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Impact of an Atmosphere Rich in Sulfur Dioxide on a Foliose Lichen Species Flavoparmelia Caperata (L) Hale from the El Kala National Park (Northeast Algerian)

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ABSTRACT

Lichens are often exposed in their natural habitat to anthropogenic pollutants, including those from vehicles and industrial and agricultural activities. In the absence of outer protective barrier, such that the waxy cuticle has evolved in vascular plants. The thallus of these organisms is sensitive to the penetration of suspended substances, some of which are essential for the functioning of cells, while others can be toxic. Accordingly, lichens can disappear within and around urban and industrial areas. Lichens can be used for early detection of stress caused by air pollution. To better characterize the impact of SO2 pollution in lichens. We began our study by highlighting the accumulation of sulfur dioxide by the thallus of foliose species Flavoparmelia caperata (L) Hale using the immersion method designed by Deruelle and Lallemand, which involves immersing lichens in SO2 -saturated atmosphere at different concentrations . Thereafter, we performed morphological observations and measurements of some physiological parameters (chlorophyll, total protein and as biomarkers of oxidative stress such as antioxidant enzymes system).

Our results indicate physiological and biochemical morphological alterations of lichens exposed to SO2, including

- discolorations and necrosis weaknesses frond splitting them into small clusters
- Disturbances contents chlorophylls (a, b and (a + b))
- Activation detoxification systems (APX, CAT and GPX);
- Increased levels of proteins.

Keywords: Lichen, Biomarkers, SO2, Oxidative Stress, Pollution.

I. INTRODUCTION

The importance of lichens grew thanks to their quality biological indicators of air pollution. Their sensitivity to phytotoxic agents exceeds that of vascular plants for several reasons (1) , because it does not have stomata or cuticle . Their gas exchange with the atmosphere takes place freely and quickly across the surface of the thallus , the limited amount of chlorophyll and slow metabolism of lichens makes them vulnerable to phytotoxic agents. They are limited to a slow recovery, when the damage is reversible. All these factors confirm the sensitivity of lichens in polluted environment (2).

Therefore, lichens are good examples for assessing air pollution (3). Several approaches are proposed according to the scale of observation considered (4). Thus, there is ecological scale biological response of individuals (bioindicators) or communities (biointegration), scale geochemical by the accumulation of contaminant (bioaccumulation).

To estimate the impact of SO2 pollution at the Annaba region fig 1 that is under the influence of several sources of much anthropogenic and natural pollution such as urbanization , a strong industrial sector presented by

various complexes such as iron and steel complex METAL STEEL and complex FERTIAL phosphate fertilizer.

Given the limited number of chemical sensors in our region , their failure common in recent years and their very expensive cost , we preferred to use the lichen bio-monitoring which reveals today a complementary tool in order to estimate the impact of pollution.

While submitting lichens polluted atmospheres (immersion method recommended by (5), we can evaluate the effect of pollution on lichens that results in a change of morphology, physiological and biochemical which also justifies their use as bioindicators .

II. METHODS AND MATERIAL

A. Plant Material

The lichen species used for this study is Flavoparmelia caperata (L.) Hale, a Ascolichen foliaceus developing on zeen oak in the forest of Bougous part of the family Parmeliacea.

Figure 1 : Ascomycota - Lecanoromycetideae - Lecanorales - Parmeliaceae

Chemistry: Medulla and soralies K ± yellow, C-, KC ± red, P + yellow-orange to red.

Thallus: foliose green yellowish, sometimes greenish yellow frankly, ± rosette up to 20 cm in diameter, with broad lobes, those of the periphery rounded, 0.5 to 1.3 cm wide, upper surface ± irregularly wrinkled, especially towards thallus central lower side on the free edge of rhizines black except at the margin of the lobes where it is brownish few mm. No isidia; soralies facial granular surface and rather coarse (especially towards the center of the thallus) Photosymbiote: trébouxioïde. Apothecia not observed. Very rare. Single spores, hyaline, 15-20x9-10 microns.

Habitat: arboreal on hardwoods, rarely on conifers, sometimes on rocks or on non-calcareous soils, usually in forests, photophile not nitrophilous. Very common species except in cold regions and high mountains. **(6) .**

B. Original Site

The Flavoparmelia caperata harvested at the El Kala National Park and specifically in foret Bougous on zeen oak.

El Kala National Park. "belongs to the northeastern part of the Algerian Tell, It covers an area of about 76,438 ha, bounded on the north by the Mediterranean Sea, to the east by the Algerian-Tunisian border and south by the mountains of Medjerda . The western boundary of the Park is drawn to encompass the watershed of Oued Bougous to the south and the city of El Tarf further north, then continues west along the RN44 road to the village of Bouteldja and extends north to the immediate west of Cape Rosa. (Fig 1)

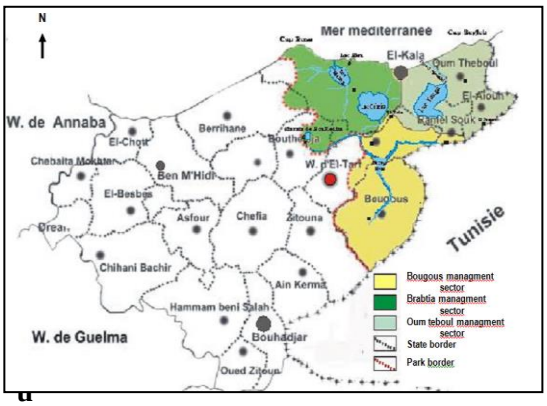

Figure 2 : Situation of El Kala National Park (Source: PNEK, 2005)

C. Study Method

1) Immersion lichens in a solution of SO2: Knowing that sulfur dioxide (SO2) gas which is lichens are more sensitive . We stated our case this gas dissolved in distilled water through an immersion method **(5)**.

This is to introduce a known mass of sodium disulfite ($Na_2S_2O_5$) powder in water and immersing the lichen . Knowing that the SO2 concentration of an aqueous solution to a value equivalent to gaseous SO2 thousand times less, we performed serial dilutions:

We conducted serial dilutions to submit Flavoparmelia caperata the following concentrations Flavoparmelia caperata the following concentrations:
Crystallizer 1: 5.10^{-3} mol/L
Crystallizer 2: 5.10^{-5} mol/L
Crystallizer 3: 5.10^{-6} mol/L
Crystallizer 4: distilled water (control)

2) Analytical Method:

Determination of Protein

The proteins were quantified according to the method of **(8)** using Coomassie Brilliant Blue (G 250 , Merck) as the reagent and bovine serum albumin (BSA , Sigma) as standard protein . The absorbances are read at a wavelength of 595 nm .

Determination of Chlorophyll

We used for the extraction of chlorophyll by the method established **(9)**.

Weigh 1 g which is cut into small sheet pieces and when milled in a mortar with 20 ml of 80% acetone (CH3COCH3) and pinch of calcium carbonate (CaCO3) total after grinding , and the solution was filtered kept in a dark black boxes to prevent oxidation of chlorophyll by light.

The assay is done by the removal of 3 ml of the solution in the tank spectrophotometer.

In the end it will play the two wave lengths of 645 and 663 nm, and calibration of the device is made by the witness acetone solution at 80 %.

The calculation value of chlorophyll is by the formula in **(10)**:

Chl.a = 12.7 (DO 663) - 2.69 (DO 645)
Chlb= 22.9 (DO 645) - 4.86 (DO 663)
Chl.a +Chlb = 8.02 + (DO 663) + 20.20 (DO 645)

Enzyme Assays

Preparation of enzyme extract

The appropriate method to obtain the enzyme extract of the plant is that of **(11).** After 4 months our transplants and control samples were harvested and cold crushed using a mortar in a phosphate buffer (50ml NaK , pH = 7.2) at a rate of 1 ml buffer per 1 g of MF . the homogenate is then filtered using an adequate web prior to centrifugation at 12000 g for 20 min to cold (-16K centrifuge 3 sigma) . supernatant extract obtained will be used as for the determination of different enzyme extracts.

Determination of ascorbate peroxidases activity (APX)

Ascorbate peroxidase activity is carried out according to the protocol adopted by **(12)** . The final reaction volume of 3 ml contains: 100µl of enzyme extract to 50µl of 0.3 % H2O2 and 2850µl NaK phosphate - ascorbate buffer (50 mM NaK , 0.5 mM ascorbate , pH = 7.2) . The calibration of the unit is done in the absence of the enzymatic extract . The reading is taken at 290 nm (Spectrophotometer JENWAY 63000) for 1 min and a molar extinction coefficient ε = 2800M - 1.cm -1, APX activity is expressed in nmol / min / mg protein.

Determination of catalase activity (CAT)

The catalase activity (CAT) is performed according to the method of **(13)** . The decrease of absorbance is recorded for three minutes by a spectrophotometer (JENWAY 6300) for a wavelength of 240 nm and a molar extinction coefficient ε = 39400 M- 1.cm - 1.The To a final volume of 3ml , the reaction mixture contains : 100µl of the crude enzyme extract , 50µl of hydrogen peroxide and 0.3% H202 in 2850µl of phosphate buffer (50mM , pH = 7.2) . The calibration of the unit is done in the absence of the enzymatic extract. The reaction is initiated by the addition of hydrogen peroxide. The catalase activity was expressed in nmol / min / mg protein.

Determination of peroxidase activity gaïacols (GPX)

The activity gaïacols peroxidase (GPX) is determined using the method of **(14)**. For a final volume of 3 ml, the reaction mixture contains: 100µl of enzyme extract , 50µl of 0.03% H2O2 and 2850µl of phosphate - Guaiacol (50 mM NaK , guaiacol 8mM, pH = 7.2) buffer . The calibration of the unit is done in the absence of the enzymatic extract. The reaction is initiated by the addition of hydrogen peroxide. The absorbance reading is performed at 470 nm (spectrophotometer Jenway 6300) and to a molar extinction coefficient linear ε = 2470 M- 1.cm -1 . The GPX activity is expressed in nmol / min / mg protein.

Quantification of spectrophotometric measurements: The following formula is used in the quantification of different spectrophotometric measurements following enzymatic assays of GPX , APX and CAT **(15)**

Act. = (ΔA.Vt) / (ε.Δt.Ve.p)

Act: Enzyme activity in nmol / min / mg Protein
ε : molar extinction coefficient of linear M
Δ : Difference in average absorbance
Vt : total volume of the reaction mixture in ml
Ve: Volume of enzyme extract in ml
L: width of the measurement vessel in cm
P : Protein content in mg
T: Reading time in min

III. RESULT AND DISCUSSION

A. Determination of chlorophyll (a, b & a + b)

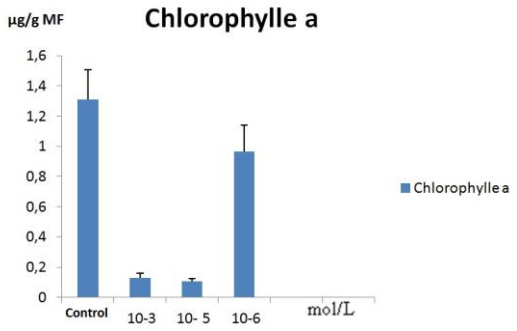

Figure 2: Variations of average chlorophyll a in different experimental batches of *Flavoparmelia caperata*

Fig 2 shows the variations in the rate of chlorophyll at different experimental batches Flavoparmelia caperata shows a decrease in chlorophyll a at all batches compared to control. However this decrease is very clear in the treated group at a concentration of 5.10^{-3} mol/L and 5.10^{-5}mol/L .

According to the ANOVA test, comparing the average grade of the level of chlorophyll a in different batches of the Flavoparmelia caperata shows that variations in average thereof are highly significant in space (0.000 *).

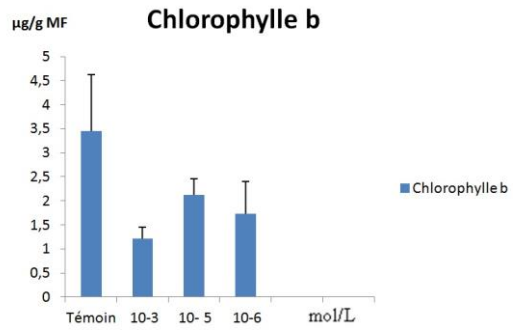

Figure 3: Variations of average chlorophyll b at different experimental batches of *Flavoparmelia caperata*

According to Fig 3, we observe changes in mean values of chlorophyll b but still down from the witness and that the degradation of chlorophyll b appears much more like a concentration 5.10^{-3}mol/L.

According to the ANOVA test, comparing the average grade of the rate of chlorophyll b in different batches of the Flavoparmelia caperata shows that variations in average thereof are highly significant in space (0.000 ***).

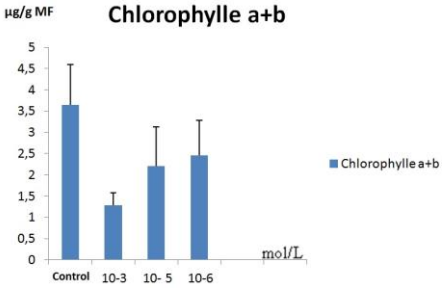

Figure 4: Variations of average chlorophyll a + b in the various experimental groups of Flavoparmelia caperata

Our results show that chlorophyll (a + b) follows the same trend as the other two chlorophylls a and b, a low production of chlorophyll pigments in all treated groups compared to the control and this decrease appears especially at a high concentration of SO_2 (5.10^{-3}mol/L) (Fig4).

According to the ANOVA test, comparing the average grade of the level of chlorophyll a + b in different batches of the Flavoparmelia caperata shows that changes in averages that are very highly significant in space (0.000 ** *).

B. Determination of the rate of total protein

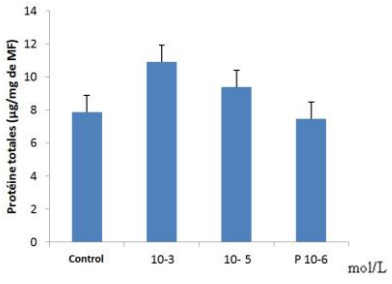

Figure 5: Variations of averages total protein at different experimental batches of *Flavoparmelia* caperata

We see from the results in increased levels of total protein in all treated groups compared to the control. According to the ANOVA test, the comparison of changes in protein levels in different batches of *Flavoparmelia caperata* shows that these variations are highly significant in space (0.000 ***) (Fig5).

C. Determination of the activity of different enzymes

1) Measurement of ascorbate peroxidases activity(APX):

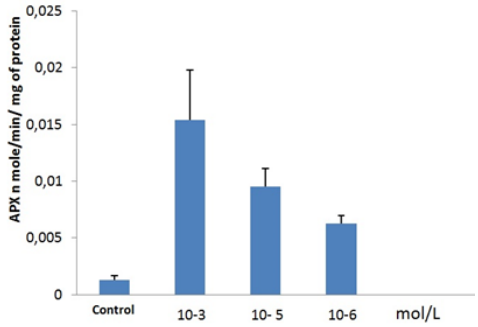

Figure 6 : Variations of averages Ascorbate peroxidase activity at different experimental batches of Flavoparmelia caperata

We note(Fig6), that the rate of ascorbate peroxidase activity is very high in lots immersed in a solution of SO2 concentration 10-3 mol / L with an average of 0.0153 nmol / min / mg protein According to the ANOVA test, comparing the average of ascorbate peroxidase activity in different batches of *Flavoparmelia caperata* shows that variations in average APX are highly significant in space (0.000 ***) .

2) Measurement of Guaïcol peroxidases activity (GPX)

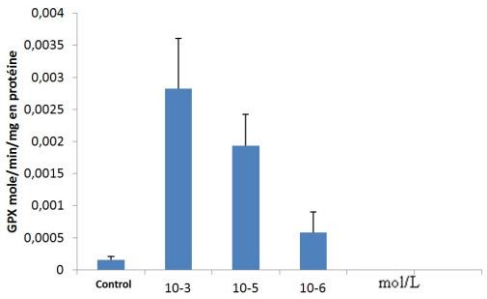

Figure 7: Variations of averages Guaïcol-peroxydase activity in different experimental batches of *Flavoparmelia caperata*

Our results show a clear increase in -peroxydase activity in lots immersed in a solution of $SO_2$to 10^{-3} and 10^{-5}mol/L concentration, however, a slight increase of Guaïcol activity was observed in the treated immersion (10^{-6} mol/L) (Fig7).

According to the ANOVA test, comparing the average grade of the GPX activity in different batches of the Flavoparmelia caperata shows that variations averages of GPX are highly significant in space (0.000 ***)

3) Measurement of catalase activity (CAT)

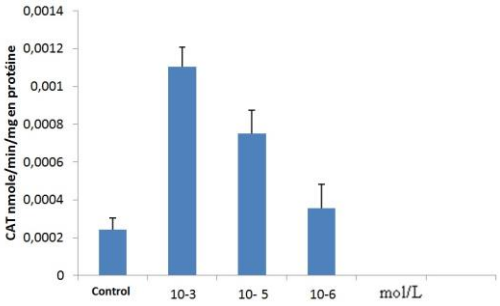

Figure 8: Variations of average Catalase activity at different experimental batches of Flavoparmelia caperata

From Fig 8 we see an increase in catalase activity in all treated versus control. Indeed the rate of CAT Password 0.0002 nmol / min / mg protein in the control to 0.001 nmol / min / mg protein in the treated concentration of 5.10^{-3} mol/L

Comparing the average grade of CAT activity in all treated groups of Flavoparmelia caperata shows that variations of CAT activity are highly significant in space (0.000 ***).

D. Observation Under Binocular Of Thallus Immersed:

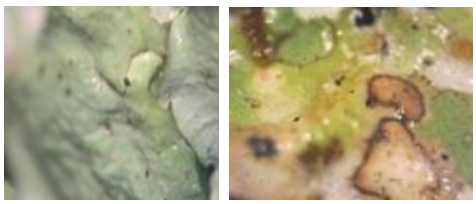

Figure 9 : Control 5.10^{-3} mol/l Flavoparmelia caperata(L.) Hale

When the thallus Flavoparmelia caperata observed treated with a solution of 5.10^{-3} mol / l compared to the thallus witness, we note that there are significant morphological changes, among these changes: Color change to clear (chlorosis) on the majority of thallus accompanied by lines on the thallus;

Presence of several brown spots (necrosis) on all of thallus

Figure 10 : Control. 10^{-5} mol/l *Flavoparmelia caperata* (L.) Hale

Thallus Treaty *Flavoparmelia caperata* by 5.10^{-5} mol / l solution becomes wrinkled green with some brown spots (necrosis) surrounding the lobes.

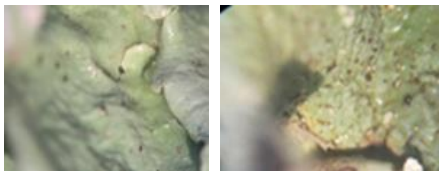

Figure 11 : Control 5.10^{-6} mol/l *lavoparmelia caperata* (L.) Hale

Thallus Treaty *Flavoparmelia caperata* by 5.10^{-6} mol / l solution becomes wrinkled green with some brown spots (necrosis) surrounding the lobes. At frond which are processed by the solution concentration of 5.10-6 mol / l, there is not a big change compared to the control; fronds are green .

According Arora et al 2002 , pollutants such as ozone(O_3), nitrogen oxide (NOx) and sulfur dioxide (SO2), involved in the formation of free radicals that promote stress oxidative . ROS can be harmful for most cellular components **(17)**. Cells have developed strategies of defense against ROS, they have significant antioxidant systems such as photosynthetic pigments, proteins and enzymes.

According to our results, the higher the concentration of the pollutant decreases, signs of disturbance are minimal. The chlorophyll degradation is generally related to the intensity of the pollution and the high concentration of sulfur dioxide. The air pollutants affect lichens through destruction of chlorophyll. This hypothesis was verified by **(18)**, upon exposure to some foliose lichens a concentration of 5 ppm SO2 for 24 hours.

Our results indicate a significant increase in total protein levels in all treated groups Flavoparmelia caperata . Based on analyzes of variance (ANOVA) statistically significant relationships were observed, the accumulation of total protein results from a general stress suffered by the cell. An important synthesis of soluble proteins accompanies or completed the acquisition of resistance to stress, it results from slower growth and storage of molecules in the hyaloplasm or in some organelles (chloroplasts, mitochondria) . It seems that the synthesis of specific proteins is necessary for the hardening **(19)** .

Increasing protein levels can also be due to activation of a set of genes for the synthesis of specific proteins associated with stress proteins such as "LEA" which provide protection of all vital cell proteins **(20)**. According to **(21)**. The increase in total protein could be linked to induction of detoxification brought into play by a control system which consists of enzymes, protein and antioxidant molecules.

Oxidizing enzymes help protect cells against free products physiological manner during normal cellular metabolism radicals. Catalase, the APx and GPx belong to the primary defense mechanism against reactive oxygen species "ROS" by catalyzing the conversion of hydrogen peroxide H2O2 to H2O **(22)**, **(23)**. Our results reveal a very highly significant increase in ascorbate peroxidase activity, guaiacol peroxidase and catalase in batches treated by different concentrations of SO2, this increase is probably due to increased antioxidant activity in the cells. Indeed, APx is the only enzyme for decomposing H2O2 in chloroplasts **(17)**

The other enzyme studied in our study is the GPX. The gaïacol peroxidase enzyme is a key enzyme of the detoxification mechanism. The latter is localized in the cytosol and has an important role in the decomposition of hydrogen peroxide and the reduction of toxic hydroperoxides**(24)** . The components of the antioxidant system and especially catalase are known for their involvement against oxidative stress **(25)**.

Exposure of lichens to sulfur compounds **(26)**. is clearly reflected by morphological changes and several authors confirmed , according to **(27)**. The whole thallus dies shortly after the algal cells are damaged. **(28)** during a study of lichen transplantation in 3 different macro environments (Arambagh semi- urban , urban and industrial area in Burdwan Durgapur India) shows that damage the algal component of the thallus is a testament to its discoloration.

IV. CONCLUSION

Currently, immersed lichens, are used to estimate the damage caused by pollution and are then compared to those in the field. Actually comparing the results which we have achieved with a recent study (Serradj & al.,

2014) on the same lichen species Flavoparmelia caperata transplanted in areas with different levels of pollution in the region of Annaba (North East Algerian), we again see that the urban and the various sources of SO2 emissions have harmful and obvious influence on lichen vegetation of our area, this influence diminishes gradually and probably with the remoteness of these anthropogenic sources, on the other hand that the antioxidant enzymes remain early indicators of environmental stress by transplantation method or immersion lichens in polluted atmospheres.

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