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Impact of Two Herbicides (Zoom and Sencorate) on Physiological Parameters (Chlorophyll, Proline and Total Protein), Enzymatic (CAT and APX) and Non-Enzymatic Biomarkers (MDA and GSH) of the Species *Orthotrichum affine*

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Authors' contributions

Al the work of the paper was caried out among the authors. All the authors made corrections, read and aproved for final publication mutualy.

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ABSTRACT

The bryophytes of the genus *Orthotrichum* (*O. affine*) are treated in hydroponic conditions with 125, 250, 500, 1000 and 1500 mg/L of two herbicides (Zoom and Sencorate) for 3, 7 and 14 days. The signs of toxicity are manifested by disturbances in chlorophyll contents (*a*, *b* and *a* + *b*), accompanied by an increase of the content of proline (3 and 7 days) and total protein. Our results also showed a decrease in the GSH level with an increased rate of proline. Regarding the CAT and APX, we showed low activity of these enzymes seems insignificant for the majority of time reflecting the high tolerance of this species to pollutants.

Keywords: Orthotricum affine; chlorophyll; proline; protein; enzymatic activity; biomarkers; sencorate; zoom.

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1. INTRODUCTION

The implications of such disturbances (mass production of thousands of new molecules, lack of waste and overconsumption) often result in irreversible loss of biodiversity, highlighting the dysfunction of ecosystems and their capacity of evolution. Plant communities, because of their sedentary lifestyle, are found first exposed to contamination [1].

The presence of pesticides as organic pollutants in soils, surface water and groundwater is a recurring problem in the world. With the growing awareness of the risks that they can generate for the environment (water, soil, air, food, etc.) and the human health, the systematic and large-scale use of pesticides is questioned [2].

This contamination is characterized by the diversity of organic pollutants and high number of molecules and their metabolites can be as toxic as the parent molecules. If pesticides are first appeared beneficial, their harmful side effects were quickly highlighted. Their toxicity related to their molecular structure, is not limited in effect to those species that one wishes to eliminate. They are particularly toxic to humans [3,4,5].

In parallel with the diversity of antioxidant molecules, there is a complex and highly regulated antioxidant enzymes present in several cellular compartments in order to maintain a sustainable level of ROS in the organism [1].

Generally, the increased activity of enzymes involved in defense responses appear to be related to the species or plant variety, the physiological conditions of the plant, or the type of oxidative stress applied [6].

This study aims at determining the fate of two xenobiotics group of herbicides (Zoom and Sencorate), which are widely used in agriculture, a living organism (plant) which has been selected as a biological model and the ability of the selected model to adapt to the presence of increasing concentrations of xenobiotic .

2. MATERIALS AND METHODS

2.1 Chemical Material

Our chemical material comprises two herbicides, the first (Zoom) is a combination of two active substances (Dicamba and Triasulfuron) and the second (Sencorate) is a triazinone herbicide family.

2.1.1 Zoom herbicide

Zoom is an herbicide used to control broadleaf weeds in wheat and barley. Containing 4.1% ofTriasulfuron and 65.9% of Dicamba as water dispersible granules (datasheet).

Triasulfuron is a radicular herbicide of sulfonylurea family wich is typically absorbed by the rod so that the roots of the plant. The rapid translocation of triasulfuron to the growing points of roots and stems resulting in inhibition of cell division in vulnerable species, where it inhibits the acetolactate synthase (ALS), an enzyme responsible for the biosynthesis of amino acids [7].

Dicamba is a selective systemic herbicide belongs to the "hormone herbicides" or phytohormones. The leaves and roots, with rapid translocation throughout the plant via its symplastic and apoplastic systems (via phloem) with a half-life of about 14 days in most conditions, absorb it. Dicamba works as a growth regulator in the form of a synthetic auxin to create a hormonal imbalance within targeted weeds [8].

2.1.2 Sencorate herbicide

Sencorate is an herbicide that contains 75% metribuzin, belonging to the chemical family triazinones used for weed control in potato and tomato (datasheet).

Metribuzin is a photosynthetic electron transport inhibitor at the photosystem II acceptor side.This chemical disrupts photosynthesis and disturbs plant growth, which eventually leads to death (datasheet).

2.2 Biological Material

Our choice is focused on a bryophyte species: *Orthotrichum affine* which is the commonest *Orthotrichum* on trees and shrubs in districts with clean air [9].

This common moss forms loose, slightly branched, dull, mid-green or yellowish-greentufts 0.8–3.5 cm tall. Individual leaves are about 3 mm long, have recurved margins, and end in an acute tip [9].

2.3 Choice of Sampling Site

Our choice of the sampling site is focused on the Ain synour region (SoukAhras, Northeast of Algeria) (Fig. 1). The geographical coordinates of the site are 36º19'11 "North and 7º52'13" East, with a latitude of 36.319773 and Longitude of 7.870218 (in decimal degrees) (Direction Of Agriculture, 2013).

2.4 Collection and Analysis Methods

2.4.1 Sampling strategy

Sampling was performed in January and February 2013. Several thalli of this species were collected on the bark of trees of several stations under standardized conditions (height of samples is 1.50 to 2 m to the ground) [10]. Then, the samples were stored in tightly closed plastic bags to reduce water loss through evapotranspiration.

2.4.2 Method of growing foams

Both herbicides (Zoom and Sencorate) are used for the impregnation of *Orthotrichum affine* samples. They are dissolved in distilled water at concentrations of 125, 250, 500, 1000 and 1500mg/liter. Approximately 1g of this species thallus is soaked in 100 ml of the solution during three treatment periods: 3, 7 and 14 days (with n = 5 for each concentration and for each periods) [11] (Fig. 2).

Fig. 1. Geographical location of the sampling site"Ain Seynour" (Direction of Agriculture, 2013)

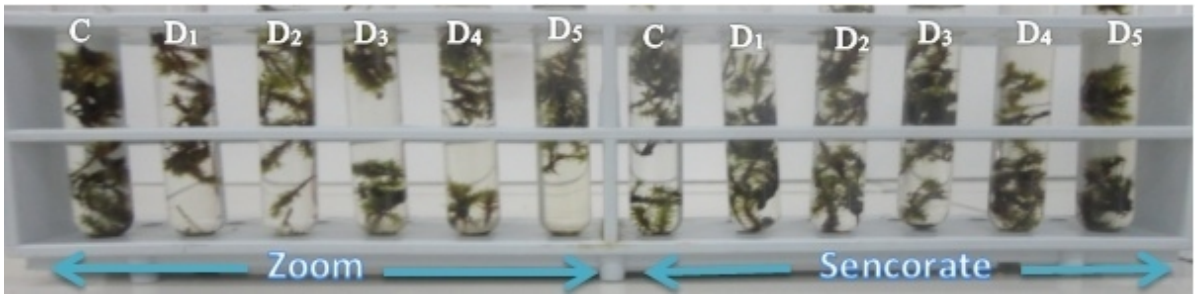

Fig. 2. Culture of the species *Orthotrichum affine* in the solutions prepared with both herbicides (Zoom and Sencorate) (n=5).C: control, D1 125, D2 250 and D3 500, D4 1000, D5: 1500 mg/ l

2.4.3 Determination of physiological parameters

The method used for the extraction of chlorophyll is determined by the traditional method of Holden [12], which consists of a maceration of the plant in acetone. Proline was quantified by the method of Monneveux and Nemmar [13].Total protein was quantified by the method of Bradford [14], using bovine serum albumin (BSA) as a standard (Merk).

2.4.4 Determination of biomarkers

The method used for the extraction of enzyme is the method of Loggini [15]. The catalase activity (CAT) (EC 1.11.1.6) is performed according to the method of Cakmak and Horst [16]. The ascorbate peroxidase activity (APX) (EC 1.11.1.11) is performed according to the protocol adopted by Nakano and Azada [17].

The concentration of reduced glutathione(GSH) was assayed by the method of Weckberker and Cory [18]. Lipid peroxidation was estimated by changing the content of malondialdehyde (MDA) determined according to the method described by Alia et al. [19].

2.5 Data Analysis

The processing and data analysis was performed using specialized statistical software: Statistica ® 8.0 (Statsoft: www.statsoft.com) where a statistical description was given for each studied variable (physiological, biochemical and enzymatic) calculating medians ($\tilde{x}$);

Our statistical analysis is based on the following points:

- The non- parametric Kruskal Wallis test was used to test the effect "dose" on the two herbicides (Zoom and Sencorate) compared to control batches (intra - herbicide comparison);
- A post- hoc analysis presented by the multiple comparison test (*z*' values) or pairwise comparison to classify different doses according to the " herbicide " effect ;
- Mann-Whitney U test was used to test the overall effect of inter-and intra- herbicide ;
- The coefficient of Spearman correlation was used to test the sharpness of linear relationships between all quantitative variables used in this work.

The conditions of application of statistical tests were checked and met as recommended reported in several statistical literature as: Dagnelie [20,21] and Scherrer [22,23].

3. RESULTS AND DISCUSSION

The exposure of the species *Orthotrichum affine* to two herbicides (Zoom and Sencorate) shows that the dose effect is remarkable in different periods of treatment for the majority of measured parameters. To confirm this hypothesis, we applied the non-parametric test of Kruskal Wallis (Table 1) and the multiple comparison test (z' values) of Kruskal Wallis which classifies the doses of the two herbicides and group those that have the same effect on *O. affine* (Table 2).

The chlorophyll content is often used to assess the impact of many environmental stresses (toxicity of herbicides). The exposure of *O. affine* during all periods of treatment (3,7 and 14 days) to those herbicides shows significant differences of chlorophylls (*a*, *b* and *a* + *b*) (Table1).

Fig. 3a shows the disruptive effect of Zoom herbicide on *O. affine* where a dependent-dose decrease was observed at the beginning of the treatment (3 days), followed by an increase with low doses (125, 250 and 500 mg / l) on the 7th day then with high doses (1000 and 1500 mg / l) at the day 14. This effect is consistent with the work of Groppa et al. [24,25] and Mysliwa-Kurdziel et al. [26]. As those of Puritch and Barker [27], highlighting a disruptive effect of the ammonium on chlorophyll biosynthesis. Other authors Shraddha et al. [28] report a common degeneration of chlorophyll and carotenoids amount in plants exposed to different concentrations of heavy metals.

However, the application of Sencorate (Fig. 3b) induced a lowering of chlorophyll content (*a, b* and *a* + *b) i*n the three treatment periods. This is consistent with numerous studies that reported a decrease in chlorophyll content under the effect of Cd [24,25]and heavy metals in general [26,29]. The reduction of chlorophyll is a primary events in plants subject to metal stress and results from the inhibition of the enzymes responsible for the biosynthesis of chlorophyll [26].

Thus, the work of Maizi et al. [30] show that the levels of chlorophyll (*a* + *b*) in *Funaria hygrometrica* tend to decrease in highly polluted sites due to the disruption of the photosynthetic process.

The accumulation of amino acids due to environmental stress can be explained by the breakdown of certain proteins sensitive to stress or by the synthesis of new amino acids [31,32]. Among the amino acids, proline, although not constituting less than 5 % of free amino acids [29,33], is probably one of the metabolites of the most common stress. Its content, which was 4.40% of total amino acids is significantly increased in *Orthotrichum affine* following treatment with both herbicides from the 3rd day (Table 1) whose samples treated with Zoom (Fig. 4a) shows proline contents higher than samples treated with Sencorate (Fig. 4b). This explains why this treatment period is sufficient for this species to detect stress due to the addition of both xenobiotics (herbicides) in the culture medium.

An increase of proline under the effect of a xenobiotic (Cd) has been reported for various plants such as lupine [34], rice [35,36], radish [37] and soybean [38]. Such an increase in the proline has also been observed under the effect of other metals such as Mn [29], Zn, Pb, Co, Cu [19,32,39,40], but also under the influence of other types of stress: Saline [41], water [42], UV radiation [43], thermal [42].

This effect is consistent with a protective function of membranes against free radical attack, agreeing with the observations of Smirnoff and Cumbes [44] suggest that the proline reacts with hydroxyl radicals to generate non toxic hydroxyproline and those of Matysik et al. [33] and of Alia et al. [45] that show a singlet oxygen scavenging by proline. In addition, increased chlorophyll content could result from protection by proline of thylakoid membranes against ROS attack as reported by Kavikishor et al. [46].

Our results also show a significant increase in the amount of protein (3 and 7 of exposure to Zoom and 3, 7 and 14 days of exposure to Sencorate) (Fig. 5). Which is explained by the fact that the presence the xenobiotic inside plant tissues stimulates protein synthesis of many other enzymes between those involved in detoxification, this is in perfect agreement with the results of Shraddha et al. [28], which shows that the accumulation of proteins is proportional to that of metal.

Our results are also consistent with the work of Sanchez et al. [47], who found a significant increase in amino acid levels and protein in green beans (*Phaselous vulgaris* L.cv.strike) treated with 24mM of NH_4NO_3. Thus, the work of Khaldi [48] showed an increased rate of proline and total proteins in foams (*Leucodon sciuroides*) and in lichens (*Ramalina farinacea*) treated with different concentrations of fertilizer NH_4NO_3.

Table 1. The results of "*Kruskal Wallis"* Test of the effect of Zoom and Sencorate on *O. affine* for the three periods of treatment (*H* and *p* values)

Herbicides	Parameters	3 days		7 days		14 days	
		H	***p***	***H***	***p***	***H***	***P***
Zoom	Chl*a*	27,2761	0,0001	28,2258	0,0000	27,1213	0,0001
	Chl*b*	28,1019	0,0000	28,1019	0,0000	27,2452	0,0001
	Chl*a+b*	27,6374	0,0000	28,2258	0,0000	27,6477	0,0000
	Prol	27,1455	0,0001	25,2603	0,0001	25,4063	0,0001
	Prot	24,7224	0,0002	27,7682	0,0000	26,9415	0,0001
	CAT	2,7187	0,7433	2,2026	0,8205	6,2594	0,2818
	APX	7,3242	0,1976	3,4506	0,6309	12,8813	0,0245
	GSH	18,8477	0,0021	24,7884	0,0002	27,2297	0,0001
	MDA	9,9703	0,0761	25,6400	0,0001	8,7265	0,1205
Sencorate	Chl*a*	28,1019	0,0000	27,6374	0,0000	28,2258	0,0000
	Chl*b*	25,6245	0,0001	26,1452	0,0001	28,2258	0,0000
	Chl*a+b*	27,0748	0,0001	26,5536	0,0001	28,2258	0,0000
	Prol	26,7097	0,0001	21,6897	0,0006	24,1878	0,0002
	Prot	23,6128	0,0003	27,0189	0,0001	26,6109	0,0001
	CAT	3,6890	0,5950	3,4185	0,6358	4,9897	0,4171
	APX	12,0607	0,0340	3,4185	0,6358	4,0009	0,5493
	GSH	27,3587	0,0000	16,7444	0,0050	26,1361	0,0001
	MDA	23,8697	0,0002	24,8813	0,0001	18,1344	0,0028

Table 2. Classification of concentrations of Zoom and Sencorate herbicides involved in the treatment of *O. affine* (multiple comparison (*z' values*)). (The grading is done only for significant results)

Herbicides	Parameters	3 days	7 days	14 days
Zoom	Chl*a*	C D1 D2 D3 D4 D5	C D1 D4 D5 D2 D3	C D1 D2 D3 D5 D4
	Chl*b*	C D2 D1 D3 D4 D5	C D1 D4 D5 D2 D3	C D2 D1 D3 D4 D5
	Chl*a+b*	C D1 D2 D3 D4 D5	C D1 D4 D5 D2 D3	C D1 D2 D3 D5 D4
	Prol	C D1 D2 D3 D4 D5	C D1 D2 D3 D4 D5	C D2 D3 D4 D1 D5
	Prot	C D1 D2 D3 D4 D5	C D1 D2 D3 D4 D5	D3 D2 C D1 D4 D5
	GSH	C D1 D2 D3 D4 D5	C D1 D2 D3 D4 D5	D5 C D4 D3 D1 D2
	MDA	----	C D1 D2 D3 D4 D5	----
Sencorate	Chl*a*	D4 C D1 D3 D5 D2	D2 D1 C D3 D4 D5	C D1 D2 D3 D4 D5
	Chl*b*	C D3 D4 D5 D1 D2	D2 D5 C D1 D4 D3	C D1 D2 D3 D4 D5
	Chl*a+b*	D4 C D1 D3 D5 D2	D2 D1 C D4 D3 D5	C D1 D2 D3 D4 D5
	Prol	C D1 D2 D3 D4 D5	C D1 D2 D3 D4 D5	D1 C D2 D3 D5 D4
	Prot	C D1 D2 D3 D4 D5	C D1 D2 D3 D4 D5	C D4 D3 D1 D2 D5
	GSH	C D1 D2 D3 D4 D5	C D1 D2 D4 D3 D5	C D1 D2 D3 D4 D5
	MDA	C D1 D2 D5 D3 D4	C D1 D2 D3 D4 D5	C D1 D2 D3 D4 D5

To fight against oxidative damage, plants establish enzymatic and non-enzymatic antioxidants defense systems that play a role in regulating the levels of ROS [49]. Indeed, an increased synthesis of detoxification enzymes (SOD, CAT or APX) has been observed following the use of fungicides in barley [50].

The subsequent application of the treatment with both types of herbicides (Zoom and Sencorate) does not significantly affect the enzyme biomarkers (CAT and APX), where the Fig. 6 illustrate that the analysis of these enzyme activities involved in the management process of oxidative stress throughout the treatment does not show significant differences compared to controls. Except APX of the 3rd day (Sencorate) and of the 14th day (Zoom) (Table 1). Our results confirm the strength of the foams to pollutants (herbicides), but also emphasizes the fact that they are also very dependent in this case of the nature of the substrate in particulary (Zoom and Sencorate).

In our work, we demonstrated a dependent-dose decrease in GSH manner (Fig. 7a) in the presence of both herbicides (3 and 7 days for Zoom and 3 days for Sencorate) in *O. affine*

species. This depletion can be explained by the direct bond of glutathione to xenobiotic atoms as glutathione has a carboxylic acid group, an amine group, a sulfhydryl group (-SH) and two peptide grafts may be involved in reactions with other atoms. Its -SH functional group play an important role in binding to the xenobiotic [51]. These results are consistent with the works of Cao et al. [52], Freeman et al. [53], Khaldi et al. [54,55].

Our results are also consistent with those of Durcuix et al. [56] in which the level of GSH is reduced with the increase of the tolerance to accumulation of pollutant for low concentrations and also with those observed by Khaldi [57] where the level of GSH decreases in response to the stress induced by high concentrations of pollutant.

Lipid peroxidation is a mechanism of cell damage well known and used as an indicator of oxidative stress. Lipoperoxides are the aberrations of the polyunsaturated fatty acids. They are unstable and decompose to form carbonyl compounds, the most abundant is the malondialdehyde (MDA). That is why the MDA is often used as an indicator of lipid peroxidation [58].

Malondialdehyde (MDA), which is a good marker of lipid peroxidation, is markedly increased in this species of bryophytes treated with different concentrations of both herbicides when the

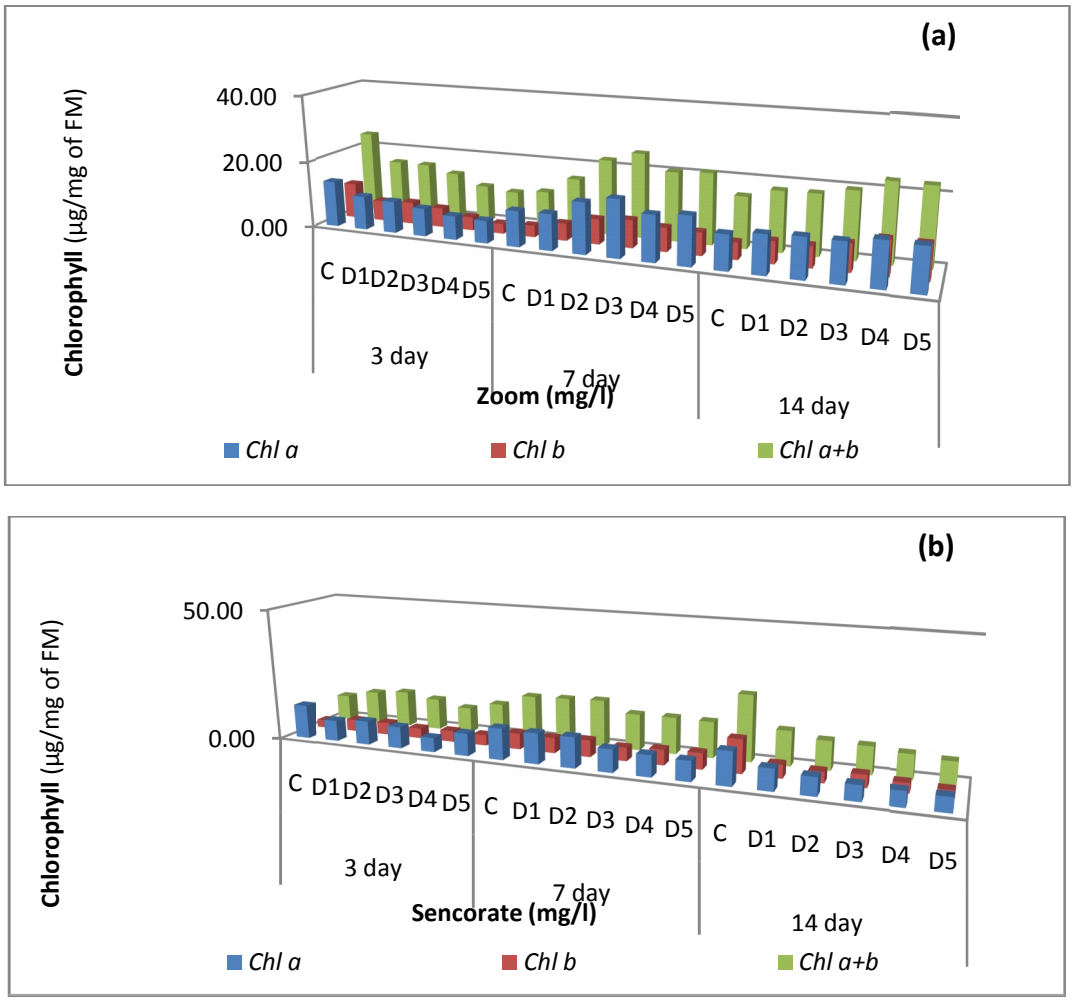

Fig. 3. Effect of Zoom (a) and Sencorate (b) on chlorophyll pigment levels (median) in the species *O. affine* after 3,7 and 14 days of exposure (*Kruskal Wallis* test, n = 30). C: Control, D1: 125, D2: 250, D3: 500, D4: 1000 and D5: 1500 mg / L. (FM: fresh matter)

application of Sencorate (Fig. 7b) shows a significant increase and dose dependent MDA levels while the application of Zoom (Fig. 7b) gives significant results at the 7day of treatment (Table 1). These results are confirmed in foams treated with fertilizer [11], bryophytes or lichens contaminated by metals [59,60].

To compare the effect of the two herbicides on *O. affine*, we applied the non-parametric test U of Mann-Whitney (Table 3).

During the treatment of three days, we did not detect significant differences between the two herbicides for all metabolites, enzyme biomarkers and MDA (p> 0.05). By against chlorophyll pigments *a*, *b* and *a* + *b* and GSH showed significant differences. After 7 days of exposure, significant differences are noted for chlorophyll pigments *a, b,* and *a* + *b*, proline, GSH and MDA. While the total proteins, CAT and APX have no significance (p> 0.05). The treatment of 14 days indicated the existence of significant differences ($p < 0.001$) between the two herbicides remarkable for the majority of the parameters measured in *O. affine* (chlorophyll pigments and metabolites). However, the CAT shows a lower significance (U = 264, p = 0.006), the latter is absent for APX (U = 417, p = 0.63), GSH (U = 382, p = 0, 31) and MDA (U = 334, p = 0.09) (Table 3).

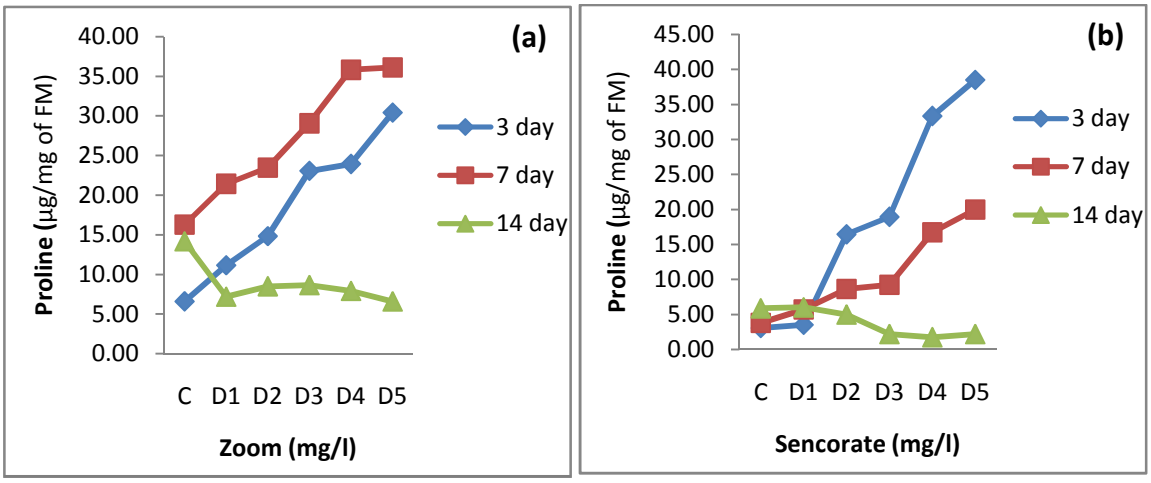

Fig. 4. Effect of Zoom (a) and Sencorate (b) on the levels of proline (median) in *O. affine* after 3,7 and 14 days of exposure (*Kruskal Wallis* test, n = 30). C: Control, D1: 125, D2: 250, D3: 500, D4: 1000 and D5: 1500 mg / L. (FM: fresh matter)

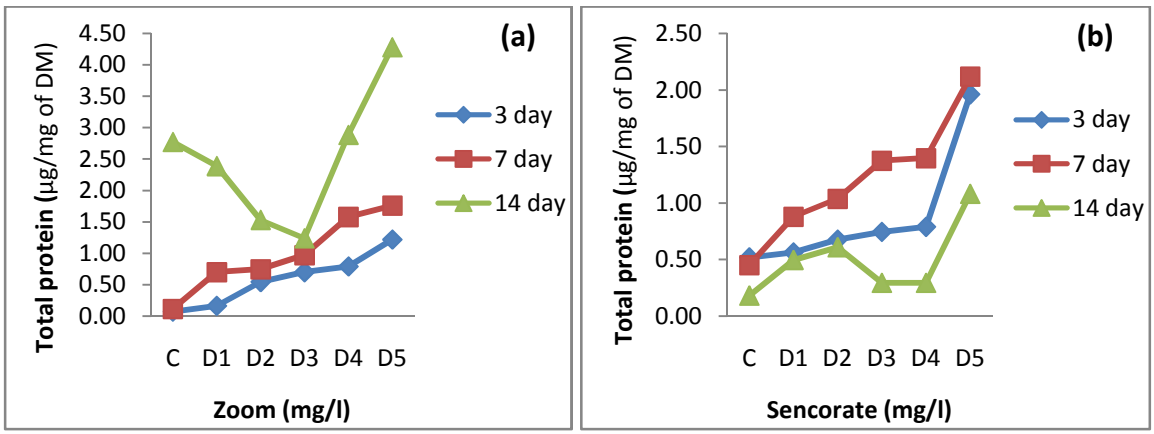

Fig. 5. Effect of Zoom (a) and Sencorate (b) on the levels of total protein (median) in *O. affine* after 3,7 and 14 days of exposure (*Kruskal Wallis* test, n = 30). C: Control, D1: 125, D2: 250, D3: 500, D4: 1000 and D5: 1500 mg / L. (DM: dry matter)

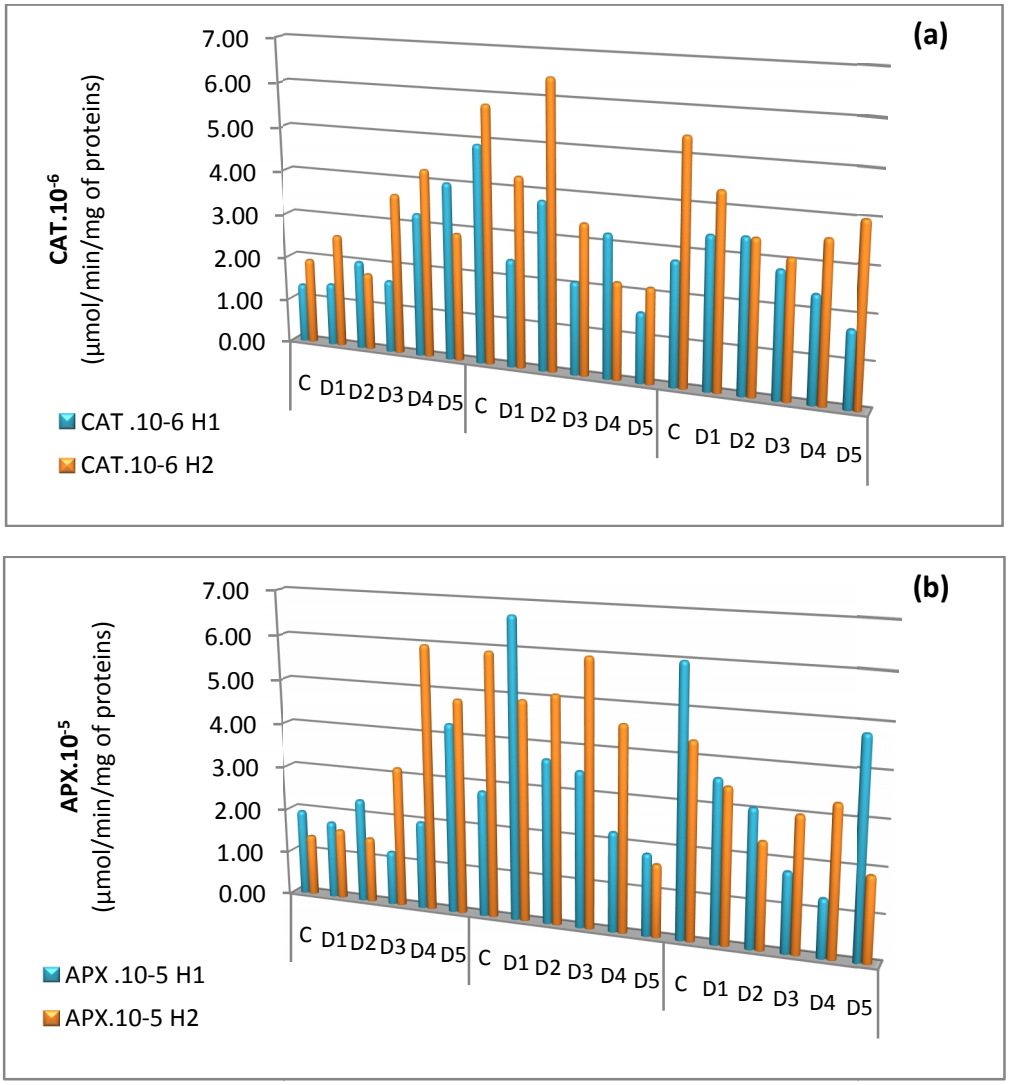

Fig. 6. Effect of Zoom (H1) and Sencorate (H2) on the enzymatic activity (CAT (a) and APX (b)) (median) in *O. affine* after 3,7 and 14 days of exposure (*Kruskal Wallis* test, n = 30). C: Control, D1: 125, D2: 250, D3: 500, D4: 1000 and D5: 1500 mg / l

Table 3. Inter-herbicide comparison in the species *O. affine* (*U* and *p*values) as a function of exposure time (Mann Whitney U test) (H1:Zoom, H2: Sencorate)

O. affine	3 days		7 days		14 days	
H1/H2	***U***	***p***	***U***	***p***	***U***	***p***
Chl*a*	250,0	0,003108	194,0	0,000154	104,0	0,000000
Chl*b*	238,0	0,001723	285,0	0,014711	159,0	0,000017
Chl*a+b*	203,0	0,000260	225,0	0,000880	150,0	0,000009
Prol	449,0	0,988204	48,5	0,000000	18,0	0,000000
Prot	379,0	0,293859	348,5	0,133455	2,5	0,000000
CAT	400,0	0,459773	446,0	0,952842	264,0	0,005962
APX	436,5	0,841802	432,0	0,790147	417,0	0,625626
GSH	0.0	0,000000	197,5	0,000189	382,0	0,314727
MDA	403,5	0,491783	278,0	0,010993	334,0	0,086343

According to the Table 3, Overall, we can conclude that the difference between the effect of the two herbicides on *O. affine* increases with the exposure time.

To complete the statistical analysis between and within herbicides, we analyzed the correlation between physiological, metabolic and enzymatic parameters studied in *Orthotrichum affine*. Table 4 shows that the contents of chlorophyll pigments *a* and *b* of *O. affine* are highly correlated (r = 0.85) and with the total chlorophyll (*a* + *b*). Conversely, they are negatively correlated with glutathione. Furthermore, there is a reasonable correlation between the total protein content and the rate of MDA up to 54%.

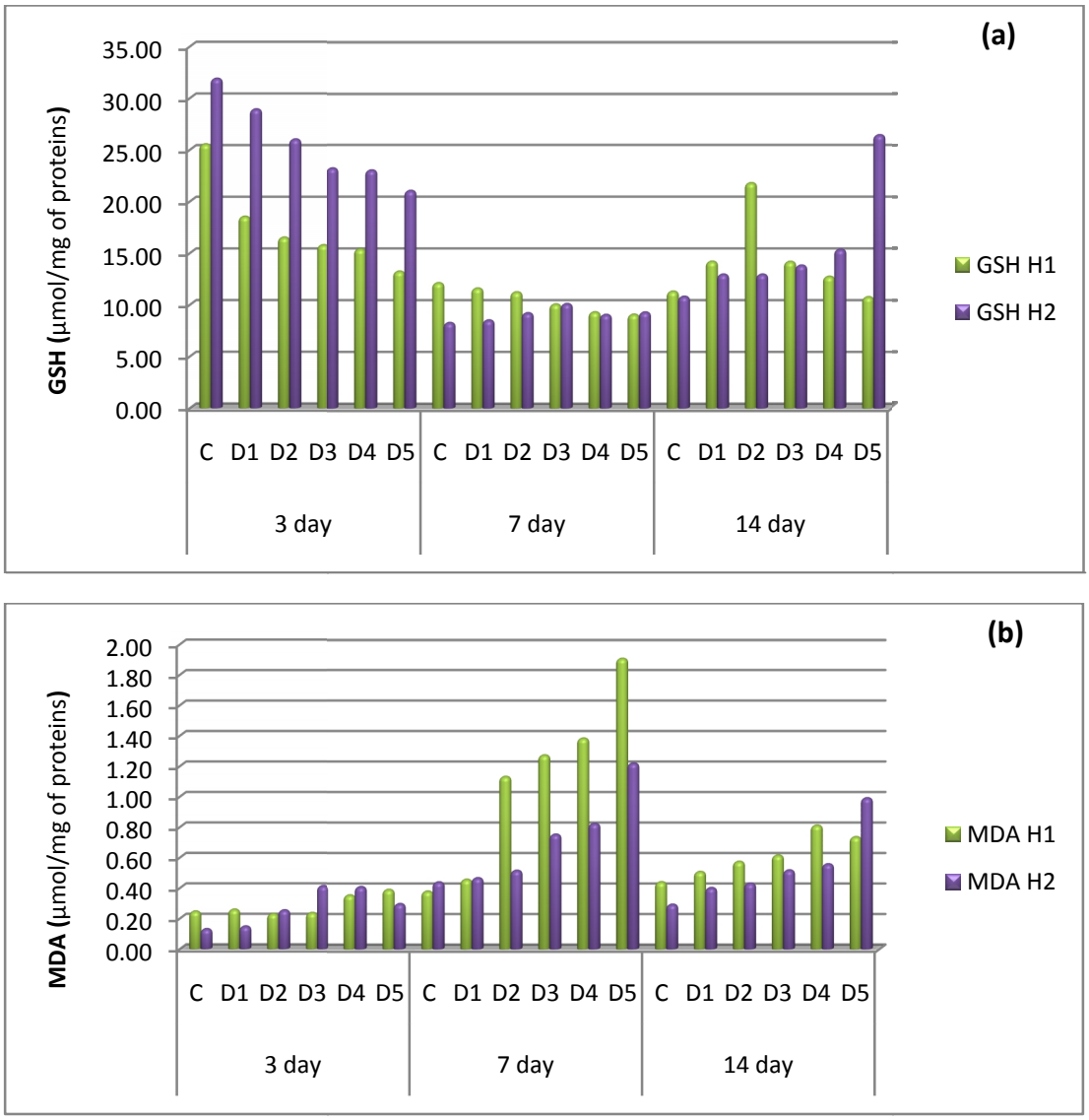

Fig. 7. Effect of Zoom (H1) and Sencorate (H2) on levels of glutathione (GSH) (a) and malondialdehyde accumulation (MDA) (b) in *O. affine* after 3, 7 and 14 days of exposure (*Kruskal Wallis* test, n = 30). C: Control, D1: 125, D2: 250, D3: 500, D4: 1000 and D5: 1500 mg/L.

Table 4. Spearman correlation matrix of the various parameters measured in the species *O. affine*

O. affine	Chl*a*	Chl*b*	Chl*a+b*	Prol	Prot	CAT	APX	GSH	MDA
Chl*a*	1								
Chl*b*	0,847	1							
Chl*a+b*	0,968	0,944	1						
Prol	0,178	0,044	0,105	1					
Prot	0,093	0,090	0,094	0,274	1				
CAT	-0,119	-0,134	-0,128	-0,024	-0,095	1			
APX	0,071	0,019	0,063	0,082	-0,017	0,125	1		
GSH	-0,506	-0,450	-0,511	-0,166	-0,284	0,050	-0,197	1	
MDA	-0,033	0,002	-0,023	0,169	0,737	0,021	-0,047	-0,359	1

4. CONCLUSION

As part of this work, we try to make our contribution on the use of plants as bio-indicators of pollution and thereby even their behavior versus probably harmless molecules but at the same time controversial as Zoom and Sencorate where we followed the evolution of the parameters of vitality (metabolites, chlorophyll and enzyme biomarkers) throughout the exhibition. This study showed a better tolerance of *Orthotrichum affine* against xenobiotic.

It appears that the mechanisms developed in this species of foam to withstand difficult environmental conditions (decreased concentration of GSH, increased levels of proline and the absence of enzymatic activity (CAT and APX)), confer tolerance capabilities to particularly important contaminants.

To continue studying the effects of herbicides (Zoom and Sencorate) on this species (*Orthotrichum affine*), we could move towards higher concentrations of these xenobiotics in the culture medium of these biological models.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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