dehydrated by the addition of disodium sulphate. The optical density was determined by a spectrophotometer at a wavelength of 528 nm and the calibration curve was established by different concentrations of proline from a stock solution of 2 mg/100 mL of 40% methanol. Proline contents were determined using equation established by known concentrations of proline (y=28.0 x, R^2=0.9911); the results were expressed in µg g^{-1} FW.

Soluble sugars assay

The soluble sugar contents were quantified by the method of Dubois et al. [33]. 100 mg of fresh lichen material were extracted in 3 mL of 85% ethanol for 48 h in the dark, then filtered and recovered with 20 mL of distilled water, 1 mL of 5 % phenol and 5 mL of 1.8 N sulfonic acid were added to 1 mL of the filtrate. After incubation for 15-20 min in a water bath adjusted to 30 °C, the optical densities were determined at the wavelength 490 nm. The calibration curve was established by glucose at different concentrations prepared from a stock solution of 250 mg/L. Soluble sugar contents (µg.g^{-1} FW) were calculated using equation established by known concentrations of glucose (y=0.731x + 0.001, R^2=0.999).

Statistical analysis

Three repetitions were performed at each concentration, so we can calculate the standard deviation (SD). The statistical study was performed using the ORIGIN 6.0 system using the test univariate variance (one way ANOVA). For the study, the results were expressed as mean±SD. The difference was considered to be not significant when $p>0.05^{(NS)}$, significant when $0.01<p<0.05$ (*), highly significant when $0.001<p<0.01$ (**) and very highly significant when $p<0.001$ (***).

Correlation matrices between NaF, $Pb(NO_3)_2$ and different studied parameters were analyzed by STATISTICA Version 10 software.

RESULTS AND DISCUSSION

MDA accumulation

Results of the MDA contents in the thalli of *X. parietina* treated by different concentrations of NaF and $Pb(NO_3)_2$ during 0, 24, 48 and 96 h are presented in Fig. 1. Correlation matrices between NaF / MDA, $Pb(NO_3)_2$ / MDA are presented in Fig. 2.

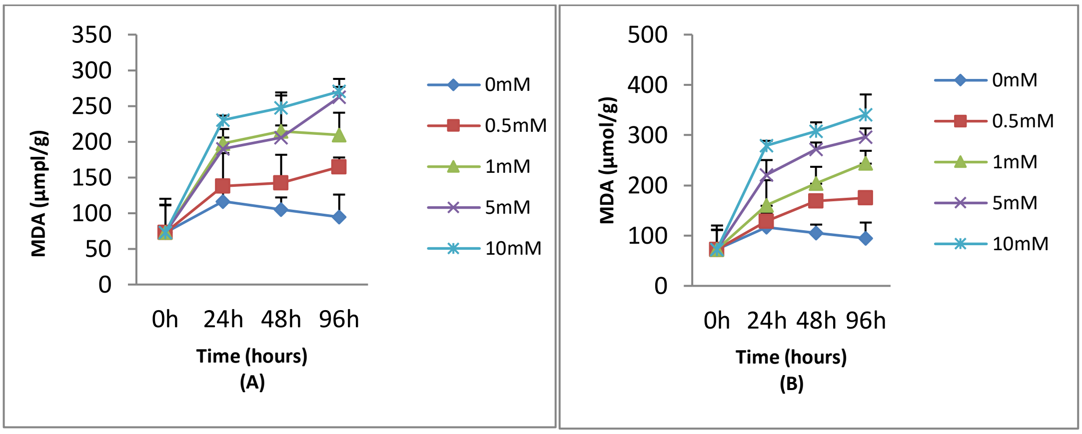

Fig. 1. *MDA accumulation in X. parietina treated with different concentrations of (A): NaF, (B):* $Pb(NO_3)_2$

According to Fig. 1, no significant increase in the MDA content was noted as a function of the concentrations of NaF (p=0.179^{NS}) and $Pb(NO_3)_2$ (p=0.109^{NS}). However, depending on exposure time, a significant increase of MDA content was noted with treatment by NaF (=0.005**) and $Pb(NO_3)_2$ (p=0.014*).

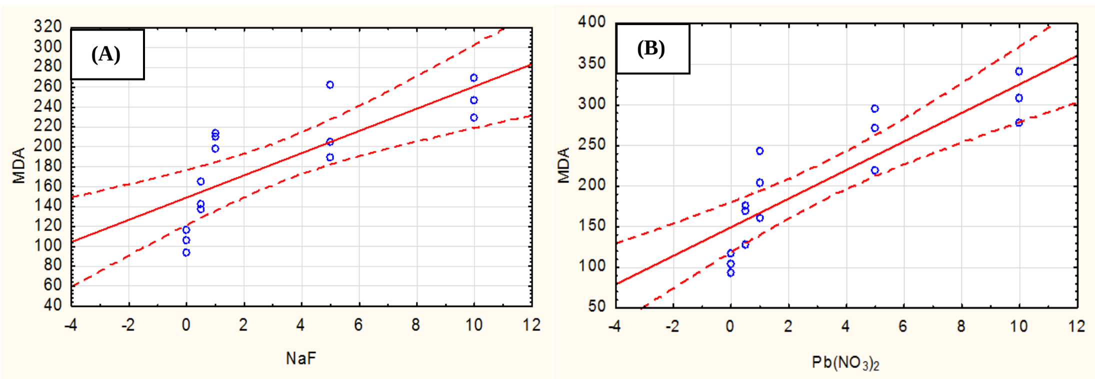

Fig. 2. *Correlation matrices between NaF / MDA (A),* $Pb(NO_3)_2$*/MDA (B). (A): r=0.773, p=0.000712***, (B): r=0.865, p=0.000031****

From the data presented in Fig. 2, the statistical analysis results shows a significant positive correlation between MDA (Fig. 2 A) and NaF concentration, and between MDA and $Pb(NO_3)_2$ concentration (Fig. 2 B).

During lipid peroxidation, a wide variety of aldehydes are formed, among these aldehydes is MDA. MDA is used as an interesting biomarker and diagnostic for lipid oxidative damage under drought stress [34, 35]. MDA is also accumulated in plants under heavy metal stress such us mercury [36]. Compared to the control test, our results show that the exposure of *X. parietina* to NaF and $Pb(NO_3)_2$ solutions caused a significant increase in MDA content (p<0.05*) where we found a positive correlation between MDA contents in *X. parietina* and increasing concentrations of NaF and $Pb(NO_3)_2$ (r=0.773, p=0.000712*** and r=0.865, p=0.000031***respectively). The same results were obtained by Dzubaj et al. [37] and Pisani et al. [38] who showed that *X. parietina* reacts against fluorine, boron and lead-induced stress by increasing of the MDA content. Our results are in agreement with those of El-Shora et al. [39] which showed that lead stress increased MDA contents in the treated plants, and those of Alsherif et al. [40] which indicated that heavy metal contamination resulted in significant increases in MDA in plants. Also, our results are in the same line with those obtained by Fan et al. [41] who show that MDA content increased in *Festuca arundinacea* Schreb after high concentration of fluorine treatment. Kacienė et al. [42] showed that oxidative stress induced by stress factors of different origin-ozone, ultraviolet (UV)-B radiation, drought, cadmium and copper, causes the increase in the content of MDA in barley. Likewise, Gutiérrez-Martínez et al. [43] noticed that the MDA content increases with increasing concentrations of cadmium accumulated in the leaves and roots of *Phaseolus vulgaris* plants under cadmium stress.

Chlorophyll integrity variations

Variations of the OD_{435}/OD_{415} ratio in *X. pareitina* are presented in Fig. 3. Correlation matrices between NaF/OD_{435}/OD_{415} ratio, $Pb(NO_3)_2/OD_{435}/OD_{415}$ ratio are presented in Fig. 4.

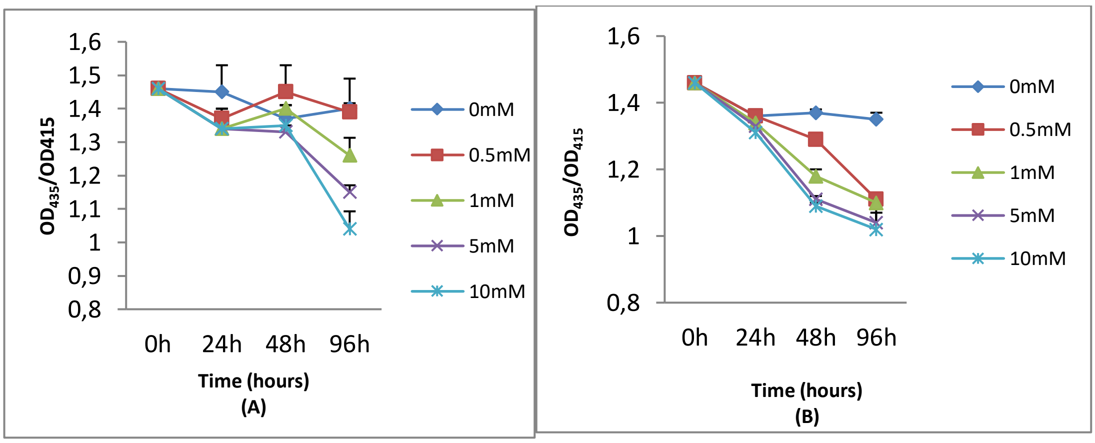

Fig. 3. *Variations of the OD_{435}/OD_{415} ratio in X. parietina treated with different concentrations of (A): NaF, (B): $Pb(NO_3)_2$*

Depending on exposure time, Fig. 3 (A) shows that the variations of OD_{435}/OD_{415} ratio in the thalli treated with the different concentrations of NaF are significant (p = 0.009**), but depending of concentrations they are not significant (p=0.422^{NS}).
A slight decrease in OD_{435}/OD_{415} ratio in thalli treated with the different concentrations of $Pb(NO_3)_2$ is recorded after 24 h of treatment (p>0.05^{NS}), while between 24 h and 96 of treatment, a significant decrease in this ratio was noticed (p=0.036*).

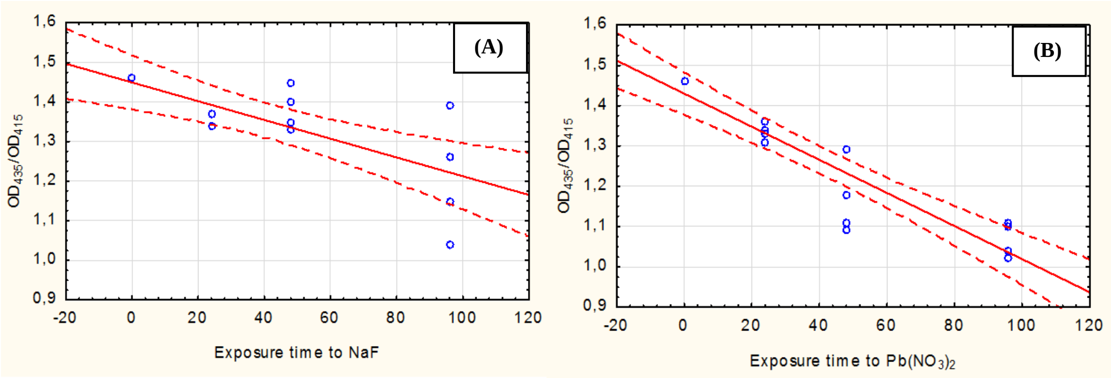

Fig. 4. *Correlation matrices between NaF/OD_{435}/OD_{415} ratio (A), $Pb(NO_3)_2$/OD_{435}/OD_{415} ratio (B). (A): r=- 0.737, p=0.0011**, (B): r=- 0.926, p<0.0001****

From the data presented in Fig. 4, the statistical analysis results shows a significant negative correlation between OD_{435}/OD_{415} ratio and exposure time of NaF (Fig. 4 A) and between OD_{435}/OD_{415} ratio and exposure time of $Pb(NO_3)_2$ (Fig. 4 B).
The most commonly used metric to quantify chlorophyll degradation is the ratio of optical density of chlorophyll samples read at 435 and 415nm. A ratio of 1.4 informs about chlorophyll integrity, any reduction in this value indicates the degradation of chlorophyll to provide stress to the organism [44, 45]. Ours results show that a ratio of 1.4 was obtained in lichen thalli treated with distilled water (control), but for those treated with different concentrations of NaF and $Pb(NO_3)_2$, a decrease in this ratio was noted. According to this results, we found that the decrease of the OD_{435}/OD_{415} ratio is in correlation with increasing exposure time of *X. parietina* to NaF and $Pb(NO_3)_2$ (r=- 0.737, p=0.0011** and r=-0.926, p<0.0001*** respectively). Our results are in agreement with those obtained by Shukla and Upreti [46], who reported that OD_{435}/OD_{415} ratio values decreased with the increase in the amount of Cu, Pb and Zn in the lichen P*yxine subcinerea Stirton* and of Bajpai et al. [47]; Sharma and Singh [48] and Chetia et al. [49] who indicate that chlorosis increases in lichens under heavy metals stress and with those obtained by Panda [50], who reported that chlorosis is one of the symptoms of fluoride toxicity in plants.

Proline accumulation

Treatment with increasing exposure time to increasing concentrations of NaF and $Pb(NO_3)_2$ caused a general increasing accumulation of proline in *X. parietina* (Fig. 5). Correlation matrices between NaF/proline, $Pb(NO_3)_2$/proline are presented in Fig. 6.

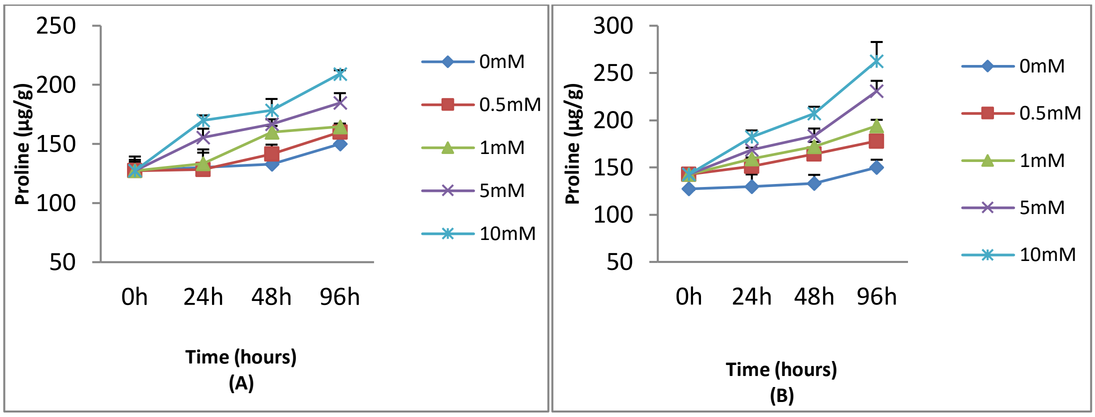

Fig. 5. *Accumulation of proline in X. parietina treated with different concentrations of (A): NaF, (B): $Pb(NO_3)_2$*

According to the Fig. 5 (A), it was noticed that the accumulation of proline in thalli increases significantly with increasing NaF concentrations (p=0.0052**) but not significantly with increasing exposure time (p=0.177^{NS}).

According to Fig. 5 (B), no significant accumulation of proline in the thalli treated with the 0.5, 1 and 5 mM concentrations of $Pb(NO_3)_2$ was noted (p=0.08^{NS}), however with the high concentration of $Pb(NO_3)_2$ (10 mM), proline accumulation was significant (p=0.016*). Depending on exposure time, significant accumulation of proline (p=0.013*) after 96 h of treatment with all concentrations of $Pb(NO_3)_2$ was noted. Therefore, it can be concluded that the accumulation of proline varies much more with time than with concentration.

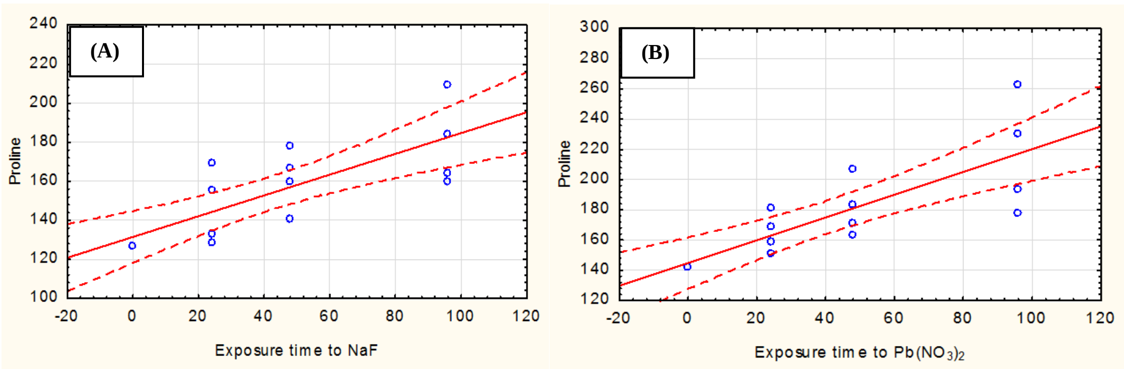

Fig. 6. *Correlation matrices between NaF/proline (A), $Pb(NO_3)_2$/proline (B). (A): r=0.783, p=0.00032***, (B): r=0.811, p=0.00013****

From the data presented in Fig. 6, the statistical analysis results shows a significant positive correlation between proline and exposure time of NaF (Fig. 6 A), and between proline (Fig. 6 B) and exposure time of $Pb(NO_3)_2$.

Proline is part of a general adaptive syndrome to adverse environmental conditions [51, 52]. The accumulation of proline can be considered as a biomarker of stress [34] which varies depending on the plant species. Plants react against stress by accumulating proline to protect the structure of its macromolecules [53]. Amri and Layachi [54] reported that exogenous application of proline on a Faba bean (*Vicia faba*) plant cultived under cadmium stress helped the plant recover from the cadmium stress-induced physiological changes. The determination of proline in lichens is a detective method of the various possible stress phenomena. Our results show a significant increase in proline content in *X. parietina* correlating with increasing exposure time to NaF and $Pb(NO_3)_2$ (r=0.783, p=0.00032*** and r=0.811, p=0.00013***respectively), the same result was obtained by Li et al. [55] who reported that proline contents increased in maize varieties under Cd stress. Likewise, our results are similar to those obtained by Koleva et al. [56], which were noted that *Phaseolus vulgaris* seedlings under cadmium-induced stress exhibited an increased level of proline. Several other studies indicate also that proline increases under the action of other types of stress: salt stress [53], water limitation [57], changing climate conditions [52], UV radiation [58], heat stress tolerance [59] and nutrient deficiency [28].

Soluble sugars accumulation

The effect of different concentrations of NaF and $Pb(NO_3)_2$ on the content of soluble sugars in *X. parietina* are shown in Fig. 7. Correlation matrices between NaF/soluble sugars, $Pb(NO_3)_2$/soluble sugars are presented in Fig. 8.

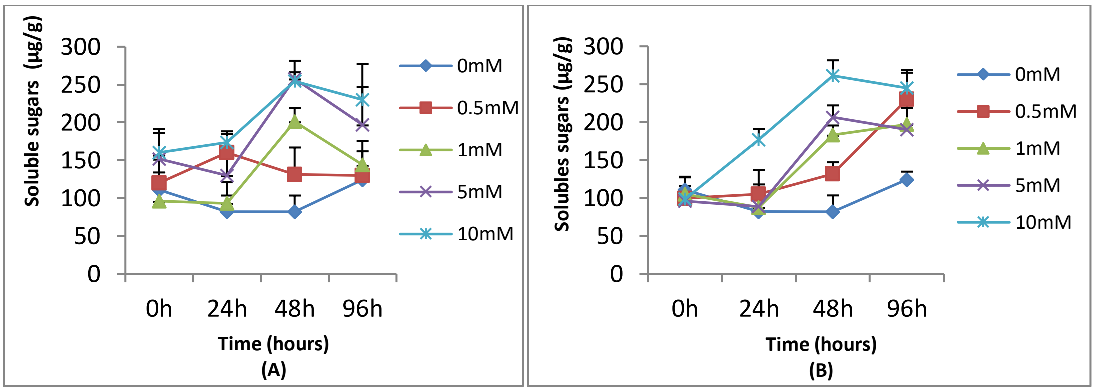

Fig. 7. *variations of soluble sugar contents in X. parietina treated with different concentrations of (A): NaF, (B):* $Pb(NO_3)_2$

According to the results presented in Fig. 7, the soluble sugar contents generally increase in thalli treated with different concentrations of NaF and $Pb(NO_3)_2$, but these contents decrease slightly after 96 h of $Pb(NO_3)_2$ treatment with 5 mM and 10 mM concentrations and with all concentrations of NaF (0.5 mM, 1 mM, 5 mM, and 10 mM). Fig. 7 (A) shows a significant increase in soluble sugar contents in thalli treated with all concentrations of NaF (p=0.01*), but depending of exposure time, the variations in soluble sugar contents were not significant ($p=0.23^{NS}$). Unlike NaF, Fig. 7 (B) shows no significant increase in soluble sugar contents depending of concentrations ($p=0.238^{NS}$), and a significant increase depending of exposure time (p=0.010*).

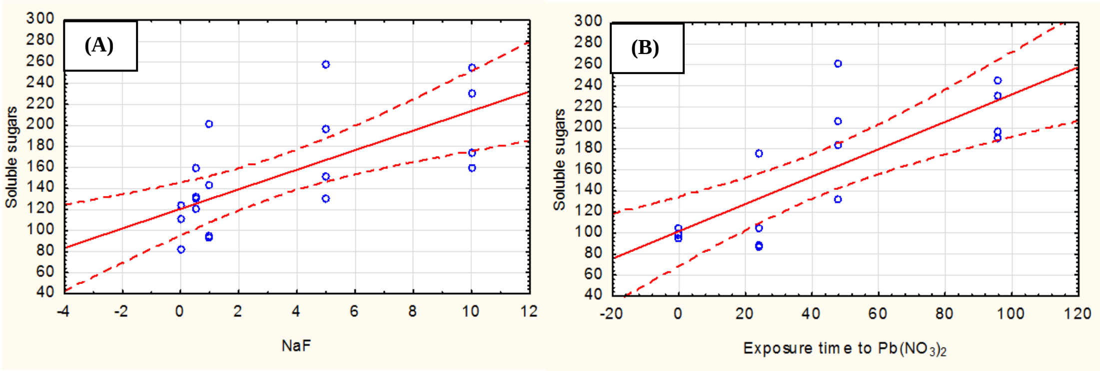

Fig. 8. *Correlation matrices between NaF/soluble sugars (A), Pb(NO$_3$)$_2$/soluble sugars (B). (A): r=0.678, p=0.0010***, (B): r=0.780, p=0.00036****

From the data presented in Fig. 8, the statistical analysis results shows a significant positive correlation between soluble sugars (Fig. 8 A) and NaF concentration, and between soluble sugars contents (Fig. 8 B) and exposure time of Pb(NO$_3$)$_2$.

Like proline, soluble sugars are part of the adaptation strategies used by plants to combat and tolerate stressful conditions [27, 34, 60]. The results obtained show a significant increase in the content of soluble sugars correlating with increasing concentration of NaF (r=0.678, p=0.0010***) and correlating with increasing exposure time of *X. parietina* to Pb(NO$_3$)$_2$ (r=0.780, p=0.00036***); these results are in agreement with the results obtained by Gandonou et al. [61] who showed that soluble sugars accumulate in two sugarcane cultivars under salt stress. According to Abbaspour et al. [62], the increase of the salinity stress resulted in the increasing concentration of soluble sugars in three pistachio cultivars. Also, several other studies showed that drought stress increased the contents of soluble sugars in the leaves of soybean seedlings [63] and in *Sophora davidii* (Franch.) [64]. Our results are also concomitant with those obtained by Aldoobie and Beltagi [65], who reported that contents of total soluble sugars increased in common bean (*Phaseolus vulgaris L*. cv. Nebraska) plants in response to lead, cadmium and nickel stress.

According to Ahmad et al. [66], plants accumulate soluble sugars as a defense mechanism against stressful conditions caused by drought and water scarcity, varying temperature from minimal to maximum level, and accumulation of salt and heavy metals.

Our results show that chlorosis, lipid peroxidation, accumulation of proline and soluble sugars in *X. parietina* under Pb(NO$_3$)$_2$ treatment are more intense than under NaF treatment. Based on our statistical analyses, it was concluded that lead is significantly more toxic than fluoride (p=0.02*).

CONCLUSION

The results of the present study showed a significant increase of MDA content in *X. parietina* correlating with increasing concentrations of NaF and Pb(NO$_3$)$_2$, and a significant increase of OD$_{435}$/OD$_{415}$ ratio and proline accumulation correlating with increasing exposure time of NaF and Pb(NO$_3$)$_2$, results showed also that soluble sugar contents increased correlating with increasing concentrations of NaF and correlating with increasing exposure time of Pb(NO$_3$)$_2$. Despite the fact that lead was more toxic than fluoride, *X. parietina* has a very high sensitivity to fluoride, we were able to draw the conclusion that fluoride must be classified among the most toxic air pollutants, and therefore to open the field to other works to study and compare the toxicity of fluorine with that of heavy metals on the various other ecosystems.

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Conflict of Interest. The author declared that there is no conflict of interest.

Authorship Contributions. Concept: O.B., N.B., E.L., Design: O.B., N.B., E.L., Data Collection or Processing: O.B., N.B., E.L., Analysis or Interpretation: O.B., N.B., E.L., Literature Search: O.B., N.B., E.L., Writing: O.B., N.B., E.L.

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