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Lead-Induced Oxidative Stress and Antioxidant Defence Responses in *Mentha aquatica* L.: Implications for Phytoremediation of Polluted Water

Laib Besma[1], Zekri Jihane[1], Laib Abir [2], Chekkal Raghda[3], Yaiche Fatma[4], Benamara Sara[5]
[1]Department of Ecology and Environment, Natural and Life Sciences Faculty, University Mostefa Benboulaid Batna 2. Route de Constantine, 53. Fesdis, Batna 2, Algeria.05000.
[2]University of Paris-Est Créteil, 61 Avenue du Général de Gaulle, Créteil Cedex, France 94010.
[3]Environmental Biomonitoring laboratory, Faculty of Sciences, Badji-Mokhtar University, Annaba, Algeria.
[4]Department of Common Core Studies, Faculty of Natural and Life Sciences, University of August 20, 1955, Skikda, Algeria.
[5]Cellular Toxicology Laboratory, Faculty of Sciences, Badji-Mokhtar University, Annaba, Algeria.

[*]**Corresponding Author: besma.laib@univ-batna2.dz**

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ABSTRACT

Water pollution poses a number of problems for living organisms and ecosystems. With this in mind, we carried out research to analyze the impact of a trace metal (lead) on a purifying aquatic plant (*Mentha aquatica* L.), and to determine its influence on improving the quality of polluted water, by evaluating various biochemical and enzymatic parameters. The data collected indicate that the incorporation of this toxic compound, at various concentrations during 7 and 14 days of treatment, stimulates the accumulation of total proteins, proline and glycine betaine in the roots. There was also a marked increase in stress biomarkers such as malondialdehyde (MDA) and glutathione (GSH). Similarly, assessment of the enzymatic activity involved in the detoxification process reveals a notable stimulation of catalase (CAT), peroxidase (APX) and guaiacol peroxidase (GPX) activity. These enzymes have a significant impact in reducing oxidative damage, demonstrating the ability of our plant model to resist this xenobiotic and its potential application as a solution for phytoremediation.

INTRODUCTION

Trace metal contamination of surface and groundwater is a major environmental issue. Although these metals are naturally present in water through geochemical phenomena, it is human activity that is responsible for the release and spread of large quantities of these elements via industries, fertilizers and pesticides **(Hargreaves *et al.*,**

2018). The freshwater aquatic environment is the main recepient for these discharges **(Islam *et al.*, 2018)**.

The removal of toxic contaminants is essential to reduce the risk to human and environmental health. The extraction of heavy metals by various techniques, such as reverse osmosis **(Al-Alawy *et al.*, 2017)**, precipitation **(Huang *et al.*, 2017)**, chemical adsorption, and solvent extraction **(Burakov *et al.*, 2018)**, and ion exchange involves considerable operational and maintenance costs and is often not environmentally friendly **(Kulkarni *et al.*, 2018; Levchuk *et al.*, 2018)**. Conventional methods of removing heavy metals are generally expensive and time-consuming, with these capital-intensive treatment systems ultimately raising the issue of sludge disposal **(Grandclément *et al.*, 2018)**. It is crucial to have a technology that is both environmentally friendly and economical for purifying wastewater laden with heavy metals **(Shahid *et al.*, 2018)**.

Recent research highlights a promising ecophytoremediation method for wastewater purification, which uses plants and micro-organisms to remove, bind or decompose pollutants present in the immediate environment **(Tee *et al.*, 2016a; Pakdel, 2018b)**. In other words, phytoremediation is based on the idea that a living plant can act as a photosynthetic pump, capable of effectively removing pollutants such as metals and metalloids from the environment and water **(Peligro *et al.*, 2016)**. More specifically, aquatic plants play an essential role as natural absorbers in the phytoremediation of heavy metals and pollutants, thanks to their extensive root systems, which make them ideal for absorbing contaminants through their roots **(Calzadilla *et al.*, 2011)**.

Due to its proven effectiveness in reducing environmental pollution, phytoremediation technology is attracting interest from researchers and government institutions around the world. Among the various species of floating aquatic plants are the phytoremediators such as *Azolla*, *Eichhornia*, *Lemna*, *Spirodela*, and *Mentha aquatica* **(Verma *et al.*, 2022)**. Numerous studies have demonstrated their effectiveness in eliminating aquatic pollutants through bioaccumulation in their plant tissues **(Huang *et al.*, 2017)**. It should be noted that *Lemna minor* and *Pistia stratiotes* are particularly used for the elimination of metal ions present in aquatic ecosystems **(Burakov *et al.*, 2018)**. For example, *Eichhornia crassipes* has the ability to break down inorganic contaminants by focusing on different metal cations, such as copper (Cu), cadmium (Cd), lead (Pb), and zinc (Zn). It is also capable of removing various pollutants such as total dissolved solids (TDS), total suspended solids (TSS), biological oxygen demand (BOD) and chemical oxygen demand (COD) from industrial wastewater **(Shaari *et al.*, 2022)**.

In addition, the use of plants such as *Lemna minor* and *Pistia stratiotes* has been shown to be effective in reducing BOD, COD, chloride and sulphate in wastewater. This

is the context of our research, which aimed to assess the toxic effect of a trace metal on the antioxidant defense of upper aquatic vegetation (*Mentha aquatica* L.).

MATERIALS AND METHODS

Biological materials

The biological material used in this study is the aquatic plant *Mentha aquatica* L., a rhizomatous aromatic perennial. Its toothed, oval leaves are rich in organic matter. It generally has an upright or ascending stem. Water mint is renowned for its high polyphenol content, which gives it antioxidant activity **(Poul & Ferdinand, 1977)**.

Chemical equipment

This study focused on trace metals and lead carbonates (Pb CO_3), which accumulate in aquatic plants and can have adverse effects on their growth and metabolism **(Garnier, 2005)**.

Trial conduct and processing

The plants were harvested during April from Lake Tonga, located in El Kala National Park in eastern Algeria, which is rich in aquatic plants. This freshwater lake is part of an abundant lake ecosystem with nearly 100 plant species, including characteristic plants such as *Iris pseudoacorus* and *Mentha aquatica*.

Prior to treatment, the plants were kept in an optimal environment to ensure their viability. We maintained a temperature of around 20–25°C and provided sufficient but not excessive light, with a controlled day-night cycle and good quality water that was oxygenated, rich in minerals and had an appropriate pH (6.5).

The plants were grown in four sterile glass jars, large enough to accommodate the plant and its roots. A draining layer of gravel was placed to prevent water stagnation and promote root aeration, then a thin layer of charcoal was added to the gravel to limit mold formation and keep the water clean; the jars were filled with mineral water until the roots were submerged, but without drowning the plant. These cultures were treated with increasing concentrations of lead for 7 and 14 days.

Table 1. Different concentrations of applied metal

C	**Control =0 µg/L**
C1	Low Concentration= 25 µg/L
C2	Medium concentration= 50 µg/L
C3	High concentration= 100 µg/L

The concentrations used are realistic and correspond to those found in the lake from which the samples were taken (heavy metal analysis was performed). Then, the concentration was increased experimentally to study the effect of this element on the plant.

Studied parameters

Total protein assay

The concentration of proteins in solution was assessed using the method of **Bradford (1976)**, which is based on spectroscopic analysis. This involved reducing 0.1g of plant material to a powder and mixing it with 10ml of distilled water. After filtration, 0.2ml of the supernatant was removed and 2ml of BBC (Bradford reagent) was added. This method is based on the interaction of the Comassie Blue dye with the basic and aromatic residues of the proteins, resulting in a blue coloration that signals the presence of the proteins. Absorbance was measured at a wavelength of 595nm using a spectrophotometer.

Proline level assay

The proline analysis technique used was that of **Troll and Lindsley (1955)**, modified by **Dreier and Goring (1974)**. 100mg of fresh material (roots) was taken and weighed, then placed in clean control tubes to which 2ml of 40% methanol was added. The mixture was heated in a water bath at 85°C for one hour. After cooling, 1ml of the solution was extracted, to which 1ml of acetic acid (CH_3COOH) and 1ml of a mixture consisting of 120ml distilled water + 300 ml acetic acid + 80 ml orthophosphoric acid and 25mg ninhydrin were added. The solutions were boiled for 30 minutes, turning red. Once cooled, 5ml of toluene was added and the mixture was stirred to separate the upper and lower phases. Calculation of the optical density of the samples at a wavelength of 528 nm, using a standard range prepared from a proline stock solution

Determination of glycine betaine content

This analysis is based on the method of **Gieve and Grattan (1983)**, which was modified by **Bacha *et al.* (2015)**. A 0.5g sample of finely ground fresh material (roots) was taken, to which 20ml of distilled water was added. The resulting solution was incubated for 48 hours at a temperature of 25°C. The samples were then filtered and diluted in sulphuric acid (2N), and 0.5ml of the resulting solution was cooled in ice-cold water for one hour. Absorbance was measured at 365nm using a UV-visible spectrophotometer. Betaine glycine standards were prepared in 2N sulphuric acid.

Dosage of malondialdehyde (MDA)

Lipid peroxidation was estimated by the evolution of the malondialdehyde (MDA) content determined according to the method defined by **Alia *et al.* (1995)**. Fresh roots were ground in 5% trichloacetic acid (TCA) at a rate of 10 ml per 1g of plant tissue, followed by centrifugation for 15 minutes at 12,000g. An equivalent volume of 0.5% thiobarbituric acid (TBA) prepared in 20% tri-chloroacetic acid (TCA) was added to the upper liquid phase. The mixture was incubated at 100°C for 30 minutes. The reaction was then stopped by placing the tubes in an ice bath. The absorbance of the supernatant, obtained after centrifugation at 10,000g for 5 minutes, was measured at 532nm.

Determination of glutathione (GSH) levels

The enzyme extract was homogenized in a 0.02M ethylene diamine tetra-acetic acid (EDTA) solution and deproteinized with 0.25%. Following centrifugation at 2000g for 10 minutes, the supernatant was used for spectrophotometric assay with 0.01M dithiodibenzoic reagent (DTNB) at a wavelength of 412nm. The method of **Weckbeker and Cory (1988)** was used to evaluate GSH concentrations, expressed in µMol/mg protein.

Measurement of catalase activity (CAT)

Catalase (CAT) activity was measured using the method of **Cakmak *et al.* (1991)**. 50µl of H_2O_2 (300mM) and 2.85ml of 50 mM phosphate buffer (pH = 7.2) were added to 100µl of enzyme extract. The rate of H_2O_2 decomposition was determined by evaluating the decrease in absorbance at 240nm over a period of one minute. Catalase activity is expressed in µMol/min/mg protein.

Ascorbate peroxidase (APX) activity measurement

The activity of ascorbate peroxidase (APX) was measured according to the method of **Nakano and Asada (1981)**. The reaction mixture consists of 100µL of enzyme extract, 0.5mM ascorbate, 50mM phosphate buffer (pH 7.2), and 50µL of H_2O_2 (300mM). The oxidation of ascorbate is determined by measuring the change in absorbance at 290nm.

Measurement of guaiacol peroxidase (GPX) activity

Guaiacol peroxidase activity was assessed using the technique of **Putter (1974)**, modified by **Ali *et al.* (2011)**. This method involved mixing 100µl of enzyme extract, 100µl of guaiacol (1.5% v/v), 100µl of H_2O_2 (300 mM) and 2.7ml of phosphate buffer (25mM) with 2mM Ethylene diamine tetra acetic acid (pH=7.0), for a total volume of 3ml. The absorbance was measured at 470nm using a spectrophotometer.

Statistic study

The results obtained were expressed as the mean plus or minus the standard deviation (m±sd). Means from the same series were compared with each other, using the statistical test, two-factor analysis of variance (ANOVA), as a function of time and increasing concentrations of the metal applied, with a significance level (*P*). For each analysis, an appropriate calibration curve was used to quantify the concentrations.

RESULTS

Effect of lead on total protein content

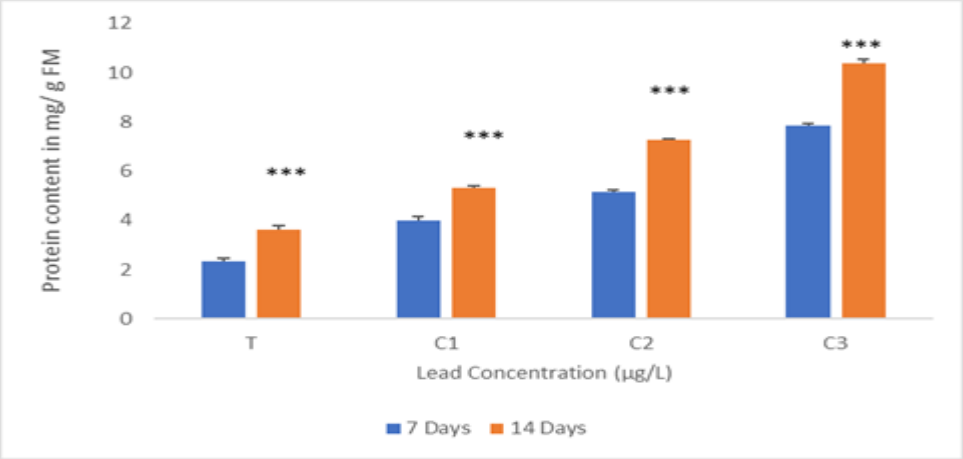

Fig. 1. Total protein content in water mint (*Mentha aquatica* L.*)* roots treated with different concentrations of lead. The values are means of a at least three replications ± standared deviation (SD)

The results obtained show a significant increase ($P \leq 0.001$) in protein levels in *Mentha aquatica L.* roots subjected to increasing lead concentrations after 7 days of treatment. This increase is of the order of 7.85mg/ g MF in roots treated with the highest dose and 2.01mg/ g MF in control roots, a five-fold increase. The highest value, 11.23mg/ g MF, was recorded in the roots treated with the highest dose, while in the control roots it was only 3.82mg/ g MF.

Effect of lead on proline content

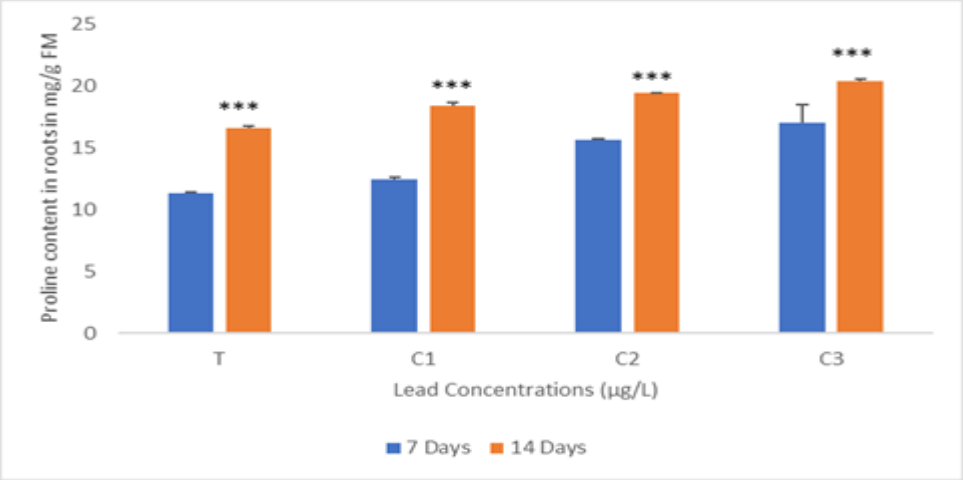

Fig. 2. Proline levels in water mint (*Mentha aquatica* L.) roots treated with different concentrations of lead. The values are means of a at least three replications ± standared deviation (SD)

The results show that proline levels increase significantly ($P \leq 0.001$) and in a dose-dependent manner in roots exposed for 7 days of treatment, compared with control roots, reaching 16.432 mg/g MF in roots treated with the highest dose of xenobiotic applied (C3) and 11mg/g MF in control roots. However, the increase in proline levels recorded after 14 days of treatment is greater than those obtained after 7 days of treatment, rising from 16 mg/g MF in control roots to 22.324 mg/g MF.

Effect of lead on glycine betaine levels

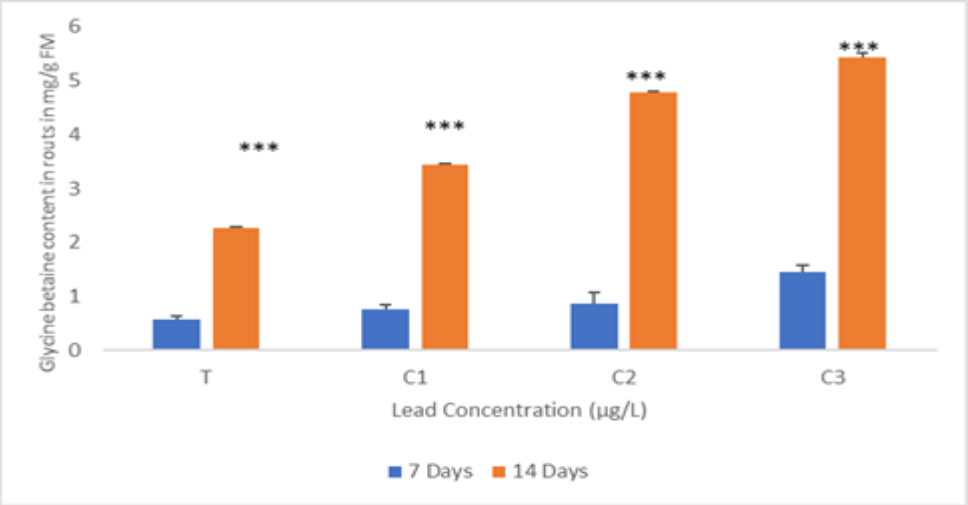

Fig. 3. Glycine betaine levels in water mint (*Mentha aquatica* L.) roots treated with different concentrations of lead. The values are means of at least three replications ± standared deviation (SD)

The results show that treatment with increasing concentrations of lead induced a highly significant ($P \leq 0.001$) increase in glycine betaine (GB) content in the roots of treated plants, compared with control roots, after 7 days of treatment. This increase was mainly observed in roots exposed to high concentrations, where it reached 1.34mg/ g MF, respectively, in roots treated with the highest dose of metal applied. However, the increase in glycine betaine levels after 14 days of treatment was greater than that obtained after 7 days of treatment.

Effect of lead on malondialdehyde (MDA) levels

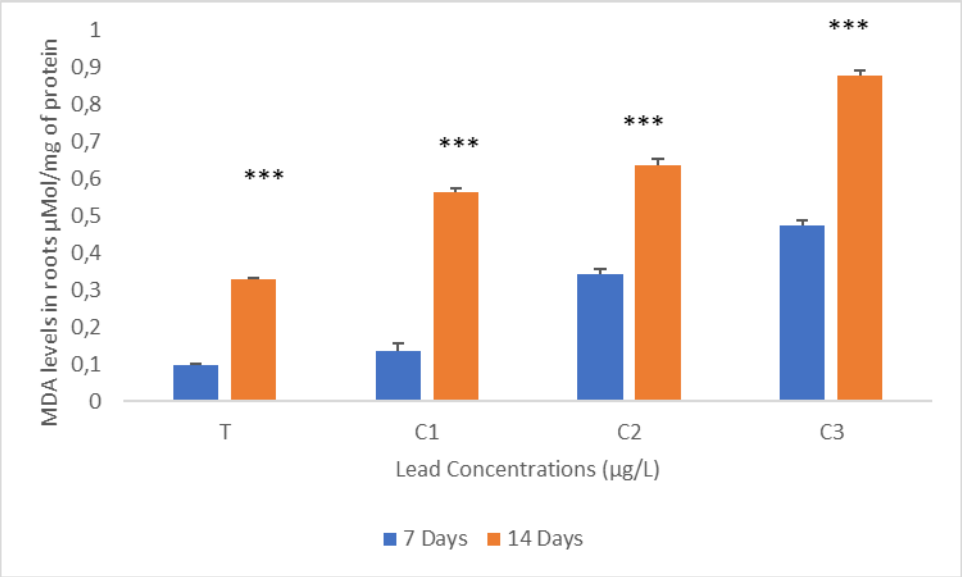

Fig. 4. Malondialdehyde (MDA) levels in water mint roots (*Mentha aquatica* L.) treated with different concentrations of lead. The values are means of at least three replications ± standared deviation (SD)

The results show that MDA levels increase significantly ($P \leq 0.001$) and in a dose-dependent manner in roots treated with increasing concentrations of lead after 7 days of treatment, from 0.098 µMol/mg of protein in control roots to 0, 47 µMol/mg of protein in roots treated with the highest concentration, or five times the control. After 14 days of treatment, the MDA assay reveals a significant increase ($P \leq 0.001$), which is significantly higher than that obtained after 7 days of treatment, when a value of 0.89 µM/mg of protein was recorded at the highest concentration.

Effect of lead on glutathione (GSH) levels

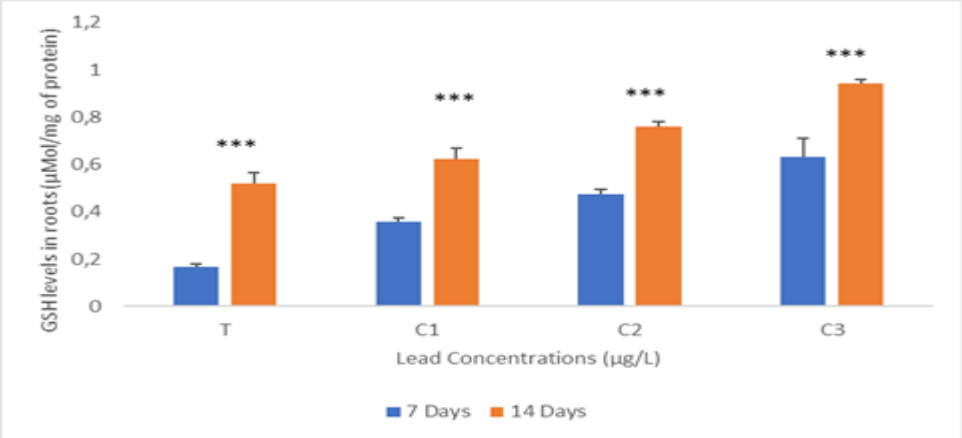

Fig. 5. Glutathione levels in lead-treated water mint (*Mentha aquatica* L.) roots. The values are means of at least three replications ± standared deviation (SD)

Glutathione levels in water mint roots showed a significant increase ($P \leq 0.001$) after 7 days of treatment, reaching 0.66 µMol/mg of protein in roots treated with the highest applied dose of the metal. However, the increase in glutathione levels after 14 days of treatment was greater than that obtained after 7 days of treatment, where a value of 0.89 µMol/mg of protein was recorded at the highest concentration.

Effect of lead on catalase (CAT) activity

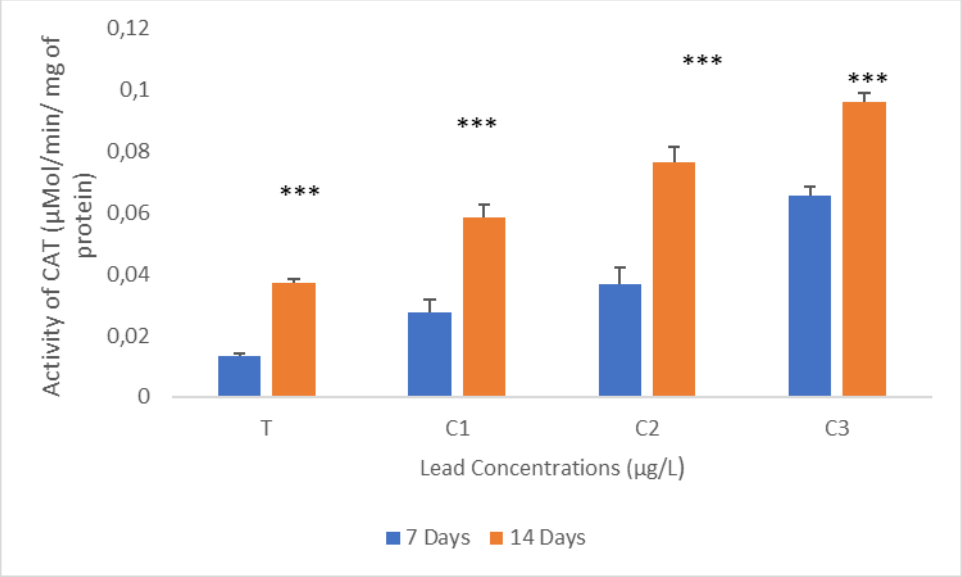

Fig. 6. Catalase activity in water mint roots *(Mentha aquatica* L.*)* treated with different concentrations of lead. The values are means of at least three replications ± standared deviation (SD)

Our results reveal an induction of CAT activity in *Mentha aquatica L.* roots treated with increasing concentrations of lead, from the lowest concentration tested, compared with control roots after 7 days of treatment. Indeed, this highly significant induction ($P \leq 0.001$) at root level reaches a rate of 0.073 µMol/min/mg of protein in roots treated with the highest concentration, whereas it is only 0.018 µMol/min/mg of protein in controls. Similarly, an induction of catalase activity was recorded after 14 days of treatment, this increase went from 0.038 µMol/min/mg of protein in control roots to 0.1 µMol/min/mg of protein in roots treated with the highest dose of xenobiotic.

Effect of lead on ascorbate peroxidase (APX) activity

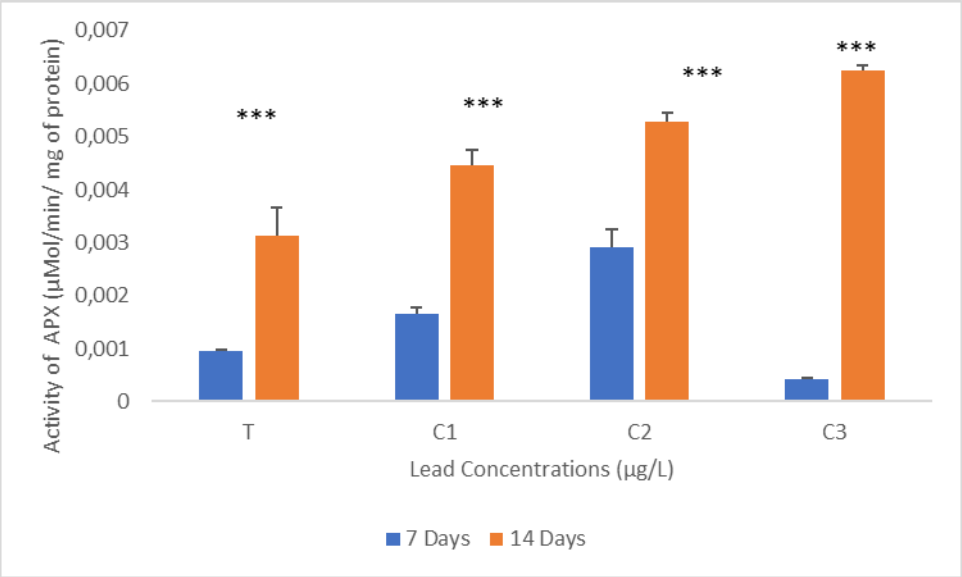

Fig. 7. Ascorbate peroxidase activity in water mint roots (*Mentha aquatica* L.*)* treated with different concentrations of lead. The values are means of at least three replications ± standared deviation (SD)

The results obtained demonstrate an intensification of APX activity in water mint plants at root level, after 7 days of treatment in the presence of increasing lead concentrations, compared with control plants. Ascorbic activity increased very significantly ($P \leq 0.001$) in treated roots, multiplying from the lowest concentration and increasing until it reached a peak at medium concentration, followed by a decrease at high concentrations, compared with control batches. Similarly, after 14 days of treatment, we observed a highly significant increase in ascorbate peroxidase activity, which reached a maximum level in treated plants in the presence of the highest lead concentration, with a value of 0.0066 µMol/ min/mg of protein, compared to 0.0033 µM/min/mg of protein in controls.

Effect of lead on guaiacol peroxidase activity (GPX)

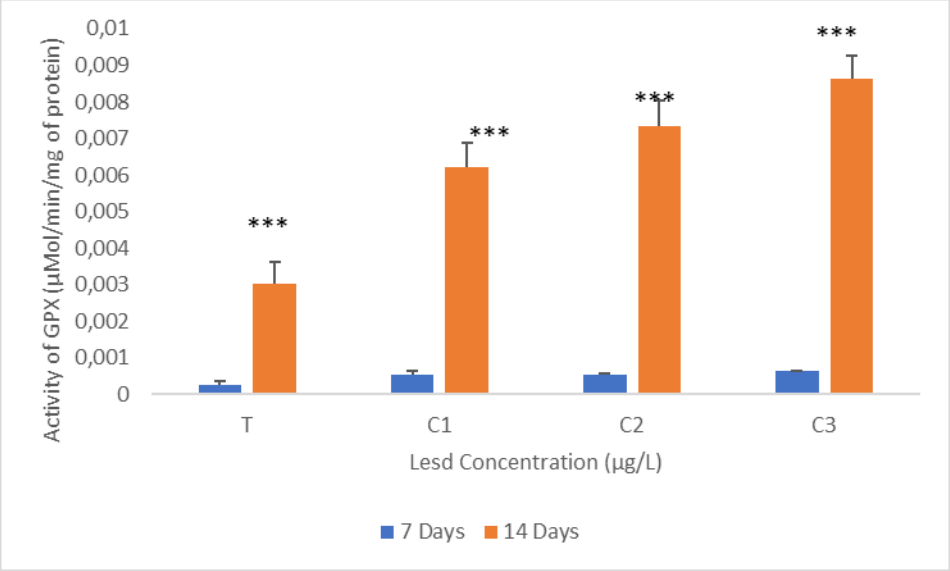

Fig. 8. Guaiacol peroxidase activity in water mint roots (*Mentha aquatica* L.) treated with different concentrations of lead. The values are means of at least three replications ± standared deviation (SD)

Measurement of guaiacol peroxidase activity in water mint roots treated with increasing concentrations of lead reveals a significant increase ($P \leq 0.001$) in this enzymatic activity compared to control organs after 7 days of treatment. The highest value is recorded respectively (0.0018 µMol/min/mg of Protein) at the high concentration (C3) compared with the control value of around (0.00089 µMol/min/mg of protein). Measurement of GPX activity after 14 days of treatment revealed a significant increase ($P \leq 0.001$) as a function of the doses applied, from (0.003 µMol/min of/mg of protein) in control roots to 0.0085 µMol/min/mg of protein in roots treated with the highest dose.

DISCUSSION

Heavy metals introduced into the aquatic environment are known to be extremely toxic to aquatic organisms **(Siwela *et al.*, 2009)**. The impact of these toxic metals causes physiological, biochemical and ultrastructural changes in aquatic organisms, which can be used as biomonitoring instruments to assess metal pollution in aquatic ecosystems **(Zhou *et al.*, 2008)**.

According to our studies, exposure to lead caused a relative increase in protein levels in the roots of water mint (*Mentha aquatica* L.). This rise in protein concentration is due to the action of reactive oxygen species which, by interacting with proteins, disrupt signaling molecules, leading to a change in the expression of certain genes and regulating the production of new proteins specific to the stress response **(Verma *et al.*, 2003)**. Our

results corroborate those of **Bensaid *et al.* (2017)**, who observed an increase in protein levels as a function of increased metal stress applied to *Typha latifolia* roots. The insertion of different concentrations of heavy metals into the culture medium stimulates total protein production, particularly at root level, as has been found in *Elodea canadensis*, *Lemna minor* **(Tlidjen *et al.*, 2012)** and *Phragmites australis* **(Kleche *et al.*, 2013)**.

Analysis of proline showed an increase in roots treated with various concentrations of lead. Many authors, such as **Zerroumda (2012)**, have reported that this amino acid is among the most stable solutes commonly found in plants subjected to biotic or abiotic stress. The rise in proline levels concomitant with the increase in zinc nanoparticle (ZnO NPs) concentrations in *Brassica juncea* roots and leaves implies an association between the generation of free radicals and their uptake by proline **(Rao & Shekhawat, 2014)**.

Our findings agree with those of **Kandziora-Ciupa *et al.* (2017)**, who demonstrated a significant effect of cadmium and zinc on proline accumulation in *V. myrtillus* leaves. Similarly, **Cheraitia *et al.* (2020)** reported an increase in proline levels in marsh iris treated with two types of fungicide.

Glycine betaine is an organic compound that is essential for the survival of plants and their ability to adapt to changing and difficult environmental conditions **(Bhatti *et al.*, 2013)**. Glycine betaine acts synergistically with membrane lipids, contributing to their stability and protection against deterioration caused by adverse environmental conditions such as metal stress **(Hanana *et al.*, 2011)**. In our research, we found that elevated lead concentrations resulted in increased glycine betaine levels at the root level.

Bhatti *et al.* (2013) made similar observations, showing an elevation in glycine betaine concentration in wheat leaves exposed to heavy metals. Similarly, **Bacha *et al.* (2015)** reported an elevation of glycine betaine in *Solanum lycopersicum* L. subjected to salt pressure. Furthermore, **Subbarao *et al.* (2001)** and **Mouhaya (2008)** assessed that the notable accumulation of glycine betaine is due to the activation of the expression of the CMO gene that encodes choline monooxygenase.

Cell membrane lipid peroxidation is one of the most devastating effects of reactive oxygen species (ROS) within cells. The malondialdehyde (MDA) indicator is essential in the process of lipid peroxidation in plants under stress and is often used to assess lipid peroxidation of cell membranes in plants **(Sun *et al.*, 2008; Xue *et al.*, 2009)**. Numerous studies have shown the degree of lipid peroxidation. In our study, we aimed to evaluate this parameter and observed a dose-dependent increase in MDA levels in *Mentha aquatica* L. roots treated with various concentrations of lead. This finding is consistent with that of **Ma *et al.* (2015)**, who demonstrated that silver nanoparticles (Ag NPs) increase MDA levels in *Crambe abyssinica*. This result was also obtained by **Laib *et al.* (2020)**, who found that MDA levels rose in *Cicer arietinum* roots treated with copper and nickel.

glutathione (GSH), a non-enzymatic antioxidant, is a low molecular weight thiol that plays a key role in many metabolic processes. It is also a vital defense system for plants against environmental stresses, including those linked to heavy metals **(Hossain *et al.*, 2013)**. According to **Jozefczak *et al.* (2015)** and **Parrotta *et al.* (2015)**, GSH is a precursor of phytochelatins (PCs), which have the ability to chelate cadmium, thereby reducing its toxicity. This research showed that exposure of water mint (*Mentha aquatica* L.) to different concentrations of lead leads to a significant increase in GSH levels in roots compared to control samples. These results corroborate studies that have indicated that exposure to bisphenol leads to an increase in GSH levels in *Lemna minor* **(Wang *et al.*, 2015; Liang *et al.*, 2022)**. Several researchers have also observed an increase in GSH levels in *V. faba* leaves grown in soil contaminated with lead and zinc **(Nadgorska-Socha *et al.*, 2012)**.

Catalase is an enzyme that promotes the conversion of hydrogen peroxide into water and oxygen molecules **(Arora *et al.*, 2002)**. An increase has been detected in catalase activity due to plant protection products, with this action being closely linked to the increase in hydrogen peroxide concentration within cells in stressful situations **(Ferfar *et al.*, 2016; Nohatto *et al.*, 2016)**. Our results are in line with those of **Cheraitia *et al.* (2020)**, who observed an increase in catalase activity in the roots of marsh iris (*I. pseudacorus*) exposed to different concentrations of fungicides. Similarly, **Bensaid *et al.* (2017)** highlighted an increase in catalase activity in the roots of an aquatic purification plant, *Typha latifolia*, subjected to metal stress.

Plants have a very effective enzymatic defence system, which helps them to protect themselves against excess reactive oxygen species and preserve their cells from oxidative damage **(Gill *et al.*, 2010)**. In this study, our results showed an increase in ascorbate peroxidase activity in the roots of water mint *(Mentha aquatica* L.). Research by **Souahi *et al.* (2016)** postulated that ascorbate peroxidase activity increases following the application of recorded doses of the sulfonylurea herbicide (Sekator OD), used on a soft wheat variety. This finding is consistent with the work of **Shaw *et al.* (2014)**, who observed an increase in APX activity in *Hordeum vulgare* L. barley leaves treated with CuO NPs. This also applies to the work of **Shaw and Hossain (2013)**, who noted an activation of APX activity in young rice shoots exposed to nanocopper (CuO) stress, or that of **Cheraitia *et al.* (2020)**, who observed a stimulation of APX activity in the roots and leaves of an aquatic plant (*I. pseudacorus*) treated with two fungicides; results perfectly corroborate those obtained in this study.

In addition, our study revealed a significant increase in the activity of guaiacol peroxidase (GPX), a biomarker of metal stress in plants, in *Mentha aquatica* L. roots exposed to different concentrations of lead. This suggests that this enzyme plays a role in neutralizing the excess H_2O_2 produced within aquatic mint roots, which could represent an adaptive mechanism for managing these trace metal elements and protecting root cells

from the effects of reactive oxygen species **(Semane *et al.*, 2007)**. Our results are consistent with those of **Labrada *et al.* (2019)**, who observed an increase in GPX activity in leaf tissue of tomato plants subjected to salt stress. Similarly, the results presented by **Laib *et al.* (2020)** demonstrated an increase in GPX activity in *Cicer arietinum* roots treated with different concentrations of copper and nickel.

CONCLUSION

The aim of this research was to demonstrate the various responses of water mint to metal stress. At the end of our experiment, we observed several results. Of particular note was the identification of oxidative stress in response to xenobiotic accumulation in the roots of this plant. This led to an increase in total proteins, proline and glycine betaines, as well as the activation of certain non-enzymatic (GSH, MDA) and enzymatic (CAT, APX, GPX) stress biomarkers. The defensive reactions triggered by this plant illustrate its ability to cope with this type of stress, and could constitute a promising model for the restoration of aquatic environments contaminated by heavy metals.

It is essential to supplement this study by examining the effects of co-cultivation with other aquatic and microbiological species, such as heavy metal-accumulating bacteria, which could promote phytoremediation or enhance collective resistance to toxic elements through synergistic interactions, such as competition or cooperation.

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