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Research Article

Oxidative stress induced by fluorine in *Xanthoria parietina* (L.) Th. Fr.

Ouahiba Benhamada[1,*], **Nabila Benhamada**[2], **Essaid Leghouchi**[2]

[1]Laboratory of Biotechnology, Environment and Health, Jijel University, Faculty of Nature and Life Sciences, Department of Applied Microbiology and Food Sciences, Algeria
[2]Laboratory of Biotechnology, Environment and Health, Jijel University, Faculty of Nature and Life Sciences, Department of Cell and Molecular Biology, Algeria

Abstract: In our work we were interested in the toxicity of fluorine on the various parameters of stress: chlorophyll, proteins, and antioxidant system in the lichen *Xanthoria parietina* (L.) Th. Fr., and for this purpose, lichen thalli were treated by sodium fluoride (NaF) at concentrations of 0, 0.5, 1.0, 5.0 and 10.0 mM, for time scale 0, 24, 48 and 96 h. The analysis results obtained revealed that all the parameters evaluated showed significant variations compared to those of the controls. From the analysis results obtained, it was noted that chlorophyll a (C_a), chlorophyll b (C_b) and total chlorophyll (C_{a+b}) decreased correlating with exposure times to NaF (r = -0.785, $p < 0.001$; r = -0.955, $p < 0.001$; r = -0.899, $p < 0.001$, respectively), with a significant increase of $C_{a/b}$ ratio ($p = 0.00572^{**}$) showing that C_b was more affected than C_a. However, hydrogen peroxide (H_2O_2) increased (r = 0.949, $p < 0.001$). In correlation with NaF concentrations, Glutathione (GSH) increased (r = 0.969, $p < 0.001$), while proteins decreased (r = -0.872, $p < 0.001$). Furthermore, results showed that catalase activity (CAT) increased correlating with increasing exposure time of *X. parietina* to increasing concentrations of NaF. Long-term exposure (48 h -96 h) caused a significant decrease in GSH content ($p = 0.02^{*}$) followed by total destruction at time 96 h.

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1. INTRODUCTION

In their habitat, lichens are exposed to severe abiotic stresses such as desiccation and temperature extremes (Beckett *et al*., 2021), salinity (Chowaniec & Rola, 2022), heat (Kraft *et al*., 2022) heavy metals (Rola, 2020), and fluoride (Roberts & Thompson, 2011).

Plants endured significant biochemical and physiological changes as a result of the stressful environment. Chlorophyll degradation is the most common metric used to assess the toxicity of air pollution on lichens (Sujetovienė, 2015).

Fluoride is found in a variety of environmental matrices and even at low quantities and is one of the most phytotoxic chemical elements for plants (Banerjee & Roychoudhury, 2019). It affects the metabolic activity of plants by decreasing nutrient uptake, germination,

*CONTACT: Ouahiba Benhamada ✉ wahibabenhamada@yahoo.fr Jijel University, Faculty of Nature and Life Sciences, Department of Applied Microbiology and Food Sciences, Algeria

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photosynthesis, growth, and productivity (Sharma & Kaur, 2018). Fluoride toxicity has also a negative impact on enzyme activity, protein synthesis, gene expression patterns, and the formation of reactive oxygen species (ROS) (Choudhary *et al*., 2019).

The most important adaptation mechanism used by lichens for tolerance to stressful conditions is the scavenging of ROS. To prevent ROS-induced damage, plants synthesize many enzymatic components like catalase (Lei *et al*., 2022) and non-enzymatic components like glutathione (Hasanuzzaman *et al*., 2020), and change their protein composition (Amnan *et al*., 2022). In response to abiotic stress, plants also produce H_2O_2 as one of the ROS (Zhang, 2022). Under stressful conditions, GSH levels rise in plants (Nahar *et al*., 2017), and it is one of the plant's adaptive methods for combating and tolerating stress (Gong *et al*., 2018). Catalase activity also increases in plant under fluoride stress correlating with high concentration (Sharma & Kaur, 2019) and with exposure time (Sharma *et al*., 2019).

The study's aim is to explore the harmful effect of fluorine which is in the form of sodium fluoride (NaF) on the lichen *Xanthoria parietina*, by measuring contents of chlorophyll, proteins, catalase, hydrogen peroxide (H_2O_2), and reduced glutathione (GSH) as stress biomarkers.

2. MATERIAL and METHODS

2.1. Lichen Material

The *X. parietina* lichen thalli were collected in a rural area far from any urban or industrial area south of Jijel (Algeria) during the spring season 2017. Samples were transported to the laboratory in clean closed boxes. The thalli were then separated from their supports and kept in the laboratory until their use.

2.2. Fluorine Treatment

The lichen thalli of *X. parietina* were incubated in NaF solutions at 0.5, 1.0, 5.0, and 10.0 mM concentrations at room temperature in comparison with a control test which consists of a treatment in distilled water. These solutions were then kept at room temperature for 0, 24, 48, and 96 h in the dark. After treatment and before each analysis, the samples were washed three times with distilled water to remove excess NaF solutions attached to thalli surfaces.

2.3. Chlorophyll Analysis

Chlorophyll a (C_a), chlorophyll b (C_b), and total chlorophyll (C_{a+b}) contents were assayed according to the method described by Lichtenthaler (1987). The fresh lichen sample was macerated in 80 % acetone, and the maceration extract was then filtered and read at 663 nm and 645 nm using a spectrophotometer. Chlorophyll contents were calculated using the following equation:

$$C_a = 12.7 \times A_{663} - 2.69 \times A_{645}$$

$$C_b = 22.9 \times A_{645} - 4.68 \times A_{663}$$

$$C_{a+b} = 20.2 \times A_{645} - 8.02 \times A_{663}$$

Where A_{663}, A_{645} absorbance at 663 and 645 nm, respectively. Results were expressed in $\mu g.g^{-1}$. To measure the physiological activity of algal cells, the chlorophyll a/b ($c_{a/b}$) ratio was calculated.

2.4. Proteins Assay

Protein contents were tested using Bradford's method (1976). 100 mg of fresh weight lichens were homogenized in 2 ml of 0.05 M phosphate buffer pH 6.8 and centrifuged for 20 min at 12000 t/min at 4 °C. An amount of 2 ml of Bradford's solution was added to 50 µl of supernatant. After 10 min, a reading of 595 nm was recorded. BSA's equation ($y = 28.9$ x, $R^2 = 0.9911$) was used to quantify protein levels ($mg.g^{-1}$).

2.5. Catalase Activity Assay

Catalase activity was measured using the Chance and Maehly's method (1955). An amount of 50 mg fresh weight of lichens was homogenized in 2 ml of 0.05 M phosphate buffer at pH 7, and then centrifuged at 15000 t / min at 5 °C for 20 min. 50 µl of the supernatant was added to 2.95 ml of 0.015 M H_2O_2 in the phosphate buffer. A first reading was taken at 240 nm right away, and a second was taken 3 min later. The following formula was used to calculate catalase's enzymatic activity:

$$k = 2.303 / T \times \log (A_1/A_2)$$

of which:

K: the reaction rate constant**.**

T: Time interval in min.

A_1: Absorbance at t = 0.

A_2: Absorbance after 3 min.

Results were expressed in IU/g of proteins.

2.6. H_2O_2 Assay

H_2O_2 concentration was assayed according to the method described by Sagisaka (1976). An amount of 2 ml of 5 % trichloroacetic acid (TCA) was used to homogenize about 1g of fresh lichen material. The resultant mixture was centrifuged for 20 min at 0 °C at 14000 g. 1.6 ml of supernatant was mixed with a mixture of 0.4 ml TCA (50 %), 0.4 ml ferrous ammonium sulfate (1 %) and 0.2 ml of thio potassium cyanate (1 %). The amount of H_2O_2 in the supernatant was calculated using the optical density at 480 nm. The concentration of H_2O_2 ($mmol.g^{-1}$) was calculated using an equation based on known H_2O_2 standard concentrations ($y = 0.1864 \times + 0.2281$, $R^2 = 0.09691$).

2.7. GSH Assay

GSH assay was carried out by the colorimetric method described by Ellman (1959). GSH is oxidized by producing thionitro-benzoic acid (TNB), which absorbs at 412 nm, in the presence of 5,5'-dithiobis 2-nitrobenzoic acid (DTNB). Lichen thalli were homogenized in 50 mM phosphate buffer (pH 6.5) and centrifuged at 12000 g for 15 min at 4 °C. The absorbance at 412 nm of a combination containing 100 µl of supernatant and 1200 µl of DTNB solution was measured. The results were represented in $mmol.g^{-1}$ using an equation based on known GSH standard concentrations ($y = 0.2012 \times + 0.3852$, $R^2 = 0.9573$).

2.8. Statistical Analysis

Three repetitions were performed at each concentration, so that we could 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 this study, the results were expressed as mean ± SD. The difference was considered to be not significant when $p > 0.05$ (NS), significant when $0.01 < p < 0.05$ (*), very significant when $0.001 < p < 0.01$ (**), and highly significant when $p < 0.001$ (***).

Correlation matrices between NaF and different studied parameters were analyzed by STATISTICA Version 10 software.

3. RESULTS

3.1. Chlorophyll Contents Variations

Variations in C_a, C_b, and C_{a+b} contents in *X. parietina* are shown in Figure 1 (a, b and c, respectively), whereas, $C_{a/b}$ ratio variations are presented in Table 1.

Figure 1. Chlorophyll content variations in *X. parietina* after treatment of thalli by NaF solutions, (a): C_a, (b): C_b, (c): C_{a+b}

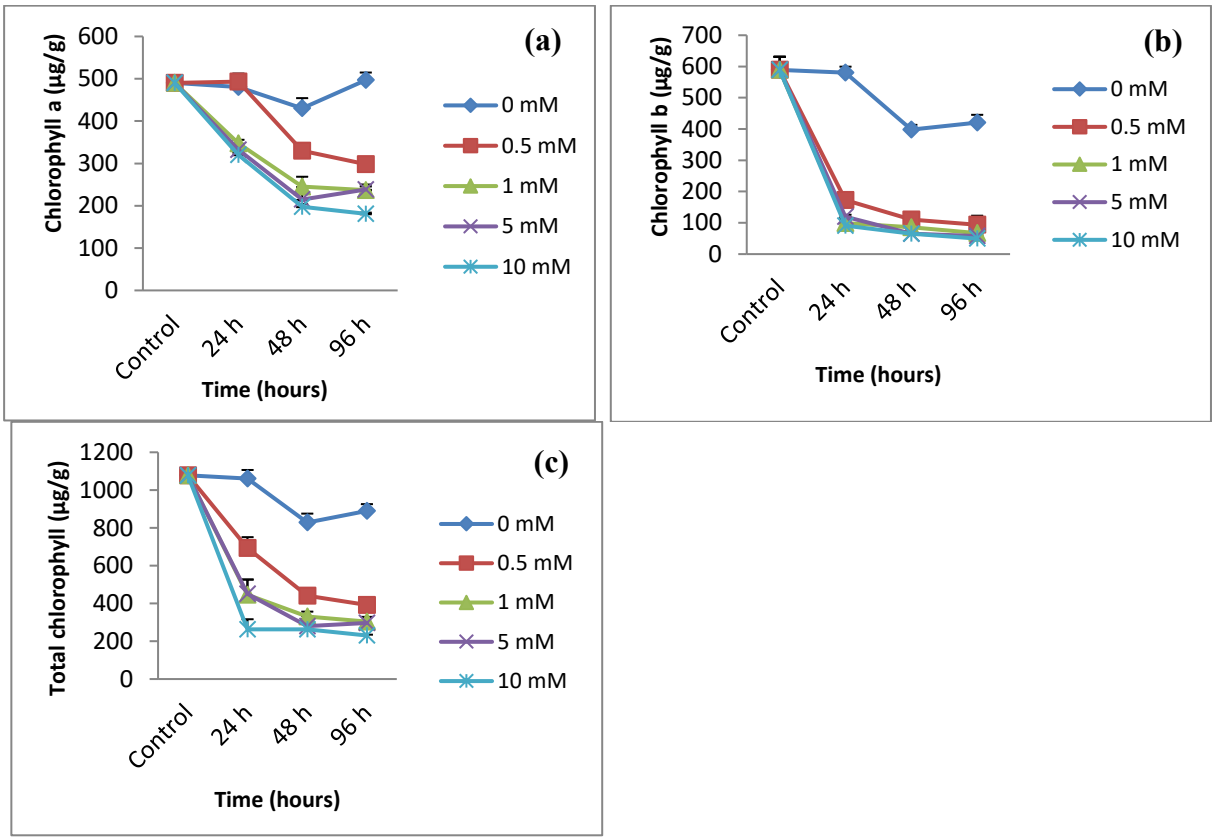


Figure 1 (a) shows a significant decrease in C_a content as a function of different concentrations of NaF ($p = 0.0052^{**}$) and as a function of exposure time ($p = 0.0031^{**}$), variations in C_a levels are not significant between 48 h and 96 h of exposure time ($p > 0.05$).

From the Figure 1 (b), it was noticed that all the concentrations of NaF exhibit the same effect on C_b content, where a very significant decrease was observed ($p = 0.007^{**}$). According to the exposure time, a significant decrease in the content of C_b was also noted in the 24 h following the treatment ($p = 0.0037^{**}$), between 48h and 96 h of treatment, C_b content variations were not significant ($p = 0.755^{NS}$).

Figure 1 (c) shows that the decrease in C_{a+b} content is significant as a function of exposure time ($p = 0.0012^{**}$) as well as a function of NaF concentrations ($p = 0.020^{*}$).

The results presented in Figure 1 allowed us to deduce that the variations in chlorophyll contents (C_a, C_b, and C_{a+b}) in *X. parietina* under NaF stress are significant as a function of exposure time as well as a function of NaF concentrations. From the results presented in Table 1, it was noted a significant increase of $C_{a/b}$ ratio ($p = 0.00572^{**}$). This increase explains well that C_b is the most affected by fluorine compared to C_a.

Table 1. Chlorophyll a/b ratio variations in the lichen *X. parietina* after treatment of thalli by NaF solutions.

	0 mM	0.5 mM	1 mM	5 mM	10 mM
0 h	0.83 ± 0.02	0.83 ± 0.02	0.83 ± 0.02	0.83 ± 0.02	0.83 ± 0.02
24 h	0.82 ± 0.02	2.86 ± 0.02	2.5 ± 0.02	2.77 ± 0.1	3.51 ± 0.02
48 h	0.9 ± 0.07	2.98 ± 0.08	2.84 ± 0.04	3.27 ± 0.02	3.03 ± 0.07
96 h	1.18 ± 0.05	3.17 ± 0.02	3.52 ± 0.03	4.13 ± 0.03	3.65 ± 0.05

The data in the table are represented as the mean ± SD.

3.2. Effect of Fluorine on Proteins, Catalase, H_2O_2, and GSH Contents

Under fluorine stress, proteins contents decrease (Figure 2), whereas catalase, H_2O_2 and GSH contents increase (Figures 3, 4, and 5, respectively).

Figure 2. Protein content variations in *X. parietina* after treatment of thalli by NaF solutions.

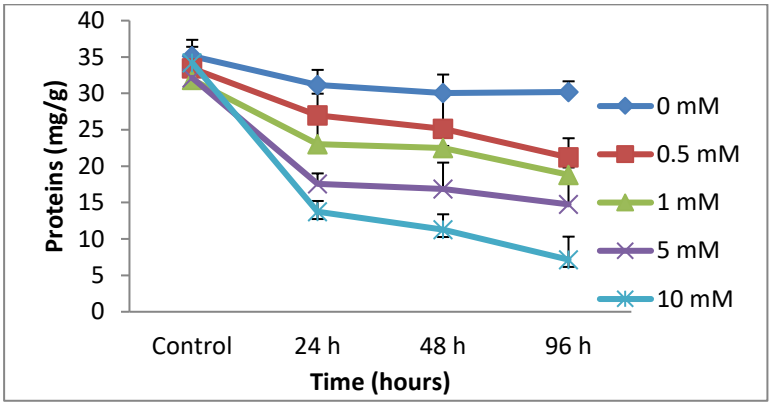
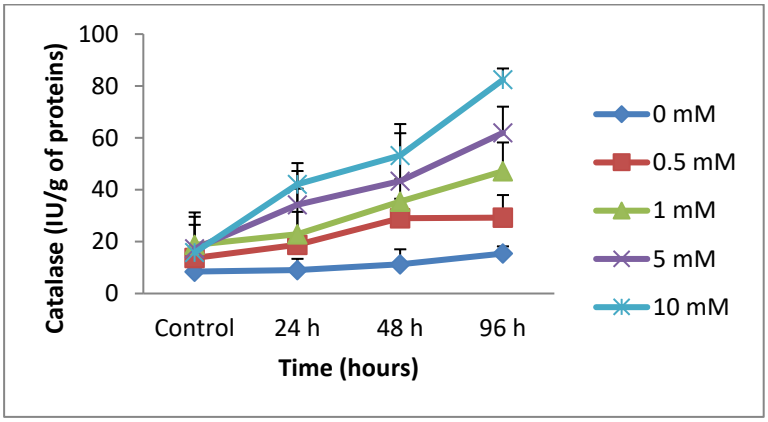


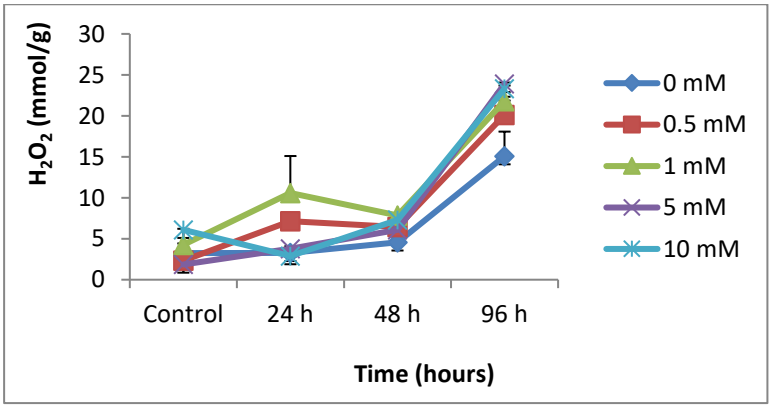
Figure 2 shows that protein levels are affected by NaF, with a significant decrease at all concentrations ($p = 0.0240^{*}$), and a significant decrease was also noted within 24 h of treatment ($p = 0.0062^{**}$); however, between 24 h and 96 of exposure time, the decrease in protein contents was not significant ($p > 0.05^{NS}$).

Figure 3. Catalase activity variations in *X. parietina* after treatment of thalli by NaF solutions.



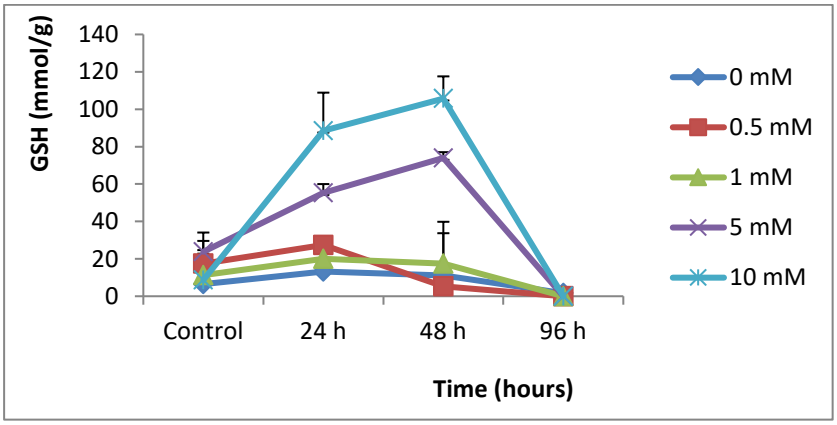
According to Figure 3, it was found that the expression of the catalase increases with increasing concentration ($p = 0.047^{*}$), and increasing exposure time of NaF ($p = 0.045^{*}$).

Figure 4. H_2O_2 content variations in *X. parietina* after treatment of thalli by NaF solutions.



According to the results presented in Figure 4, it was noticed that the variations of H_2O_2 concentrations are not significant either according to the different concentrations of NaF (p = 0.95^{NS}), or after the 48 h which follow the treatment (p = 0.16^{NS}), however, a significant increase was observed after 96 h of treatment (p = 0.017^{*}).

Figure 5. GSH content variations in *X. parietina* after treatment of thalli by NaF solutions.



From the data presented in Figure 5, it was noted that the variations in GSH content in thalli treated with low concentrations of NaF (0.5 mM and 1 mM) are negligible. Whereas, the high concentrations (5 mM and 10 mM) caused a significant increase after 24 h and a non-significant increase after 48 h of treatment (p = 0.64^{NS}). However, a significant decrease in the GSH content was noted between 48 h and 96 h of exposure time (p = 0.02^{*}) with complete degradation after 96 h of treatment.

3.3. Correlation Analyzes

Correlation matrices between NaF and different studied parameters are presented in Table 2. From the data presented in Table 2, the statistical analysis results show a significant negative correlation between C_a, C_b, C_{a+b} and exposure time of NaF and between proteins and increasing concentrations of NaF. A significant positive correlation was noted between $C_{a/b}$ ratio and exposure time of NaF, catalase and exposure time to increasing concentrations of NaF, H_2O_2 and exposure time of NaF, and between GSH and increasing concentrations of NaF. However, a non-significant negative correlation was noted between GSH and 48 h to 96 h of exposure of NaF.

Table 2. Correlation matrices between NaF and C_a, C_b, C_{a+b}, $C_{a/b}$, proteins, catalase, H_2O_2, and GSH contents in *X. parietina*

Correlation matrices	Correlation dependency	r	p	Significance
NaF / C_a	Time (0 - 48 h)	-0.785	< 0.001	***
NaF / C_b	Time (0 - 24 h)	-0.955	< 0.001	***
NaF / C_{a+b}	Time (0 - 48 h)	-0.899	< 0.001	***
NaF / $C_{a/b}$	Time	0.818	< 0.001	***
NaF / proteins	Concentration	-0.872	< 0.001	***
NaF / catalase	Time and concentration	0.784	< 0.001	***
NaF / H_2O_2	Time (48 -96 h)	0.949	< 0.001	***
NaF / GSH	Concentration	0.969	< 0.001	***
	Time (48 - 96 h)	-0.6	0.06	NS

4. DISCUSSION

Compared with the control test, and depending on the increase of concentration and exposure time to NaF, our results show a significant decrease in C_a, C_b, and C_{a+b} contents in *X. parietina*. Zhao *et al*. (2021) found the same thing, indicating that high cadmium concentrations affect photosynthesis in *Sassafras* seedlings. Wang *et al.* (2021) also found that the contents of C_a, C_b, and C_{a+b}, decrease in tall fescue under lead stress. Significant decrease of total chorophyll content was also observed in *Lonicera japonica* Thunb. in response to 150 mg kg^{-1} or 200 mg kg^{-1} of cadmium (Li *et al*., 2022).

Photosynthesis and respiration are the processes most affected by fluoride (Sharma & Kaur, 2018). According to the results of statistical analysis presented in Table 2, it was noted a significant decrease of C_a, C_b, and C_{a+b} contents correlating with exposure time to NaF (r = -0.785, $p < 0.001$; r = -0.955, $p < 0.001$ and r = -0.899, $p < 0.001$, respectively). Our results are in the same line with those obtained by Chakrabarti *et al.* (2014) who reported that chlorophyll decreased in paddy (*Oryza sativa* L.) with increasing fluoride treatment. Mondal (2017) also found pigment degradation in four widely cultivated rice (*O. sativa*) varieties treated to 5, 10, and 20 mg dm^{-3} NaF. An other study carried out by Iram and Khan (2016) showed that C_a, C_b, and C_{a+b} decreased in *Abelmoschus esculentus* (L.) Moench under NaF stress. Fan *et al.* (2022) also found a significant decrease in chlorophyll content after high concentration of NaF treatment in tall fescue (*Festuca arundinacea* Schreb). Our results show a significant increase of $C_{a/b}$ ratio correlating with exposure time to NaF (r = 0.818, $p < 0.001$). These results allowed us to conclude that C_b is more affected than C_a in thalli treated by NaF and the same results were obtained by Purnama *et al*. (2015), who showed a significant decrease in C_b content in Seagrass under lead stress, even though they also found that C_b was more affected than C_a as a result of lead effect.

Plants vary their protein composition for rebuilding, tolerance, resistance, and responsiveness to stressful situations (Amnan *et al.*, 2022). Our results show a significant decrease in protein contents in *X. parietina* correlating with increasing concentrations of NaF (r = -0.872, $p < 0.001$). The same result was obtained by Chetia *et al*. (2021), who found a decrease in total protein contents to correlate with Pb, Cd, Zn, Cu, Co, Ni, and Cr in lichens growing in differently polluted areas. Khan *et al.* (2021) found a drop in total protein contents in cultivated rice grown in lead-contaminated soil. Sharma *et al.* (2019) also showed significant reduction ($p \leq 0.05$) in protein content in *Spirodela polyrhiza* (L.) Schleiden under treatment with fluoride at all the exposure periods (24, 72, 120 and 168 h). According to Szostek and Ciecko (2017), the decrease in total protein content caused by fluoride can be explained by channeling degraded products towards metabolic pathways for energy and stress management. On the other hand, Souahi *et al.* (2021) found an increase in total protein contents in *Triticum durum* Desf. leaves and roots after treatment with 0.3 and 0.6 g/l lead acetate.

Plants increase the activities of antioxidant enzymes like catalase to trap ROS and detoxify their effects (Lei *et al.*, 2022). Ours results show that the expression of catalase in *X. parietina* increases correlating with increasing concentrations of NaF (r = 0.784, $p < 0.001$). Mondal (2017) reported similar results, demonstrating that catalase activity increased with increasing fluorine concentrations in four widely cultivated rice (*O. sativa*). Elloumi *et al*. (2017) also showed that increased catalase activity is one of the indices of oxidative stress induced by fluoride air pollution in *Eriobotrya japonica.* AL-Zurfi *et al.* (2021) found the same results, indicating that *Hydrilla verticillata* responds to cadmium stress by steadily increasing the catalase enzyme concentration. Our results are likewise consistent with those of Abu-Muriefah (2015) and Khan *et al*. (2021), who found that catalase levels increase significantly in plants exposed to lead. Sharma and Kaur (2019) also found a significant increase of catalase activity in *Spirodela polyrhiza* under fluoride stress at high concentration compared to control at a very

first exposure period of 24 h. In addition, Sharma *et al*. (2019) showed that fluoride treatment significantly increased catalase activity in exposed *S. polyrhiza* fronds when compared to control during all exposure periods (24, 72, 120 and 168 h). Ghosh *et al.* (2021) also found that during the ripening of chili fruits, treatment with chitosan and putrescine modulates reactive oxygen species metabolism, and causes an increase in catalase activity. However Chakrabarti *et al.* (2014) found that catalase activity decreased with increasing fluoride treatment. Furthermore, Orabi *et al*. (2015) indicate that the excess of H_2O_2 caused a decrease in the activity of catalase.

Plants create H_2O_2 as one of the ROS in response to abiotic stress (Zhang, 2022). According to Sofo *et al.* (2015), H_2O_2 generation is regarded as a stress marker. H_2O_2 is also necessary for plants to tolerate harsh situations (Černý *et al*., 2018). According to Hung *et al*. (2005), plants have developed complex regulatory mechanisms to adapt to various environmental stresses, the most important of which is to convert the ROS formed into hydrogen peroxide (H_2O_2). The statistical analysis results presented in Table 2 show that H_2O_2 increases correlating with increasing exposure time to NaF ($r = 0.949$, $p < 0.001$). These results are comparable with those of Panda (2007), who investigated the effect of chromium on rice and discovered that this pollutant produces H_2O_2, which is proportional to exposure time and pollutant concentration. Furthermore, those obtained by Liu *et al*. (2010), who found that contents of H_2O_2 decrease in tomato seedlings when the concentration of Mn^{2+} reached 400-600 $\mu molL^{-1}$ under hypoxia stress. Our results are also similarly consistent with those of Liu *et al.* (2021), who found that lead induces the increase of H_2O_2 in edible amaranth under simultaneous stresses of lead from soils and atmosphere, and with those of Li *et al.* (2022) who found higher levels of H_2O_2 in response to high concentration of cadmium (150 mg kg^{-1} or 200 mg kg^{-1} Cd). According to Liu *et al.* (2020), trealose treatment of tomato under cold stress causes elevated H_2O_2 levels as a way of tolerance. Our results show that the accumulation of H_2O_2 is accompanied with the decrease in protein content, the same results were obtained by James *et al.* (2022) who showed a negative correlation between H_2O_2 and proteins in bleuet Northland under hypobaric storage.

GSH increases in plants under stressful conditions (Nahar *et al*., 2017) and it is part of the adaptation strategies used by plants to combat and tolerate stressful conditions (Gong *et al*., 2018). The results obtained show that the GSH content increases correlating with increasing concentrations of NaF ($r = 0.969$, $p < 0.001$) and the same result was obtained by Li *et al*. (2022) who showed that in response to 150 mg kg^{-1} or 200 mg kg^{-1} of Cd, the antioxidants GSH increased in *Lonicera japonica* with increasing concentration of Cd, and by Pristupa *et al*. (2021) who found a decrease in GSH content in transgenic plants *Nicotiana tabacum* L. under abiotic stress conditions.

Correlating with exposure time to NaF, our results show a decrease in GSH content between 48 h and 96 h of treatment ($r = -0.6$, $p = 0.06$). According to Cempírková and Večeřová (2018), the long-term stress exposure of the individual species of green algal and cyanobacterial lichen had a significant impact on the antioxidant content resulting from high light stress. Our results are similar with those of Balarinová *et al*. (2014), who discovered that during the first 30-40 min of high light treatment, total GSH increased in two Antarctic lichens (*Usnea antarctica* and *Usnea aurantiaco-atra*), followed by a reduction at 60 min of treatment, and with those of Li *et al*. (2015) who found that increased heavy metal concentrations resulted in a considerable reduction in GSH content in both safflower roots and leaves.

According to our results, the buildup of H_2O_2 is associated with a decrease in GSH content; these results are in the same line with those of James *et al*. (2022), who found that hydrogen peroxide was negatively correlated with GSH in bleuet Northland under hypobaric storage. Arianmehr *et al.* (2022) also investigated the role of GSH in reducing arsenic (As) toxicity in *Isatis cappadocica* DESV. and *Erysimum allionii* exposed to different concentrations (0, 400,

and 800 M) of arsenic for 3 weeks, and discovered that application of GSH increased fresh weight and total chlorophyll while inhibiting H_2O_2 accumulation.

5. CONCLUSION

The results of the present study revealed that NaF stress caused a decrease in chlorophyll and protein contents, and an increase of H_2O_2, catalase, and GSH levels in *X. parietina* correlating with increasing exposure time and/or increasing concentrations of NaF. Furthermore, the obtained results show that C_b is more affected than C_a, and that high concentration of fluorine disturbed the detoxification system, resulting in total glutathione decomposition.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Ouahiba Benhamada: Investigation, Resources, Visualization, Software, Formal Analysis, Methodology and writing original draft. **Nabila Benhamada:** Analysis, Interpretation and Language revision. **Essaid Leghouchi:** Supervision and Validation.

Orcid

Ouahiba Benhamada https://orcid.org/0000-0003-2474-5739
Nabila Benhamada https://orcid.org/0000-0001-8088-8561
Essaid Leghouchi https://orcid.org/0000-0001-9087-1050

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