Egyptian Journal of Aquatic Biology & Fisheries

Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 24(7): 271 – 286 (2020)
www.ejabf.journals.ekb.eg

Evaluation of the toxicity of a systemic fungicide (FOLIETTE) in a freshwater macrophyte (*Iris pseudacorus* L.).

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ARTICLE INFO

Article History:
Received: July 27, 2020
Accepted: Oct. 24, 2020
Online: Oct. 25, 2020

Keywords:
Aquatic pollution, FOLLIETTE, *Iris pseudacorus* L., oxidative stress, tolerance.

ABSTRACT

Aquatic contaminations pose many problems for organisms and the functioning of ecosystems, mainly caused by agricultural activity including fungicides. It is in this context that we conducted an ecotoxicological study to evaluate the toxicity of **a** (FOLIETTE) fungicide, commonly used in Algeria, in a freshwater macrophyte (***Iris pseudacorus*** L.) from natural marshes in the ‘’Bourdim region, El-Tarf located in the extreme northeast of Algeria’’ by measuring certain physiological and biochemical parameters. The results obtained show that the addition of this fungicide, at different concentrations for 7 days of treatment, decreases the content of chlorophyll pigment and increases the accumulation of total proteins in different parts of the plant (roots and leaves). Similarly, a significant increase in some stress biomarkers such as total polyphenols, malondialdehyde (MDA) and glutathione (GSH). At the same time, the measurement of enzyme activity involved in the detoxification system shows a significant induction of guaiacol-peroxidase (GPX) activity, which plays an important role in mitigating oxidative damage, thus proving the tolerance of our plant model to this xenobiotic and consequently its possible use as a candidate for phytorepuration.

INTRODUCTION

Waste management in general is now a major environmental concern in many countries of the world. **(Tshala *et al*., 2017)**. In particular, agricultural wastes that have deteriorated over time the aquatic ecosystem known for its fauna and floristic biodiversity. Faced with this threat, most countries have opted for conventional contaminated water treatment systems, but this technological choice has often proven to be highly inappropriate due to the very high cost of facilities and equipment, the lack of well-prepared technicians to maintain them, and the prioritization choices made by public

powers, resulting in outdated equipment, overload and abandonment of structures **(Suwasa and Wanida, 2011)**. Furthermore, the sanitation systems to be considered must be flexible and scalable. However, to provide solutions to this problem there are known alternatives both from a technical point of view and from a socio-economic point of view such as natural methods of waste water treatment which is phytopurification. **(Bensmina-Mimeche *et al.*, 2013; Bensaid *et al.*, 2017)**. This procesus involves replicating and potentiating on a reduced scale the purification processes that take place in natural wetlands and are based on the joint action of plants, microorganisms and soil **(Vymazal, 2005)**. These are artificial wetlands or also known as macrophyte planted filters **(Suwasa and Winda, 2011)**. The installation of artificial marshes at outfalls of domestic sewage treatment plants **(Leto *et al.*, 2013)**, road runoff **(Triboit *et al.*, 2009)** or agricultural drains **(Destandau *et al.*, 2013)** is increasingly widespread **(Prost-Boucle and Boutin, 2013)**. However, this treatment system is still rarely used in Algeria to reduce any form of aquatic pollution, particularly that caused by agricultural activity. We can cite the two natural lagoon treatment plants at N'Goussa-Ouargla and the M'zab-Ghardaia valley. It is in this context that our study was carried out, which consists in assessing in semi-controlled conditions the tolerance of the studied helophyte **(*Iris pseudacorus* L.)** against systemic fungicide widely used in Algeria **(FOLIETTE)** and to test the purifing functionality of this species as an alternative solution to the treatment of water contaminated by this xenobiotic.

MATERIALS AND METHODS

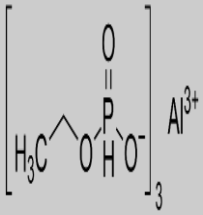
Biologicol material

The **wetar flag (*Iris pseudacorus* L.)** is a bulbous or rhizomatous perennial herbaceous plant of the Iridaceae family. It is characterized by pink rhizomes, with stems generally simple or little branched, the leaves are mainly basal, dark green, 40 to 100 cm long and 1 to 4 cm wide with thick mid-areas. This helophyte contains four to twelve flowers that are arranged in inflorescences on stems 2 to 5 cm long. The flowers are pale yellow to bright yellow and 8 to 10 cm in diameter **(Stone, 2009)**.

Chemical material

The study focused on a very widely used fungicide in Algeria **(FOLIETTE).** It is a systemic fungicide with protective and curative action against many parasitic fungi, especially of the order of phycomycetes and Oomycota. The FOLIETTE is based on 80% fosetyl-aluminum (Aluminium Tris (-O-ethyl phosphonate)), an organometallic compound, of the phosphanate group **(Bayer Jardin, 2011)**.

Table.1. Chemical structure of active substance of FOLIETTE.

Active substance	Chemical structure	Chemical formula	Molar mass
Fosetyl-aluminum		$C_6H_{18}AlO_9P_3$	354,13g/mol

The experimental device

The experimental pilot consists of four rectangular glass aquariums 40 cm long and 30 cm wide filled from bottom to top with 5cm of gravel and 15 cm of sand substrate. Carefully harvested yellow iris plants from the natural marshes of Bourdim, commune of Bouteldja, wilaya-El-Tarf, were freshly cultivated, under semi-controlled conditions, on average three plants per tank for 7 days which represents the duration of the treatment with the Foliette in the open field. The feed of the beds (the third layer), 20 cm thick, is composed of contaminated pond water with different concentrations of FOLIETTE: (0;37.72; 75.44; 150.88 µg). The latter were chosen from the regulatory standards for the use of this fungicide in agriculture from a biological and toxicological point of view.

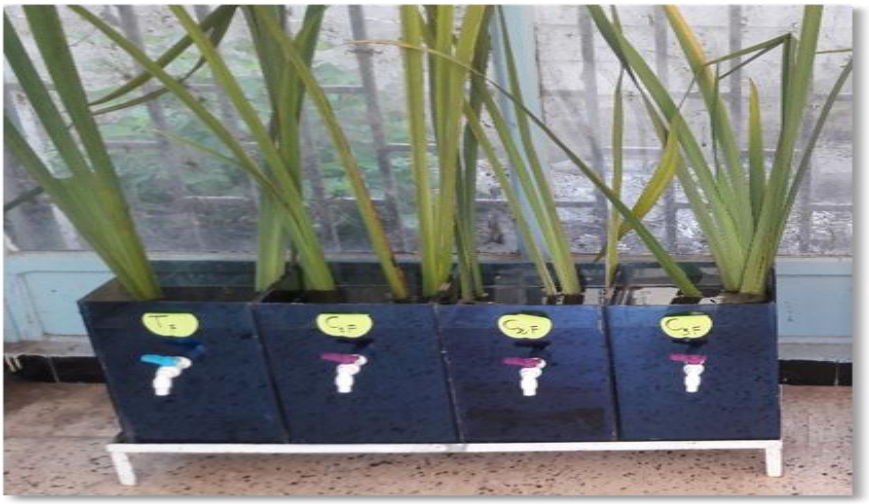


Figure.1. Experimental design.
(personal hold).

Table. 2. List of acronyms.

Aconyms	Nomenclature
C	Control =0µg
C1	Low Concentration=37.72µg
C2	Medium concentration=75.44µg
C3	High concentration=150.88µg.
Chl	Chlorophyll.
cm	Centimeter
DM	Dry material.
FM	Fresh material.
L	Leaves.
Prot	Protein.
R	Roots.
GAE	Gallic Acid equivalent

STUDIED PARAMETERS

Measurement of photosynthetic pigments: (*a*), (*b*) and (*a*+*b*)

The chlorophyll content is determined by the traditional method of **(Holden, 1975)**. For each treatment, one weighs 1g of the leaves of the plant, which is cut into small pieces and ground in a mortar with 25 ml of acetone titrated to 80% and with a pinch of calcium carbonate ($CaCO_3$). After filtration, the solution is put in black boxes to prevent oxidation of chlorophyll by light. Dosing is done by taking 3 ml of the solution from the spectrophotometer tank. Reading is done at wavelengths 645 and 663 nm, after calibration of the apparatus with the 80% acetone control solution. The concentration of these chlorophyll pigments was calculated according to the formula of **(Arnon, 1949)**, is expressed in (µg/ g of FM).

Dosage of total protein

The foliar and root proteins in ***I.pseudacorus*** are assayed by colorimetry according to the method of **(Bradford, 1976)**, which consists in measuring the concentration of proteins in solution by spectroscopic analysis, and 0,1 g of fresh vegetable material is milled with 10 ml of distilled water. After filtration, 0.2 ml of the supernatant is withdrawn and 2 ml of CBB (Coomassie Brilliant Blue) is added. The principle of the method is based on the fixing of the dye (CBB) on the proteins at the level of basic and aromatic residues, this fixing causes a transfer of its color which passes from red to blue. This color change is measured at a wavelength of 595nm, using Bovine Serum Albumin (BSA) as standard.

Determination of certain biomarkers of stress

Dosage of malondialdehyde (MDA)

Lipid peroxidation is estimated at the content of malondialdehyde (MDA) determined according to the method described by **(Alia *et al*., 1995)**. Homogenization of the plant tissue in 5% trichloroacetic acid (TCA) at 10 ml per 1 g of plant tissue is followed by centrifugation for 15 min at 12000 g. The name of the parade is added at a volume equal to thiobarbituric acid (to be determined) at 0.5% prepared in the (TCA) at 20%. The mixture is incubated at 100 ° C for 30 min. Then the reaction is by placing the tubes in an ice bath. The absorbance of the name, obtained after centrifugation at 10000 g for 5 min, was 532 nm. The concentration of MDA is used as extension coefficient: 155 $mM^{-1}cm^{-1}$. The concentrations of MDA are expressed in (µM / mg of protein).

Dosage of glutathione (GSH)

The enzymatic extract (800 µl) is homogenized in a solution of (Tris / EDTA) and de proteinized by 0. 25% sulpho-salicylic acid (SSA). After centrifugation at 2000 g for 10 minutes, the supernatant is used for the spectrophotometric assay with the reagent (DTNB) at 0.01M to 412nm. The concentrations of GSH are determined by the method of **(Weckbecker and Cory, 1988)** is expressed in (µmol / mg of protein).

Dosage of total polyphenols

The extracts of the different plant parts (roots and leaves) were prepared by maceration of 100 mg of powder of each mash in 10 ml of methanol (70 %) overnight at room temperature (20 °C to 25 °C). Finally, the maceras were filtered cold through a filter paper. The total phenol content of the extracts was determined by the method of **(Kim *et al*., 2003)**. 0.5 ml of the extract is mixed with 0.45 ml of distilled water and 0.5 ml of the Folin-Ciocalteu reagent. After 5 minutes, 5 ml of a 7% sodium carbonate (Na_2CO_3) solution was added to the mixture with stirring. After incubation in the dark for 90 min at 23 ° C the optical density of each prepared solution was read by a spectrophotometer at 750 nm. For each sample prepared five replicates were planned. Quantification of the total polyphenols was done using a calibration curve of different dilutions of gallic acid (150 ; 100 ; 80 ; 60 ; and 40 mg / l). From a stock solution (gallic acid) of 200 mg / l dissolved in water. This curve is performed under the same operating conditions as the samples. The trend curve obtained in our case is ($Y = 0.0037X + 0.0664$) with ($R^2 = 0.9516$). The polyphenol contents are expressed in mg of gallic acid equivalent per gram of dry matter (mg $GAE.g^{-1}$ of DM).

Assay of guaiacol-peroxidase (GPX) activity

The activity guaiacol-peroxidase (GPX) is determined spectrophotometrically (Jenway spectrophotometer 6300) at 470 nm by the technique of **(Hiner *et al*., 2002)**. The molar linear extinction coefficient used is = $2470M^{-1}$ cm^{-1}. For a final volume of 3 ml, 100 µl of enzymatic extract, 50 µl of 0.03% H_2O_2 and 2850 µl of phosphate-guaiacol buffer (50 mM NaK, 8 mM guaiacol, pH 7,2) GPX is expressed in nmol / min / mg of protein.

STATISTIC STUDY

The results obtained were expressed by the average of three replicates (the standard deviation). The averages of the same series were compared between them, using the statistical test, analysis of the variance to a classification factor according to the increasing concentrations of FOLIETTE, with a significance level (P). Calculations of this test were performed using statistical analysis software MINITAB version 16.0.

RESULTS

Effect of FOLIETTE on chlorophyll content in the leaves of *I.pseudacorus*

Table 3, represents the impact of FOLIETTE on photosynthetic pigments in I. pseudacorus showed a significant decrease ($P \leq 0.001$) in chlorophyll content (*a, b* and *a + b*) as a function of increasing concentrations compared to control leaves. The lowest values of chl*a*, Chl*b* and Chl (*a + b*) are recorded in the leaves exposed to C3 compared to the control values.

Tab. 3. Effect of FOLIETTE on chlorophyll content (mg/g) in the leaves of ***I.pseudacorus*** after 07days of treatment.

Samples	Chl*a*	Chl*b*	Chl(*a*+*b*)
LC	5.12± 0.04	20.80± 0.38	25.92 ±0.34
LC_1	4.61 ± 1.44	18.50± 5.75	23.10±7.21
LC_2	4.25± 1.35	16.82± 5.51	21.06±6.87
LC_3	3.96±1.32	15.62 ± 5.35	19.58±6.67

Effect of FOLIETTE on total Protein rates in the roots and leaves of *I. pseudacorus.*

The variations in total protein rates in the roots and leaves of yellow flag iris are illustrated in **figure 2**. the significant increase ($P \leq 0.001$) in total protein levels in the roots and leaves treated with increasing FOLIETTE concentrations. The highest value (118.2 µg/g of FM) is observed in roots at the highest concentration compared to the control value (58.56 µg/g of FM). On the other hand, treatment with FOLIETTE significantly decreases ($P \leq 0.001$) the protein content in stressed leaves as a function of increasing concentrations but these values remain above the control valu.

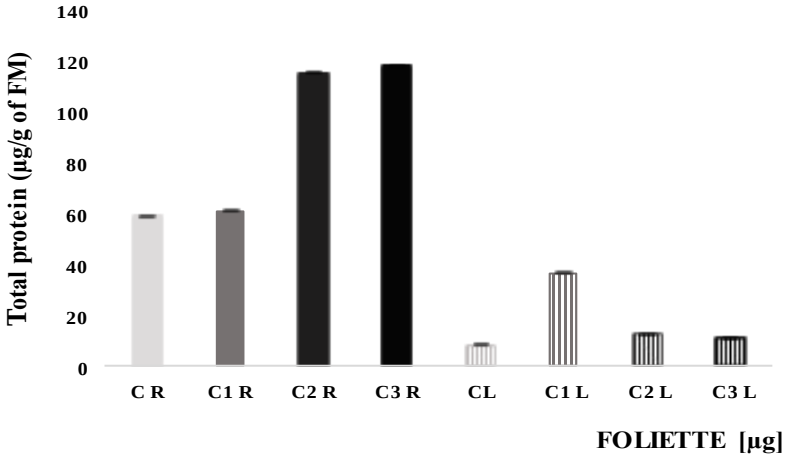


Figure.2. Effect of FOLIETTE on the total protein content in the roots and leaves of ***I. pseudacorus*** after 07days of treatment.

Effect of FOLIETTE on certain stress biomarkers

Effect of FOLIETTE on malondialdehyde (MDA) level in the roots and leaves of *I. pseudacorus*

According to **figure 3**, which represents the variations of the level of MDA according to the increase of the concentrations of FOLIETTE, one observes a significant increase ($P \leq 0.001$) of this parameter in the roots of yellow iris compared to the value witness. These variations reach successively (0.017; 0.040 and 0.069 µM / mg of prot) for C1; C2 and C3 of FOLIETTE, an increase of 4, 6 and 8 times the control value (0.009 µM / mg of prot). Likewise, a significant increase in the level of MDA is recorded in the leaves of Iris which reach its maximum (0.036 µM / mg of prot) for C3, ie twice the control value.

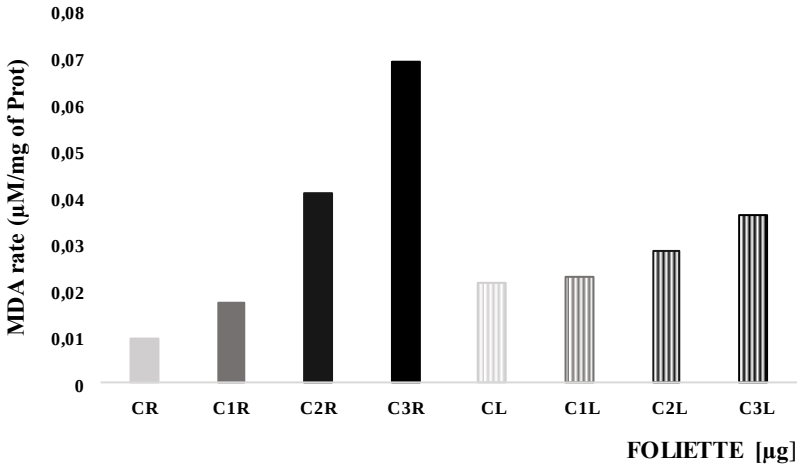


Figure.3. Effect of FOLIETTE on malondialdehyde **(MDA)** rate in the roots and leaves of the ***I.pseudacorus*** after 07days of treatment.

Effect of FOLIETTE on the quantity of glutathione (GSH) in the roots and leaves of *I. pseudacorus.*

According to **figure 4**, there is a significant increase ($P \leq 0.001$) in the level of GSH for the low and medium concentration of FOLIETTE in the roots and the leaves of iris compared to the control organs. The highest level of glutathione (0.664 µmol / mg Prot) is recorded in the leaves treated with C2 while a significant decrease is observed in the leaves treated with C3 which reaches 0.199 µM / mg Prot compared to the control value (0.485 µM / mg Prot), ie a decrease of 59%.

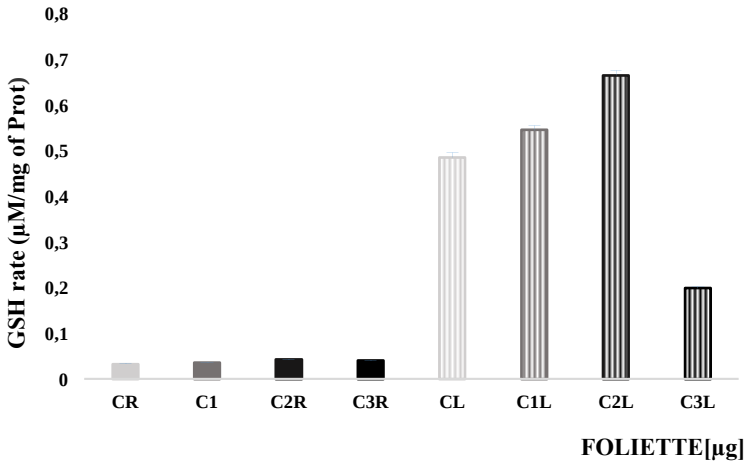


Fig.4. Effect of FOLIETTE on glutathione **(GSH)** rate in the roots and leaves of ***I. pseudacorus*** after 07 days of treatment.

Effect of FOLIETTE on total polyphenol levels in roots and leaves of *I.pseudacorus.*

Based on the results illustrated in **figure 5**, the amount of total polyphenols increased dose-dependent relative to the control in *I. pseudacorus* roots. The greatest amount of total polyphenols (436.53 mg GAE / g of DM) is recorded for the high concentration, which is twice the control value (210.13 mg GAE g-1 of DM). In addition, we observed a significant increase in the rate of total polyphenols in iris leaf treated with different concentrations and reaches its maximum (254.13 mg EAG / g of DM) for the low concentration of 68% compared to control (151mg GAE g-1 of DM).

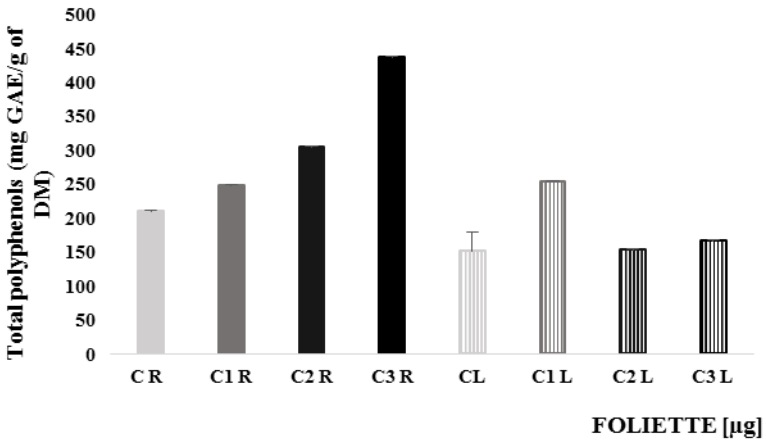


Fig. 5. Effect of FOLIETTE on total polyphenols rate in the roots and leaves of ***I.pseudacorus*** after 07 days of treatment.

Effect of FOLIETTE on gaiacol-peroxidase (GPX) activity in the roots and leaves of *I. pseudacorus.*

For this parameter, the results obtained are shown in **figure 6**, which detects a significant increase ($P \leq 0.001$) in GPX activity in roots and leaves of ***I. pseudacorus*** as a function of the increasing concentrations of FOLIETTE. The highest values are recorded for the high concentration (C_3) successively in roots and leaves (0.000206 and 0.000202 nM/min/mg of Prot) compared to the control value.

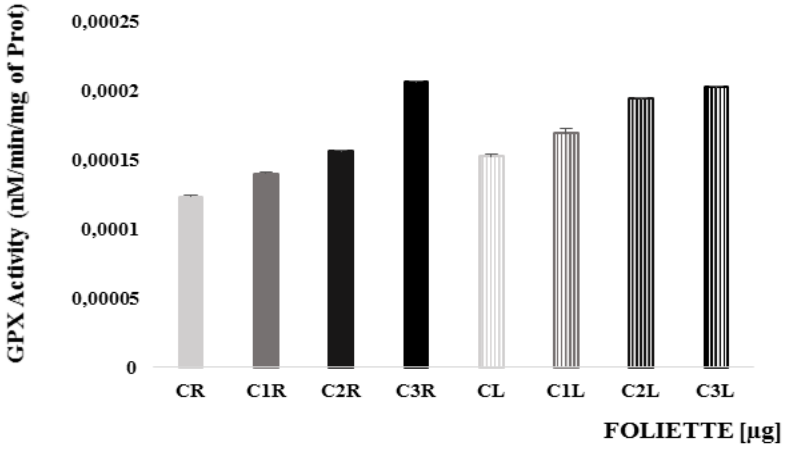


Figure. 6. Effect of FOLIETTE on gaiacol-peroxidase **(GPX)** activity in the roots and leaves of ***I.pseudacoru*** after 07 days of treatment.

DISCUSSION

In our work we tested the chlorophyll levels that are considered to be excellent biomarkers of plant toxicity knowing that there is a strong correlation between cell densities and photosynthetic fluorescence parameters in environmental pollution **(Dewez *et al.*, 2007)**. Our results clearly show a decrease in chlorophyll foliar levels in yellow iris as a function of increasing fungicide concentrations relative to control leaves. This appears to agree with **Elzbieta *et al.*, (2011)** on the phytotoxicity of a Roundup Ultra 360 SL herbicide in the waterlense: *Lemna minor* and **Moldes *et al.*, (2008)** which suggest a decrease in chlorophyll content in glyphosate-treated soybean tissues. This decrease can be attributed to the inhibition of its biosynthesis and photo-destruction of the herbicide by reducing the formation of Aminolevulinic acid (ALA) as a porphyrin precursor. Similarly, **Zaimeche, 2015 and Bensaid *et al.*, 2017** noted a decrease in chlorophyll levels, respectively, at *Lemna minor, Chlorella sp, Phragmites communis and Typha latifolia*, exposed to various concentrations of trace metallic elements, related to the reduction in energy intake from chloroplasts to the photosynthetic system.

In contrast, the addition of different concentrations of FOLIETTE in the culture medium stimulates the synthesis of total proteins, particularly in roots such as *Elodea canadensis* and *Lemna minor* **(Tlidjen *et al.*, 2012)** and **(Derradji *et al.*, 2014)**. This increase can be explained by the fact that the latter are considered the seat of resistance of the plant to different stresses because they are attached directly to the sediment through their active enzyme system **(Zouainia *et al.*, 2016)**. In addition, the work of **(Gardés-Albert *et al.*, 2003)** relates the increase in proteins to the fact that the plant seeks to protect its Morphophysiological integrity in response to the damage induced by xenobiotics. In other words, protein accumulation is a molecular stress tolerance strategy that is directly related to an overproduction of ROS **(Mishra *et al.*, 2006)**. In contrast, when stress persists and ROS are not neutralized, they can cause oxidation of proteins ranging from the simple oxidation of an amino acid to the fragmentation of the peptide chains, resulting in lower protein levels **(Sbartai *et al.*, 2015)**.

In parallel, a great deal of work has been carried out to demonstrate the action of certain stress biomarkers, in particular glutathione (GSH), malondialdehyde (MDA) and total polyphenols **(Radic *et al.*, 2009)**. GSH, which carries a thiol function, is a antioxidant system first line of defense that binds to toxic metabolites through its SH function **(Yadav *et al.*, 2010)**. During the treatment of I. pseudacorus with the low and medium concentration of FOLIETTE, we observed an increase in the level of GSH in the roots and the leaves treated compared to the controls. These results are in the same direction as those of **Fabrizio *et al.*, (2003)** who showed an increase in this rate during cadmium stress in reeds: Phragmites australis, and those of **Kamara and Pflugmacher, (2006)** in two aquatic species: Phragmites australis and Quercus suber planted in polluted waters as well as those of **(Nadgorska-Socha *et al.*, 2012; Sbartai *et al.*, 2015)** for which

the level of GSH increases with the plant's tolerance to stress. While the high concentration of the fungicide applied induces a decrease in the GSH content of the roots and leaves. These results are comparable to those reported by certain authors **(Ducruix *et al*., 2006)**, where the level of GSH decreases in response to stress induced by high concentrations of cadmium. This depression can be explained either by its direct binding to the xenobiotic or by the use of glutathione in the GST conjugation reaction **(Regoli *et al*., 1998; Canesi *et al*., 1999).**

In fact, the cascade reactions of free radicals with biological molecules such as lipids lead to lipoperoxidation of cell membranes resulting in an increase in the intracellular level of MDA, considered to be one of the products of lipid peroxidation **(Servais, 2004)**. The results of this work are comparable to those reported by **Riffat *et al*., (2007)** in Lemna polyrrhiza, which indicate an increase in the level of MDA following cadmium stress, in Lemna minor in the presence of aluminum **(Radic *et al*., 2009)**, in three varieties of durum wheat following exposure to different concentrations of ZnO nanoparticles **(Chiahi *et al*., 2016)** and in foams treated with different concentrations of NPK. Likewise, **Agostineto *et al*., (2016)** show that the application of all herbicides such as bentazon, iodosulfuron, metribuzin and metsulfuron (2,4-dichlorophenoxyacetic acid) induces lipid peroxidation.

For total polyphenols involved in several functions including plant defense mechanisms **(N'goran *et al*., 2019)**, an increase was observed in the root and leaf system of the iris. These results agree with those of **(Amensour *et al*., 2009; Aidi Wannes *et al*., 2010)** which report an increase in the biosynthesis of total polyphenols in plants stressed by some xenobiotics such as in *Sylvana potato* subjected to three phytosanitary products (herbicide , acaricide and fungicide), proving that secondary metabolites play a role in the tolerance of this species to different types of stress **(Belmahel, 2019)** and therefore their protection against free radicals **(Parida *et al*., 2004)**.

Indeed, excessive amounts of pesticides can act as toxic and induce abiotic constraints in plants **(Sunohara and Matsumoto, 2008)**. the latter have an effective enzymatic defense system to neutralize excess ROS to protect cells against oxidative damage **(Gill and Tuteja, 2010)**. Induction of GPX activity, following treatment with different concentrations of FOLIETTE, may be due to the overproduction of H_2O_2 in the roots and leaves of I. pseudacorus. This strong GPX activity clearly shows its crucial role in the defense mechanisms of plants and their tolerance to different stress conditions **(Zou *et al*., 2009)**. In addition, some authors have reported that there is a relationship between cell wall lignification, oxidative stress, and oxygen peroxide content **(Chaoui and El Ferjani, 2005)**, suggesting that the latter may be considered as the signaling molecule under stress conditions triggering thus the lignification process which has only a defense mechanism adopted by plants to improve the unbalanced state of the cellular system **(Wang *et al*., 2011)**.

CONCLUSION

Phyto-purification is an ecological, efficient and economical means of purification and many macrophytes are distinguished by their role of filtration and water purification which depends on their resistance to the active ingredients causing possible side effects on their metabolism.

At the end of our experiment a battery of results was listed, the most important of which is the installation of an oxidative stress in response to the accumulation of xenobiotics in the macrophyte tissues leading to a decrease in the levels of chlorophyll and an increase in total proteins, enzymatic activity (GPX) and certain non-enzymatic stress biomarkers (GSH, MDA and total polyphenols). These defense mechanisms triggered by these plants show that they have the capacity to resist this type of stress and could constitute an interesting model for the rehabilitation of contaminated aquatic environments (phytoremediation) by non-organic fungicides, knowing that the marsh iris is already known for its ability to absorb heavy metals. This technique can be seen as a prospect for the future, both interesting as such, but also promising given the current craze for these techniques.

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