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PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES IN MEDITERRANEAN SALTBUSH (*ATRIPLEX HALIMUS* L., AMARANTHACEAE JUSS.) TO HEAVY METAL POLLUTION IN ARID ENVIRONMENT

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Abstract

Environmental pollution as induced by mining activities is increasingly become a problem with a great concern in many developing countries around the world. This paper reports the finding of a study undertaken to investigate the physiological and biochemical effects of heavy metals. Futhermore, we evaluate the effects of Zinc (Zn), Lead (Pb), Copper (Cu), Iron (Fe), and Cadmium (Cd), on Mediterranean saltbush (Atriplex halimus L., Amaranthaceae Juss.) grown in Arid zone from Southern Tunisia. The research is conducted at 5 different sites, located at different distant populations from the Tunisian Chemical Complex (TCC) situated in Gabes. Our results showed that leaves of A. halimus were enriched following decreasing order: Cu, Zn, Pb, and Fe compared to root samples. However, and for Cd, an opposite pattern was observed and leaves showed lower mean concentration. The Bioconcentration Factor (BCF) is used to meaure the heavy metal accumulation efficiency in the plant. For Cu, and for the 5 studied sites, BCF values >1 indicate that A. halimus is a potential heavy metal hyperaccumulator species. Regarding physiological status of the plant, heavy metals affected the different parameters of *A. halimus* including relative water content, chlorophyll level, lipid peroxidation and the production of hydrogen peroxide (H2O2). Oxidative stress as induced by heavy metal initiate several biochemical responses and mechanisms in *A. halimus* and lead to the accumulation of osmolytes and the activation of both enzymatic and non-enzymatic antioxidant systems. The concentration of heavy metals in plant tissues seem to be proportional with the magnitude of physiological and biochemical processes.

Key words: Heavy metals, Bioconcentration, A. halimus, Biochemical responses, Physiological parameters.

Introduction

Metal pollution is a major environmental problem, generated by a diversity of components due to increased anthropogenic activities (such as urbanization, improper waste management and mining activities, (Nwachukwu *et al.*, 2011; Saleh & Hassan, 2022). The effect of traffic, urban waste (wastewater, sewage sludge, garbage household), agricultural (fertilizers, pesticides, insecticides) as well as mining activities lead to the generation of high quantities of heavy metal on ecosystems (Jeddi & Chaieb, 2018; Fashola *et al.*, 2016). Heavy metals (HMs) are metalloids and their accumaltion on ecosystems would inevitably pose threats to ecosystems and have harmful and deleterious effects on human health (Liu *et al.*, 2009; Solgi *et al.*, 2020; George *et al.*, 2019; Fashola *et al.*, 2016). Futhermore, severe toxicity, and the potential high accumulation ability by biological systems are among the most important factors responsible for the dangers caused by HMs (Jadaa & Mohammed, 2023). Envirenmental pollution, as induced by HMs, affects atmosphere, water, plants, and soil can cause serious problems to all organisms (Alzahrani *et al.*, 2023).

Plants as sessile biological systems depends on air, soil matrix and water to survive, and therefore, cannot avoid contaminated environments (Keyster *et al.*, 2020). Plants, continually exposed to air, were reported to be the main receptors of pollutants. In fact, plants can absorb HMs either through the aerial parts (from particles in suspension in the air) or through the roots (Kabata-Pendias, 2010; Javed *et al.*, 2017).

Several Studies were carried out, to investigate the interactions between plants and heavy metals and to understand the physiological, anatomical and biochemical mechanisms under metallic stress (Adamczyk-Szabela *et al.*, 2019; Doghbage *et al.*, 2023; Li *et al.*, 2024). HMs directly disturb photosynthetic process and gas exchange attributes in plants (Mukhtar *et al.*, 2010) causing both oxidative and osmotic stress. Heavy metals are among the dangerous environmental pollutants that seriously disturb the plant growth and development including, among others, biomass reduction yield, nutritional imbalance etc. Fe, Cu, Mn, Pb, and Cd, can induce oxidative stress which leads to the inhibition of the enzymatic activity and the disturbance of the plant attributes, in particular, photosynthesis and respiration (Van Breusegem & Dat, 2006). Furthermore, HMs can generate the oxidation of proteins, the alteration of cell membranes via the induction of lipid peroxidation phenomena, and the destruction of DNA inducing cell death (Cheng, 2003). To protect against the deleterious effects of reactive oxygen species (ROS), plant organisms have developed certain defense mechanisms, such as osmoregulators (proline, flavonoids, and sugars).

Mediterranean saltbush, *Atriplex halimus* L. (Amaranthaceae), is a perennial, halophytic and nitrophilous shrub (Walker *et al.*, 2014). The plant species is native to Iberian Peninsula, West and North Africa to Anatolia, Arabia and Horn Africa region (POWO, 2023). *A. halimus* is reported as a versatile plant species with a

prominent role in ecosystems functioning by harboring soil biota, as a food source for mammals and arthropods, to stabilize soil and minimize erosion and due to its capacity to tolerate heavy metals, Mediterranean saltbush's new uses include phytoremediation potentialities as reported by many authors (Walker *et al*., 2014; Shahrokh *et al*., 2022; Doghbage *et al*., 2023; Nedjimi., 2023). In south Tunisia, and due its drought tolerance under severe conditions, the plant species is reported as a forage for herds in traditional agricultural system (Talamali *et al*., 2001).

The Gulf of Gabes, in Southeast of Tunisia, is typically characterized by a coastal oasis agrosystem, and the region has been identified as one of the Mediterranean Sea 11 consensus eco-regions (Ismail *et al*., 2023). Tunisian Chemical Group (TCG), as a one of pivotal industrial companies of mining activities in Tunisia is focusing on the production and marketing of chemical fertilizers. In Gabes city, the industrial complex (ICG) includes many polluting activities involving mining, phosphate processing, chemicals industries, energy production etc. According to European Environment Agency (2014), in the large urban areas (including Sfax and Gabes), the air contains high levels of carbon monoxide (CO), carbon dioxide (CO2), nitric oxide (NO), nitrogen dioxide (NO2), hydrogen sulphide (H2S), hydrocarbons and dust.

By the present study, we investigate the impact of heavy metals on the biochemical and physiological responses of A., halimus grown naturally in arid environment and harsh climatic conditions and located in the vicinity of polluated zone in Gabes city.

Material and Method

Study sites: Gabes city is situated in Southeastern Tunisia (33° 53′ Nord, 10° 07′ EST). The region is subjected to many anthropogenic pressures including among others: Urbanization, industrialization and agriculture activities. In the region, environmental concerns increase regarding the pollution induced by TCG and other anthropogenic activities. The performed analyses in the present study include soil and plant sample analysis. Soil and plant samples were collected from 5 sites as summarized by Table 1. During the experimental design distance from TCG, Gabes was considered and Ghannouch (S1) situated at neighboring region from the industrial complex (2 Km), followed respectively by Tebelbou (S2; 27 Km), Ketana (S3; 52 Km), Zarat (S4; 73 Km) and Matmata (S5; 98 Km). The last one (Matatma: S5; 98 Km) is considered as the control site considering that is the most far locality and supposed to be the lowest polluted site. All sites are under the governance of semiarid to arid bioclimatic zones (Emberger, 1966; Kassah, 1996). The average annual rainfall ranges between 125 and 234 mm and the average annual temperature varies from 32°C to a of 11°C. Table 1 showed the geographic location and the main ecological traits of the five analysed sites.

Soil sampling and analysis: Soil samples were collected to the tillage depth of 0-20 cm and 3 replications were considered for each site. Samples were transported to the laboratory, homogenized, air-dried, and for soil testing, 2 mm Iron Mesh Sieves was used. Soil pH and electrical conductivity (EC) were determined in soil/water (1:2) suspension (Saturated paw method, Anon., 1987) by a pH meter and a conductivity meter.

For metals concentrations, each sample of 0.5 g of soil were treated with 10 mL concentrated HNO3 (150°C). After 3 hours, 2 mL l of concentrated HCLO4, were added. The obtained residue was treated with 2 mL of concentrated HCL for 15 min (Çelik *et al*., 2005). The obtained solutions were, then, adjusted up to 25 mL with ultra-pure water and then filtered. Concentrations of Zn, Pb, Cu and Cd were determined using an atomic absorption spectrophotometer (Avanta, GBC spectrophotometer, Australia), equipped with an air-acetylene flame. Concentrations were expressed in mg/Kg. For soil analysis experiments, all measurements were performed in triplicate.

Plant sampling and analysis: The sampling procedure was conducted during the period from January to March 2021. For the 5 studied sites, eighteen healthy plantlets were transplanted in situ in biodegradable plastic bags. Prior to transplantation step, samples of roots and leaves of each individual were conserved in plastic bags for further analyses. Plant material was washed in order to remove dust particles, oven dried at 80°C for 24 h, powdered and sieved.

Table 1. Geographic localization and bioclimatic characteristics of the 5 analysed study sites located in the vicinity of the industrial complex of Gabes, TCG, Tunisia.

Populations	Code	Zone	Variant	Rainfall (mm/year)	Altitude	Longitude	Latitude
Ghannouch	1	Higher semi-arid	Mild winter	197	22	33°97’	10° 65’
Tebelbou	2	Higher semi-arid	Mild winter	234	29	33°84’	10°31’
Ketena	3	Higher semi-arid	Mild winter	234	24	33°75’	10°2’
Zarat	4	Higher semi-arid	Cool winter	234	26	33°4’	10°24’
Matmata	5	Inferior arid	Mild winter	125	600	10°40’	33°28’

Zones are defined according to Emberger's pluviothermic coefficient: Q2 = 2000 P/(M2 – m2), where P is the average of annual rainfall (mm), M is the mean of maximum temperature (K) for the warmest month, and m is the mean of minimum temperature (K) for the coldest month. P, M, and m calculated as the average of the period from 1953 to 2003

HMs content analyses: Identification and hazard analysis of HMs detection was determined according to the protocol as described by Farahat and Linderholm (2015). Briefly, samples from each site were individually preserved for subsequent analysis. Subsequently, the plants were divided into belowground roots and aboveground shoots. To eliminate dust and other solid particles, all samples underwent a thorough washing with distilled water. Following this, they were subjected to a drying process at 80 °C for 24 hours, after which they were ground and homogenized by passing through a 0.2-mm sieve. Dry samples weighing 0.5 grams were ashed at 550°C in a muffle furnace for 3 hours and then digested using 10 ml of 2.8% HNO3.

The solutions were analyzed for Zn, Pb, Cu, Fe, Mg, and Cd using a flame atomic absorption spectrophotometer (Avanta GBC spectrophotometer, Australia). HMs concentrations in dry plant material were expressed in mg/kg and all measurements were investigated in triplicate.

Measurement of relative water content (RWC): Relative water content was estimated using the following formula as described by Scippa *et al*., (2004):

RWC (%) = (FW - DW) × 100 / (TW - DW); FW: Fresh leaf weight, DW: Dry leaf weight and TW: Turgid leaf water

Extraction and determination of chlorophyllin pigments: Chlorophyll determination was performed according to the method as described by (Torrecillas *et al*., 1984). Briefly, 100 mg of fresh leaves was macerated with 5 ml of 80% (v / v) acetone. The solution was allowed to stand in aliquots of test tubes in the darkness for 72 hours. The chlorophyll content of the samples was determined using spectrophotometry, using a double Type beam visible by UV spectrophotometer ((UV-1650 PCUV-SHIMADZU) at wavelengths 661.6nm, 664.8nm, and 470nm. The chlorophyll concentrations (Chla, Chlb and total Chl) was determined by measuring the absorbance (optical density-OD) of the extract at various wavelengths as defined by the following formulas proposed (Lichtenthaler, 1987):

Chla (mg/ g FW) = (11.24*Do661.6) - (2.04*Do644.8)

Chlb (mg/ g FW) = (20.13* Do644.8) - (4.19*Do661.6)

Chl (a+b) (mg/g FW) = (7.05*Do661.6) + (18.09*Do644.8)

Estimation of H_2O_2 lipid peroxidation: Hydrogen peroxide content of the plant parts was measured spectrophotometrically using the protocol as described by Panda (2008) and Elloumi *et al*., (2017). Briefly, H2O2 was extracted by homogenizing 0.5 g of plant tissue with 5 mL of trichloroacetic acid (TCA; 0.1%) then centrifuged for 15 min at 12000 rpm. Leaves and roots supernatant was mixed by the addition, respectively, of 0.5 mL Potassium phosphate (0.5 mM) and 1 mL of Potassium iodide (0.5 mM). As a blank probe, 0.1% TCA without plant extract was considered. The absorbance was measured at 390 nm for the estimation lipid peroxidation content.

Ascorbic acid assay: The dosage of ascorbic acid was determined according to the method described in Leskovec *et al.*, (2018). Using the coloring reagent, Dinitrophenylhydrazine-Thiourea-Copper (DTC), and a standard range of ascorbic acid, a sample of 0.2 g of fresh plant material is grounded, with a pinch of sterile and, in 5 mL of trichloroacetic acid (TCA; 10%), then centrifuged at 3600 rpm. A test portion of the supernatant was mixed with the DTC reagent. After 3 hours of incubation at 37°C, the reaction was interrupted by 65% Sulfuric acid solution (v/v). Ascorbic acid assay was determined by measuring the absorbance (optical density - OD) at 520 nm. The color intensity is proportional to the vitamin C concentration in the sample. Concentration in the sample was determined by reference to à standard curve established by blank probe (ascorbic acid).

Assay of antioxidant enzymes: All assays of antioxidant enzyme activities of plant extracts were prepared for Catalase (CAT), Superoxide dismutase (SOD), and Ascorbate peroxidase (APX). The activity of catalase, as a response to oxidative stress was estimated by observing the disappearance of H2O2. Catalase is an oxidoreductase enzyme that catalyzes the dismutation of hydrogen peroxide into water and oxygen. Its activity can be estimated by observing the decrease in absorbance at 250 nm (Aebi, 1984). The solution contained 50 mM of potassium phosphate buffer PH 6.5(KH2PO4), 10mM H2O2, and enzyme extract likewise CAT. The activity of Ascorbate peroxidase (APX) was determined by monitoring the decrease in absorbance at 290 nm. The mixture contained 50 mM K-phosphate buffer, 1 mM ascorbate, 1 mM EDTA, 2 mM H2O2 and enzyme extract.

Analyzes of biochemical parameters

Determination of sugar content: Soluble sugar content was estimated according to the method as described by Robyt & White (1990). The extraction was carried out by mixing 1 g of plant material with 10 mL (75% methanol). The extract was incubated in a water bath at 70°C for 30 minutes, followed by adding 1 mL of phenol and 5 mL of concentrated H2SO4 to 1 mL of plant extract. After stirring and chilling, absorbance at 640 nm was recorded. The standard range was established from a stock glucose solution (1 M). All measurements were performed in triplicate.

Proline determination: The determination of proline was estimated according to the procedure as described by Bates *et al.*, (1973) based on a spectrophotometrically quantification of proline-ninhydrin complex. In a test tube, a mixture of 150 mg of crushed plant material (with liquid nitrogen) and 5 mL of 40% (v/v) methanol was prepared. The ground material was incubated, for 30 min, in a water bath (80°C). One mL of plant extract was cooled and mixed with 2 mL of glacial acetic acid, 1 mL of ninhydrin solution (25 mg/mL) and 2 mL of a solution containing: distilled water (24%), glacial acetic acid (60%), and orthophosphoric acid (16%). The obtained mixture was placed in a water bath at 100°C for 30 min. The concentration of proline was determined by measuring the absorbance (optical density - OD) at a wavelength of 528 nm. The results were expressed in mg/g MF with a reference to the calibration curve for proline concentration.

Data analysis

Bioconcentration factor: To explore correlation between the HMs concentrations of soils, plants organs and biological factors were determined:

The Bioconcentration Factor (BCF) was defined by (Malik *et al*., 2010) to show correlation between HMs contained in the soil and those in plant roots:

$$BCF = \frac{\text{HMs concentration in plant roots}}{\text{HMs concentration in the soil}}$$

The transfer factor (TF) was defined by Gupta *et al*., (2008) to reveal the translocation and bioaccumulation of HMs from soil to plants.

$$TF = \frac{\text{Concentration of HMs in leaves}}{\text{Concentration of HMs in roots}}$$

Statistical analysis

All studied biochemical and physiological parameters were measured in triplicate, and the results were presented as means with Standard deviation (±SD). A one-way analysis of variance (ANOVA) was performed for the comparison the significance of the differences between parameters among the studied sites. Duncan's post hoc test was performed to compare the substantial differences in the mean values at ($p<0.05$). Data analyses were performed using SPSS statistical package (SPSS, 2023).

Results and Discussion

Soil sampling and analyzes: Analysis of HMs concentration among the 5 studied sites revealed a metal pollution gradient. As showed by Table 2, the highest levels of HMs were, globally, recorded in soil samples collected from –S1- Ghannouch (175, 36.5, 28.7, 17.54 and 5 mg/kg, respectively, for Zn, Cu, Pb, Fe and Cd). This site is very close to the emission zone of the industrial complex of Gabes, (TCG, Tunisia). Moreover, the soil samples collected from -S4- Zarat and -S5- Matmata, (60 and 75 Km respectively from ICG), showed the lowest levels of HMs (Table 2). As showed by Table 1 HMs in soil samples seem to be correlated with the distance from the emission zone of ICG as anthropogenic polluted source of mining activities. Our results corroborate previous published works (Sirven, 2006; Soodan *et al*., 2014; Yan *et al*., 2018) reported the impact of various natural and anthropogenic activities on heavy metal contaminated soils. The Zn richness of the soil samples in -S1- Ghannouch site was found to be similar with the results described by Viard *et al*., (2004) and Akhtar *et al*., (2010) and the contents of soil samples exceeded 200 ppm were reported to be correlated to the industrial activities. According to Kabata-Pendias (2010) total soil range contents of 70-400; 100–400; 60–12 and 3-8 mg. Kg-1 for Zn, Pb, Cu, and Cd, respectively are considered as lethal contamination for plants. Our findings suggest that and excluding for Zn at S1, S2, and S3 (160, 156 and 102 respectively) the Pb, Cu, and Cd average values obtained in the five studied sites did not exceed the reported lethal limits. Hence, plants grown in these sites were found to be not threatened by HMs pollution.

The pH values of the analyzed soil samples sites ranged from 6.00 to 8.32 and revealing an alkaline soil samples (Table 2). Contrariwise, to metallic concentrations, the lowest soil pH value was recorded for S1- Ghannouch. However, the highest pH value is found for -S5- Matmata with a value of 8.32 (Table 2). This suggests that the availability of metals increases with decreasing soil pH (Kabata-Pendias, 2010). Neutral and high soil pH can stabilize soil toxic elements, which lead to their leaching effects (Badr *et al*., 2012).

A significant decrease in soil salinity was observed for S1, S4, S3, S2 and S5, which was revealed by electrical conductivity (EC). The Electrical conductivity (EC) decreased from the polluted site to the control site (Table 1). These results corroborated with those reported in the literature (Jeddi *et al*., 2021). The increase of EC may affect HMs absorption as Cd (Manousaki *et al*., 2011). However, salinity soil increases the mobility of Cd^{2+} cations as well as their bioavailability for the plant.

Plant heavy metals concentration: As showed by Table 3, HMs in leaves and roots of *A. halimus* varied significantly among the analyzed sites. A decreasing concentration trend was found with the highest contamination levels in -S1- Ghannouch, which seem to be the most polluated site; and the lowest contamination levels in the site –S5- Matmata reported as a control site. Our results showed that in all studied sites HMs contamination levels do not exceed the toxic limits (Table 3). Regarding the analyzed sites, -S1- Ghannouch, -S3- Ketana and –S2- Teboilbou seem to be the most polluted sites and showed therefore the highest levels of HMs contamination for root and leaf samples. The highest content of HMs was recorded in the leaves (154.6 ppm, 56.2 ppm, 44.6ppm, and 23.7 ppm for Zn, Cu, Pb, and Fe respectively) compared to contents in roots (112.1, 50.9, 42.3, 20.4 for Zn, Cu, Pb, and Fe respectively), (Table 3). The high concentrations of HMs in Atriplex leaves may be attributed to the elevated foliar uptake, the translocation of these metals from roots to leaves, and the high rate of dust deposition (Shahid *et al*., 2017). Fe and Zn were reported to be, generally, immobile HMs in the soil and their translocations in the plant turn out to be a rather rare phenomenon (Sawidis *et al*., 2011). The main sources of Fe and Zn contamination are essentially atmospheric coming from the metallurgical industries, vehicles, and road engines (Bargagli, 1998). Zn, Fe, Mn, and Cu are minor trace elements required in small amounts for plant nutrition, and development, and participate in several metabolic reactions (Khan *et al*., 2017). However, at high concentrations, they can become lethal (Shahid *et al*., 2018).

The calculated values of the TF are presented in (Fig. 1a). The trend of TF values for Cd were <1 for the 5 studied sites. However, the values of TF for all other heavy metals were> 1. Interestingly, *A. halimus*, has accumulated high concentrations of Cd in the roots rather than transporting the contamination to the leaves. The accumulation of heavy metals in roots seems to be a protective strategy adopted by the species to defend against metallic stress (Ali *et al*., 2013). In fact, Cd is a known to be a toxic heavy metal that can damage tissue structure even at very low concentrations, which may explain its stabilization in root.

Table 2. Heavy metals concentration, PH and Electrical conductivity (EC) of soil samples collected from five sites located in the vicinity of the industrial complex of Gabes, TCG, Tunisia.

Sites	PH	EC	Heavy metals concentration (mg/kg)				
			Zn	Cu	Pb	séz	Cd
S1	6.00 ± 0.77 ^{a}	4.22 ± 0.33^{c}	175 ± 6.86^{c}	36.50 ± 7.96^{b}	28.70 ± 2^{bc}	17.54 ± 1.30^{c}	5.00 ± 0.97
S2	7.36 ± 0.23 ^{bc}	1.93 ± 0.06^{a}	156 ± 14.10^{c}	17.34 ± 4.92^{a}	24.40 ± 4.93^{c}	9.13 ± 2.90^{ab}	5.63 ± 0.65
S3	8.14 ± 0.10 ^{c}	1.94 ± 0.06^{a}	102.32 ± 13^{b}	23.36 ± 9.3^{ab}	21.15 ± 7.24^{ab}	5.24 ± 1.00^{a}	4.35 ± 1.05
S4	8.16 ± 0.29 ^{c}	3.87 ± 0.29^{b}	36.05 ± 15.96^{a}	7.44 ± 3.95^{a}	11.63 ± 3.00^{a}	6.76 ± 1.60^{a}	3.23 ± 2.55
S5	8.32 ± 0.60^{c}	1.86 ± 0.04^{a}	38.47 ± 9.93^{a}	9.17 ± 4.00^{a}	18.633 ± 4.9^{ab}	12.16 ± 2.00^{a}	2.20 ± 0.72
P	**	**	**	**	**	**	NS

Values represent means± S.D., (n = 3 samples). Different letters in the same raw denote significant differences (Tukey's HSD test at p<0.05), NS: non-significant,*: Significant at p<0.05; **: Height significant at p<0.01. S1: Ghannouche, S2: Tebelbou, S3: Ketena, S4: Zarat and S5: Matmata.

Table 3. Heavy metals concentrations (mg/Kg) in leaves and roots of *A. halimus* collected from five sites in the vicinity of the industrial complex of Gabes, TCG, Tunisia.

Sites	Zn		Cu		Pb		Fe		Cd	
	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
S1	133.3±3.5^{a}	112.1±3.3^{a}	54.9±5.1^{ab}	45.9±4.0^{a}	44.6±10.5^{b}	42.3±3.0^{c}	23.0±3.0^{bc}	15.4±1.4^{b}	2.1±0.1^{b}	3.0±0.1^{c}
S2	124.5±30.1^{ab}	110.1±13.9^{a}	56.0±3.6^{b}	50.3±5.0^{b}	21.3±3.2^{a}	15.3±4.7^{ab}	19.9±3.0^{bc}	20.0±1.6^{c}	2.1±0.1^{b}	2.7±0.3^{c}
S3	154.6±7.2^{b}	97.6±15.0^{a}	56.2±3.9^{b}	50.9±3.6^{b}	26.66±3.21^{a}	25.0±5.5^{b}	23.7±3.9^{c}	20.4±1.5^{c}	2.2±0.2^{b}	2.6±0.1^{bc}
S4	96.4±17.4^{a}	84.8±17.0^{b}	47.6±2.5^{b}	26.0±6.2^{a}	20.66±1.52^{a}	10.9±1.9^{a}	16.1±0.9^{ab}	12.1±1.65^{ab}	1.9±0.1^{b}	2.2±0.2^{b}
S5	107.0±14.0^{a}	100.5±11.0^{a}	35.0±5.5^{a}	21.7±8.0^{a}	20.66±6^{a}	9.5±2. 8^{a}	10.3±1.6^{a}	8.2±1.6^{a}	1.3±0.3^{a}	1.5±0.1^{a}
P	*	NS	**	**	*	**	**	**	**	**

Values represent means± S.D., (n = 3 samples). Different letters in the same raw denote significant differences (Tukey's HSD test at p<0.05), NS: Non-significant,*: Significant at p<0.05; **: Height significant at p<0.01. S1: Ghannouche, S2: Tebelbou, S3: Ketena, S4: zarat and S5: Matmata

The high TF values of Pb, Cu, Zn, and Fe (TF > 1) in *A. halimus* make the plant species suitable for its phytoextraction from the soil, while the low TF (TF < 1) for Mn and Cd makes it appropriate for Phytostabilization.

The calculated BCF values were in the order: Cu> Fe> Pb > Zn > Cd (Fig. 1b). Among the heavy metals studied, *A. halimus* had BCF values> 1 for Cu regarding the five studied species. Therfore *A. halimus* is found to be a plant species with a potential Copper hyperaccumulator. Semilar results were described and Mediterranean saltbush was reported to be a plant species with a great potential for phytoremediation for the Chromium (Mesnoua et al., 2018), and as a Pb accumulator in the leaves (Acosta *et al.*, 2018).

Effect of heavy metals on relative water content (RWC): Among the studied sites, the water status of A. halimus showed a significant decreasing trend in RWC (Fig. 2). An average of 28.8% in the leaves as a response to HMs contamination. The lowest value of RWC were recorded in -S1- Ghannouch with a percentage of 69.5% and the highest one in -S5- Matmata (78.7%) (Fig. 2). This finding corroborate that our hypothesis supporting the idea, as mentioned in the experimental design, where S1 is considered as the most polluted site and S5 was considered as a control site with lowest contamination level of HMs. Therfore, heavy metal accumulation in A. halimus is among the various stresses that influence RWC. The effect of HMs contamination could be accentuated by the aridity of the plant species among the studied sites as revealed by Table 1. Our results were in agreement with previous studies which reported a total reduction of RWC values in plants treated with Cd (Zouari *et al*., 2016; Khan *et al*., 2017; Ikram *et al.*, 2019). Some HMs, such as Pb, by binding to the plant cell wall can cause mineralization which leads to a change in its physicochemical properties, including Electrolyte leakage, and in their plasticity (Nawab *et al*., 2015; Parkash and Singh, 2020).

Effects of HMs on Chlorophyll Variation: A highly significant difference in the total chlorophyll contents (chlorophyll a and b) was found among the studied sites (Fig. 3). It seems that the total chlorophyll contents decreased with the contents of HMs. In fact, the highest chlorophyll contents were recorded in- S5- Matmata (4.12 mg/gFW) which has been considered as a reference site and the less contaminated locality. Moreover, the lowest chlorophyll content was found in -S1- Gannouch (2.51 mg/gFW) which is situated close to the industrial complex of Gabes and, therefore, the most contaminated site with HMs. For the other studied sites, the chlorophyll contents varied between 2.91 and 3.85 mg /gFW. These results corroborated those of (Mao et al., 2018) who showed a significant reduction in chlorophyll contents with the increase of heavy metal levels.

The decrease in chlorophyll levels in *A. halimus* exposed to heavy metals is a common phenomenon and is supposed to be preliminary to the inhibition of photosynthesis by blocking Mg, Mn, and Fe ions (Chatterjee *et al*., 2004; Joshi and Swami, 2007; Ikram *et al*., 2019). Cu seems to affect chlorophyll biosynthesis more than cadmium (Prasad *et al*., 2001). However, the reduction of chlorophyll pigments is a good indicator of atmospheric contamination of the environment since it indicates an intense reduction in the photosynthetic activity of the plant species.

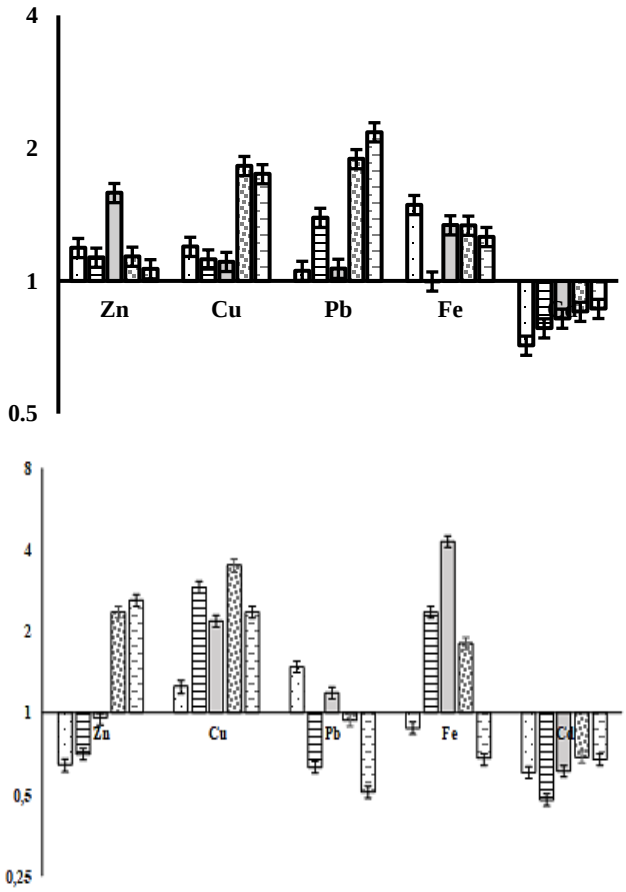

Fig. 1. a: Translocation Factor (TF) and b: Bioconcentration Factor (BCF) of Heavy metals in A. halimus leaves at five studied sites (S1: Ghannouche, S2: Tebelbou, S3: Ketena, S4: Zarat and S5: Matmata), Values represent means ± S.D, (n = 3). Results presented as Log2.

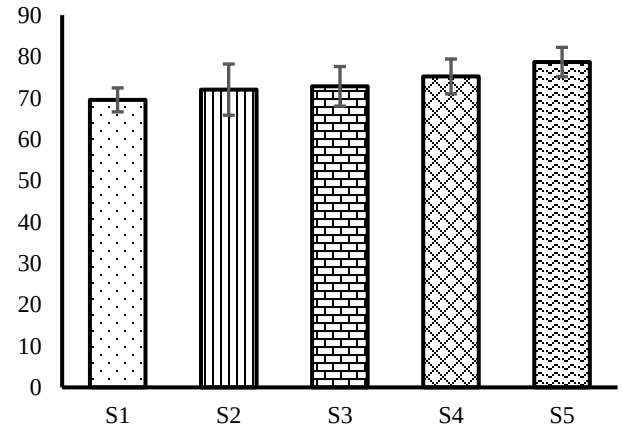

Fig. 2. Variation of RWC (%) in leaves between the study sites. (S1: Ghannouche, S2: Tebelbou, S3: Ketena, S4: Zarat, and S5: Matmata).

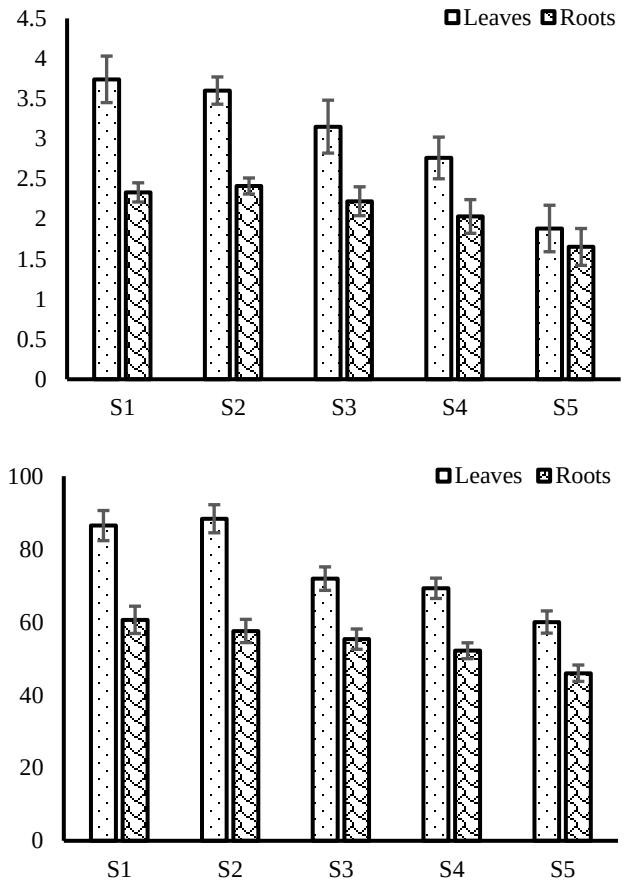

Fig. 3. Variation of total chlorophyll contents in leaves of *A. halimus* in the study sites. Values represent means ± S.D., (n = 3 samples) (S1: Ghannouche, S2: Tebelbou, S3: Ketena, S4: Zarat, and S5: Matmata)

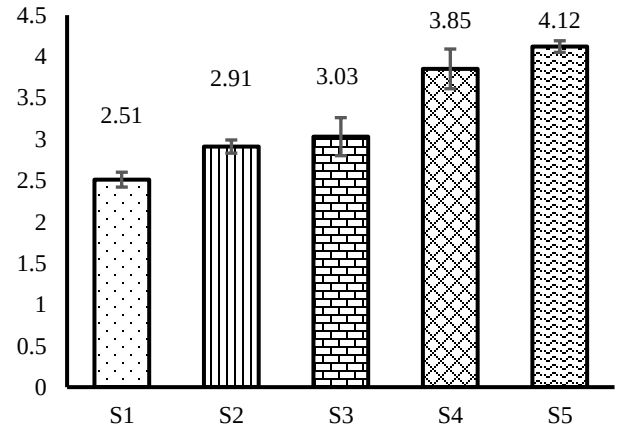

Fig. 4. Lipid peroxidation (MDA: mmol/g FW) and Hydrogen peroxidation (H_2O_2: mmol/gFW) in leaves and roots of *A.halimus* at five studied sites (S1: Ghannouche, S2: Tebelbou, S3: Ketena, S4: Zarat, and S5: Matmata).

The lipid peroxidation and H_2O_2 Production: As shown by Figure 4, both, malondialdehyde (MDA) and H_2O_2 consistently increase with heavy metals concentrations. The results obtained revealed that the increase of lipid peroxidation and H_2O_2 production was higher in leaves compared to root samples (Fig. 4). This fact could be explained by the effect of HMs accumulation in leaves, which can induce a high ROS Production. Our results were in line with those of Van Breusegem and Dat, (2006) who reported that leaves were the main sites for ROS production. The increase in O_2 production, generated by HMs, leads to high lipid

peroxidation (Mahi *et al*., 2015). A high level of MDA in A. halimus tissues seems to be a good indicator of cellular oxidative destructions (Gupta *et al.*, 2008). However, the foliar MDA content increased from the first dose of Cd used for Brassica napus (Ben Youssef *et al*., 2005). This clearly confirms the genesis of a state of oxidative stress in the leaves of the plant treated with the metal ions. Moreover, a High rate of ROS not scavenged can cause the peroxidation of membrane lipids, the attack of unsaturated lipids and protein denaturation (Rai, 2016).

Effects of heavy metals on antioxidant activities: The highest measure of antioxidant enzyme activities were recorded in leaves (Table 4). -S1- Ghannouch showed values of SOD, CAT, and APX activities two times more than the enzyme activities recorded for -S5- Matmata (control site). For root samples, the trend of enzyme activities (SOD, CAT, and APX) was similar and values of enzyme activities in S1- Ghannouch were higher than –S5- Matmata (with a ration varying from 1.61-2.7). The rest of studied sites regarding enzyme activities showed intermediate values for the analyzed samples (leaves and roots) and the trends of higher enzyme activities (SOD, CAT, and APX) seems to be conserved. The amplified activity of the antioxidant complex, as seen in Atriplex plant samples, has been mentionned in other plant species under metal toxicity. Furthermore, for Zea mays grown under Cd stress, SOD is considered as the princgipal line of resistance against ROS in plant organ, and it has been reported to activate the conversion of O_2 to H_2O_2 (Anjum *et al*., 2015). CAT and APX play an essential role in reconverting H_2O_2 in plants organ for its less toxicity behavior (Zhang and Kirkham, 2014; Mao *et al*., 2018). It is well known that the mechanism of ROS regulation of plant species plays important role in their tolerance to stress (Kumar *et al*., 2012; Huang *et al*., 2019).

Table 4. Activities of ascorbate peroxidase (APX, U mg-1 protein), catalase (CAT, mmol H2O2 min-1 mg-1 protein) and superoxide dismutase (SOD, U mg-1 protein), in leaves and roots of A. halimus collected from five sites in the vicinity of the industrial complex of Gabes, TCG.

	Enzymes	S1	S2	S3	S4	S5
Leaves	APX	6.81 ± 0.50	5.62 ± 0.29	5.49 ± 0.37	5.00 ± 0.18	3.59 ± 0.11
	CAT	7.25 ± 0.27	6.84 ± 0.20	6.12 ± 0.14	4.36 ± 0.23	3.15 ± 0.15
	SOD	41.20 ± 0.32	33.15 ± 0.18	28.9 ± 0.31	27.39 ± 0.19	19.74 ± 0.6
Roots	APX	5.49 ± 1.10	4.35 ± 1.49	4.12 ± 0.70	3.65 ± 0.78	3.12 ± 1.02
	CAT	4.80 ± 0.90	5.60 ± 1.22	5.80 ± 0.36	4.20 ± 0.126	2.74 ± 1.69
	SOD	26.25 ± 2.18	33.14 ± 1.08	26.60 ± 0.19	22.87 ± 0.18	20.27 ± 1.80

Values represent means ± S.D. (n = 3). S1: Ghannouche, S2: Tebelbou, S3: Ketena, S4: Zarat and S5: Matmata

Table 5. Proline and Soluble sugar contents in *A. halimus* organs collected from five sites in the vicinity of the industrial complex of Gabes, TCG, Tunisia.

		Sugar (mg/g FW)	Proline (mg/g FW)	Ascorbic acid (mg/g FW)
Leaves	S1	10.96 ± 0.15^{c}	33.65 ± 0.49^{c}	5.06 ± 0.75^{c}
	S2	10.70 ± 0.31^{bc}	30.13 ± 0.71^{b}	4.83 ± 0.19^{b}
	S3	9.12 ± 1.50^{abc}	31.33 ± 0.41^{b}	4.12 ± 0.64^{b}
	S4	8.79 ± 0.69^{ab}	26.00 ± 0.95^{a}	4.03 ± 0.50^{b}
	S5	8.40 ± 0.27^{a}	26.43 ± 0.55^{a}	3.15 ± 0.70^{a}
	P	**	**	**
Roots	S1	4.22 ± 0.11^{c}	20.46 ± 0.34^{d}	3.81 ± 0.21^{c}
	S2	3.53 ± 0.13^{b}	18.43 ± 0.21^{c}	3.37 ± 0.12^{c}
	S3	3.44 ± 0.32^{b}	18.44 ± 0.39^{c}	3.26 ± 0.15^{c}
	S4	2.71 ± 0.35^{a}	17.12 ± 0.11^{b}	2.29 ± 0.42^{b}
	S5	2.21 ± 0.21^{a}	15.14 ± 0.25^{a}	2.12 ± 0.40^{b}
	P	**	**	*

Biochemical responses: The biochemical analysis of leaf and root samples collected from different studied sites are shown in table 5. As previously reported regarding the effect of polluted sites, with HMs, on physiological state of *A*. *halimus*, it is clearly that the contents of proline, ascorbic acid, and soluble sugars were detected with higher concentration in more contaminated sites. Furthermore, the analyzed samples (leaves and roots) collected from -S1- Ghannouch showed concentrations, in sugars and proline, higher about 24 % and 22% in leaf samples and 25% and 48% for root samples than those recorded for the control site -S5- Matmata (Table 5). Previous studies reported that the accumulation of proline under stress conditions maintains the stabilization and turgidity of cell membranes, osmotic balance as well as antioxidant activities (Shahid *et al*., 2017).

Likewise, sugar levels demonstrate a concentration gradient deacreasing with the distance from emission source of ICG and ranging between 2.21 mg/g FW in roots and 8.40 mg/g FW in leaves (-S5- Matmata) to 4.22 mg/g FW in roots and 10.96 mg/g FW in leaves (-S1- Ghannouch) (Table 5). As reported by several authors, soluble sugars improve membrane permeability and plasticity, maintain cell turgor and water homeostasis and enhance the antioxidant protection process (Rai, 2016; Afzal *et al.,* 2021). Our results showed that proline was significantly increased in A. halimus plants growing close to the industrial complex (S1) relative to the control site (S5), by 20.46 mg/g FW (S1) to 15.14 mg/g FW (S2) in roots and 33.65 mg/g FW (S1) to 26.43 mg/g FW (S5) in leaves (Table 5). Rai (2016) reveal that an increase in Proline production is induced by a decrease in chlorophyll content and water status.

Ascorbic acid is a natural antioxidant playing an important role in the tolerance mechanisms of the plant species (Chen *et al*., 1991). It is known as an antioxidant molecule in the detoxification of atmospheric pollutants. By the present study, our results showed that the ascorbic acid content varied for leaves and roots (table 5). The highest content was recorded in S1 (3.81 in roots and 5.06 mg/g FW in leaves) and the lowest in S5 (2.12 mg/g FW in roots to 3.15 mg/g FW in leaves) (Table 5). *A. halimus* is reported to be a tolerant species since it can maintain high levels of ascorbic acid under conditions of metallic stress (Aji *et al*., 2016).

Regarding abiotic stress and in order to develop an adaptative strategy, plant species massively synthesizes osmoprotectants, such as sugars and sugar alcohols, soluble proteins, quaternary ammonium compounds, and amino acids, like proline. (Rasheed *et al*., 2014; Ozturk *et al.*, 2021).

Conclusion

By the present study, the effect of HMs contamination in the vicinity of the emission zone of the industrial complex of Gabes, (TCG, Tunisia) on some physiological and biochemical parameters of *Atriplex halimus* has been investigated. High levels of Zn, Fe, and Cu were found to be accumulated in roots while the reverse was found to be the case for Cd which seems to be a protective strategy adopted by plants against abiotic stress. Moreover, A. halimus is a Cu accumulator. The high TF values of Pb, Cu, Zn, and Fe (TF > 1) in A. halimus make it suitable for its phytoextraction from the soil, while the low TF (TF< 1) for Cd makes it appropriate for its phytostabilization potentialities. Heavy metals have harmful physiological and biochemical effects such as a decrease in relative water content, and chlorophyll content, inducing the production of ROS. The physiological changes stimulate the biochemical mechanism. Therefore, A. halimus could be a good bioindicator plant species of environmental contamination and can be used in phytoremediation program of the polluted area as induced by mining activities.

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