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RESEARCH PAPER

Screening for Heavy Metal-Resistant Clones in the Xero-Halophyte *Atriplex halimus* L.: A Prerequisite for Phytoremediation of Polymetallic Mining Pollution in Arid Areas

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Abstract

Atriplex halimus L. is a promising xero-halophyte species for phytoremediation purposes but displays high levels of genetic variability. As an attempt to select uniform material suitable for phytomanagement, five clones were established by cuttings from three non-polluted sites (Tunis, Nabeul and Sfax), one moderately Pb-polluted site (Sousse) and a highly polymetallic polluted mining site (Gafsa). Cuttings were cultivated during 90 days under controlled conditions on soil issued from the most polluted area. The clone from Gafsa accumulated higher concentrations of metals in roots (Cr) and leaves (Cd, Sr, Zn and Cu) than other clones. Gafsa showed the highest absorption efficiency, translocation factor, bioconcentration factor and bioaccumulation coefficient compared to other clones but displayed the lowest relative growth rate (RGR) value while the highest RGR was found in the clone from Sousse. Heavy metal tolerance in Gafsa was not related to a more efficient management of oxidative stress or higher concentration of phytochelatins. Total amount of Sr and Zn removed from the substrate was the highest for Sousse while removal of Cd, Cu, Cr and Ni was the highest for Gafsa. It is concluded that cuttings allow to obtain uniform material for phytoremediation by *Atriplex halimus* and that combining different clones with complementary properties is an attractive option for phytomanagement of polymetallic polluted soils by this species.

Highlights

- *Atriplex halimus* is a heterogenous halophyte species suitable for phytoremediation.
- Clones from different areas are tested for heavy metals accumulation on a polymetallic polluted soil.
- Clone from contaminated area accumulates higher shoot heavy metals concentrations.
- Heavy metal tolerance is not related to improved management of oxidative stress.
- Combining clones with complementary heavy metal accumulation and growth rate is a good option for phytomanagement.

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Introduction

Soil contamination by metallic elements affects several million sites worldwide. This ecological issue results from the overabundance of metals in the environment, primarily due to human activities such as industrial waste, factory emissions, accidental spills, inadequate agricultural practices, and wastes from consumer products (He et al. 2015). Mining activities have left an outstanding footprint in time and space regarding this type of pollution (He et al. 2015; Saxena et al. 2020; Zerizghi et al. 2022). The mining sector has long been considered the backbone of the world economy (Lindahl 2014). It has indeed played a crucial role in the development, economic progress, and social advancement of humanity. However, this sector also involves dangerous and destructive human activities in the world, with a tremendous environmental impact by producing varied, substantial, and persistent wastes, adversely affecting the quality of the life through air, water and soil pollution (Lambrechts et al. 2011; Acosta et al. 2018; Lee et al. 2023). Soil pollution by heavy metals (HMs) and metalloids stands out as a primary environmental issue consistently linked to the exploitation of metalliferous ores (Saxena et al. 2020; Zerizghi et al. 2022).

HM accumulation in soils primarily induces modifications in soil physical, chemical, and biological properties. This, in turn, affects the taxonomic, functional diversity, and microbial properties of soil, leading to the degradation of the ecosystem services provided by this non-renewable natural resource. Additionally, owing to their elevated concentrations in soils, HMs produced by mining activities can be transferred and accumulated not only in plants but also in humans, leading to numerous severe health disorders (Abdu et al. 2017; Bhat et al. 2022; Zerizghi et al. 2022; Mudgal et al. 2023). Consequently, seeking environmental solutions to manage, remediate and restore contaminated soils is of primary importance.

Different physicochemical remediation methods, such as dumping, covering, soil cleaning, leaching, electro-kinetics are effectively applied in mining areas (Karn et al. 2021; Pandey et al. 2023). Although these methods can reduce and/or stabilize HMs in the soil, they face challenges due to their high costs, the potential disruption of soil structure, and the creation of a sterile soil that is unfavorable for the survival of both animal and plant species (Lee et al. 2023). Over the past decade, there has been a growing interest in an alternative to those traditional remediation practices that could be both economically viable and environmentally friendly. This method, called "phytoremediation," harnesses the natural capabilities of plants to extract and/or stabilize HMs from soil (Bhat et al. 2022; Mudgal et al. 2023). Recent comparative tests have shown that remediating one acre of contaminated soil using plants costs only about $60,000 to $1,000,000 whereas physical remediation is at least four to six times more expensive to achieve the same result (Sharma et al. 2023). Moreover, unlike physiochemical techniques that bring about irreversible changes to ecological properties, phytoremediation has the capacity to restore the quality of contaminated mining substrate in relation to improvement of their biological, chemical and physical properies (Shen et al. 2022).

Heavy metals induce a wide range of physiological disorders in plants. Although some of these elements are required in small amounts for normal cell metabolism (Cu, Zn, Mn,…), others (Pb, Cd, Sr,…) have no known biological functions (Thakur et al. 2022). An excess of HM in plant tissues impairs plant growth, photosynthesis, regulation of plant water status and mineral nutrition, modifies the phytohormone profile and triggers oxidative stress leading to cellular structure modifications (Feki et al. 2021; Thakur et al. 2022; Riyazuddin et al. 2022). Some cytotoxic aldehydes are derived from lipid peroxides and can cause extensive damages to protein leading to changes in their function or stability (Winger et al. 2007; Liang et al. 2022). Glutathione is a powerful endogenous antioxidant but also serves as a precursor in the synthesis of phytochelatins: these small non-proteic polypeptides may bind to some heavy metals and are then sequestered in vacuole, mainly through ABC transporters (Noor et al. 2022; Riyazuddin et al. 2022; Thakur et al. 2022). Other antioxidants occur in plants and directly influence the total antioxidative properties in the various organs. Proline is frequently reported as an osmocompatible solute able to protect cellular structures, stabilize enzyme conformation and act as powerful antioxidant (Alvarez et al. 2022; Ghosh et al. 2022; Spormann et al. 2023). Plants used for phytoremediation must be able to tolerate high concentrations of accumulated HM without suffering from strong growth inhibition and should also produce enough biomass to efficiently contribute to progressive soil decontamination (Shen et al. 2022; Yang et al. 2022).

In arid and salt-affected areas, xero-halophyte plant species present numerous advantages in relation to their capacity to cope with mineral toxicities and other harsh environmental conditions (Lutts and Lefèvre 2015; Aziz and Mujeeb 2022). The *Atriplex* genus belongs to the Amaranthaceae family and comprises more than 350 species, several of them being perennial C4 xero-halophyte shrubs (Kahli et al. 2021). *Atriplex halimus* is native in arid and semi-arid Mediteranean regions and is able to spontaneously colonize HM-contaminated soils (Tapia-Fernandez et al. 2016; Acosta et al. 2018; Acuña et al. 2021; Shahrokh

et al. 2022). This plant species exhibits tolerance to Cu, Cd, Pb, Zn and As toxicities (Lutts et al. 2004; Manousaki and Kalogerakis 2009; Lefèvre et al. 2009; Tapia et al. 2013; Walker et al. 2014; Bankaji et al. 2019; Orrego et al. 2020) and may therefore be recommended as a valuable species for phytoremediation purposes. However, the major drawback of this species is related to its huge genetic variability, both between and within populations (Haddioui and Baaziz 2001; Walker et al. 2005; Ortíz-Dorda et al. 2005). Such a variability hampers the efficient use of the most adequate genotypes on large areas. To overcome this problem, Manousaki and Kalogerakis (2009) and Mancilla-Leytón et al. (2016) recommended to use stem cutting approach rather than seed sowing for plant establishment on polluted sites.

Stem cutting behavior on polymetallic mining soils was not considered until now. In most cases, studies devoted to *A. halimus* response to HM considered one single pollutant (Lutts et al. 2004; Lefèvre et al. 2009; Kahli et al. 2021) while in field conditions, HM pollution usually implies the simultaneous presence of several pollutants (Saxena et al. 2020). Moreover, it is not clear whether clones native to polluted areas may extract HM from polluted soil more efficiently than clones obtained from non-polluted sites. To address these questions, we collected clones of the xerohalophyte *A. halimus* from various Tunisian populations located in distinct pedoclimatic areas and cultivated them on a polymetallic contaminated soil issued from the arid mining region of the city of Gafsa (southwestern of Tunisia). The accumulation and distribution of accumulated heavy metals were analyzed in relation to growth, antioxidative properties, phytochelatins and proline content of the various clones.

Material and Methods

Site Geographical Location and Soil Analysis

The main contaminated area considered in this study was located in the Western part of Tunisia (Fig. 1) in the phosphate mining basin of Gafsa (Mdhilla sector). Four additional sites were considered from other areas in Tunisia: Sfax, Sousse, Nabeul and Tunis (Fig. 1). The precise location of the considered areas and their major climatic properties (annual rainfall and mean temperature) are listed in Table 1. All these sites were marginal areas which present spontaneous colonization by *Atriplex halimus.*

At each site, soil was collected from at least 6 places (0–25 cm of the top soil) with a hand auger and crushed to pass through a 10 mm stainless steel. Soil pH and electrical conductivity (EC) were determined at a soil to distilled water suspension ratio of 1:5, with a shaking time of 5 min, using glass electrodes (ISO 10390; 2005). For the determination of inorganic carbon (Ci, eq $CaCO_3$), a sample of soil (1 g), pure sand (5 g), and an acidic solution containing hydrochloric acid (HCl) with ferrous iron (Fe^{+2}) are brought into contact in a Schott flask connected to a pressure gauge. Only inorganic compounds, such as calcium carbonate ($CaCO_3$), are decomposed into carbon dioxide (CO_2), which are then detected by the pressure gauge. The obtained CO_2 was quantitatively converted (based on standards) into precise concentrations of calcium carbonate. The ferrous iron was added to prevent the transformation of organic matter into CO_2 (Baize 2018). Major cation concentrations were determined by loss on ignition method at 950 °C of soil samples with addition of lithium tetraborate and lithium metaborate. Heavy metals concentrations were obtained after acid attack of soil samples with HNO_3 68% and HF 40% as previously detailed (Lambrechts et al. 2011). Ultrapure water with a resistivity of 18 MΩ cm (Merck-Millipore, Germany) was used for sample preparation. Analysis was conducted with an inductively plasma emission spectrometry ICP-OES (Thermo Jarrel Ash Iris Advantage) by using Merck ICP standards solutions (quality: traceable to SRM from NIST) with standards concentrations ranging from 0.01 to 0.2 ppm for Cd, Sr, Zn, Cu, Cr, Ni and Pb and from 0.1 to 10 ppm for Fe. SLRS-6 was used as reference material.

The pollution metallic nature, whether it was in the form of single or complex compounds, in the soils of the native habitats of the five clones of *A. halimus*, was assessed using the single factor pollution method (P_i) and the Nemerow comprehensive pollution index method (P_n) (Tomlinson et al. 1980; Kwon and Lee 1998). These indices were derived by dividing the metal concentrations in soil (ppm) by the background metal concentration, as outlined by Government of France (1998) representing the standard levels found in uncontaminated soils and was set at 2, 300, 100, 150, 100 and 50 mg kg^{-1} for Cd, Zn, Cu, Cr, Pb and Ni respectively.

Single factor index is defined as:

$Pi = \frac{Ci}{Si}$ where P_i is the index for single-component contamination, C_i represents the measured concentration of the considered metal "I" in the soil, and S_i denotes the background concentration of this metal. The evaluation results were categorized into five grades: $P_i \leq 0.7$: safe; $0.7 < P_i \leq 1.0$: warning; $1 < P_i \leq 2$: slight pollution; $2 < P_i \leq 3$: moderate pollution; and $P_i > 3$: heavy pollution.

The Nemerow comprehensive pollution index reflects the impacts of various heavy metals on the soil, avoiding the dilution of the influence of heavy metals caused by averaging. The calculation formula is as follows:

$$Pn = \sqrt{\left(\overline{P}^2 + P_{\max}^2\right)/2}$$

P_n: composite contamination index; P: average value of the single-factor index; and Pmax: maximum value of the

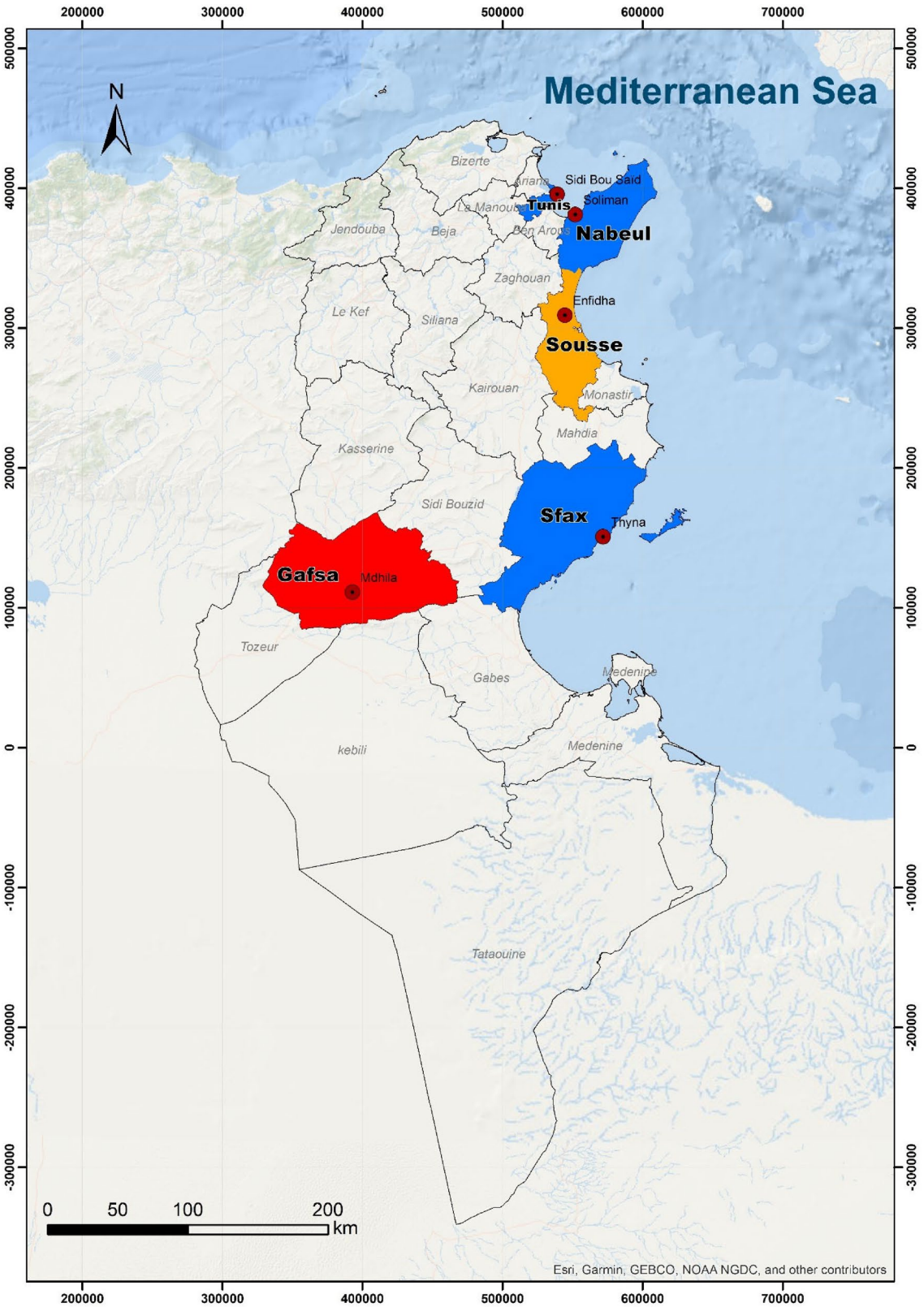

Fig. 1 Map of Tunisia indicating the five collection sites of *Atriplex halimus clones*

Table 1 Precise location, description and bioclimatic attributes of sites used for *Atriplex halimus* harvest (*P*: mean annual rainfall; *T*: mean annual temperature)

Sites	Location	GPS	Area nature	Bioclimatic stage	$\overline{P}$(mm)	$\overline{T}$(C°)
Tunis	Sidi Bou Saïd	36°51′42″ N 10°20′23″ E	Urban area	Upper semi-arid	444	19
Nabeul	Soliman	36°43′51″ N 10°28′56″ E	Protected area	Upper semi-arid	400	19
Sousse	Enfidha	36°4′55″ N 10°23′43″ E	Protected area	Lower semi-arid	340	20
Sfax	Thyna	34°39′7″ N 10°41′5″ E	Archaeological area	Upper arid	196	19
Gafsa	Mdhila	34°17′28″ N 8°44′18″ E	Mining area	Lower arid	150	20

single-factor index. The evaluation results were categorized into five levels, as previously mentioned with the single-factor index.

Bioavailability of heavy metals was assessed according to Lakanen and Erviö (1971); briefly, 8 g of soil sample were taken and mixed with 40 mL of an extraction buffer (19 mM EDTA, 500 mM ammonium acetate, and 96% acetic acid), then shaken for 30 min using a mechanical shaker. Subsequently, samples were centrifuged at 5000 × *g* for 5 min, and the resulting supernatant was filtered through a 0.45 mm filter. Residues from the first centrifugation were washed with 40 mL of distilled water. Then, samples underwent the same steps as for the first extraction, with the second supernatants being combined with the first ones, and the volume being adjusted to 100 mL with distilled water. Element quantification was conducted using inductively coupled plasma-optical emission spectroscopy (Varian, type MPX).

As the most contaminated area is located in the mining place of Gafsa (see "Results" section), soil from Gafsa was considered for subsequent greenhouse trials: *c.a.* 300 kg of soil was thoroughly homogenized and used to fill the pots (16 cm diameter × 16 cm height) with 2 kg of soil in each pot.

Plant Growth Conditions

On each site, the bulkiest bush of *A. halimus* was selected for subsequent cloning purposes. Stem segments (*c.a.* 10 cm length) were cut in T shape with two opposed nodes (50 segments per plant) and rinsed with tap water. Cuttings were then planted in plastic trays filled with a mixture of peat and sand (1:1) and watered twice a week to keep soil moisture at 60% of field capacity until they successfully established roots. After 45 d, uniform rooted plants (12 cm length; 2–3 g fresh weight, FW) were transferred to the pots containing the soil of Gafsa. At the time of transfer, five plantlets of each clone were separated in roots, stems and leaves, and each organ was dried in an oven at 72 °C during 48 h to determine the mean initial dry weight (DW1). Plants were watered twice a week with tap water (pH 7.5, EC 0.08 dS m^{-1}; Cd < 0.3 µg L^{-1}; Pb 1.1 µg L^{-1}; Ni: 0.87 µg L^{-1}; Cu 13.7 µg L^{-1}; Cr < 0.8 µg L^{-1}; Cl^- 43.1 µg L^{-1}; Fe < 10 µg L^{-1}; Sr < 1.2 µg L^{-1}; Na 26.5 mg L^{-1}).

Irrigation was conducted to maintain 60% of field capacity and plants were maintained in greenhouse conditions (16 h photoperiod, 40% mean relative humidity and mean temperature of 28 ± 4 °C during the day and 23 ± 3 °C during the night). Pots filled with substrate but without plants were irrigated in the same way and remained as unvegetated controls.

After a 90-day phytoremediation trial, leaf number, root length, and stem length were determined on each plant. Plants were then harvested and separated into roots, stems, and leaves, and their respective DW (8 plants per treatment) was estimated as previously mentioned. Fresh matter of remaining plants was frozen in liquid nitrogen and stored at – 80 °C until subsequent analysis. The relative growth rate (RGR) was calculated according to Hunt (1990): $RGR = (ln\ DW_2 - ln\ DW_1)/(t_2 - t_1)$, where DW_1 was the mean initial DW previously assessed and DW_2 was individual plant sample DW at the end of the trial and $(t_2 - t_1)$ represented the duration of the experiment.

Plant Element Content

Mineral concentrations were quantified separately in stems, roots and in leaves. Samples of dry matter were digested by incubation in 68% HNO_3 at 80 °C during 72 h on a sand bath. A mix of 37% HCl and 68% HNO_3 (1:3) was used to dissolved the mineral after full evaporation. Samples were then filtered on a Whatman n°1 filter paper. Elements were quantified by ICP-OES inductively plasma emission spectrometry (Thermo Jarrel Ash Iris Advantage) equipped with a Mainhard nebulizer. Samples were carried through an argon gas plasma (forward power of 1300 W and reflected power of 7 W). Gas flow rates of plasma was set at 16.0 L min^{-1} while auxiliary and nebulizer were both set at 1.0 L min^{-1}. The internal standard multi-element stock solution purchased from Agilent (USA) was used to control stability of the instrument while certified reference material ERM-CA713 was obtained from Sigma-Aldrich (Germany).

Phytoextraction Indices

The absorption efficiency (AE) was computed for each element using the following formula: $AE = ((Q - Metal)_{shoot} + (Q - Metal)_{root})/ DW_{root}$, Q being the total amount of the considered element.

The translocation factor (TF) indicates the plant capacity to translocate HMs from roots to shoots and was calculated as: $TF = [Metal]_{shoot}/[Metal]_{root}$.

The bioconcentration factor (BCF) reflects root ability to accumulate HMs from the soil (Arbalestrie et al. 2022). It was estimated using the following formula: $BCF = [Metal]_{root}/[Metal]_{soil}$.

The biological accumulation coefficient (BAC) represents shoot ability to accumulate HMs from the soil. It was estimated as: $BAC = [Metal]_{shoot}/[Metal]_{soil}$.

Chlorophyll, Glutathione, Non-protein Thiols and Phytochelatins Estimation

Chlorophyll (Chl a and Chl b) and total carotenoid concentrations were determined using 100 mg of fresh weight (FW) ground in a pre-chilled mortar with 8 mL of 80% acetone. After complete extraction, the volume was adjusted to 10 mL with cold acetone, and the mixture was centrifuged at 3,000 × *g* for 10 min. The absorbance of the extract was measured at 663.2, 646.8, and 470 nm using a Beckman DU640 spectrophotometer, and pigment concentrations were calculated according to Lichtenthaler (1987).

To determine reduced glutathione (GSH) and total glutathione (total GSH), 0.2 g of frozen fresh root, stem and leaf tissue were extracted and derivatized by *ortho*-phthalaldehyde (OPA) according to Cereser et al. (2001). For total GSH extraction, 0.2 g of tissue was homogenized with 1 mL dithiotreitol (DTT) and 500 µL 0.1 M Tris buffer pH 8.5 for reduction of oxidized glutathione (GSSG). After 30 min of reaction at 4 °C, 8 mL of 6% metaphosphoric acid was added and the mixture was centrifuged at 10,000 × *g* for 7 min at 4 °C. Then, 100 µL of the supernatant was adjusted with 100 µL of OPA and was left at room temperature for 5 min before adding 800 µL of 500 mM sodium phosphate (NaH_2PO_4) pH 7. GSHt was quantified after reduction of oxidized glutathione (GSSG) by DTT. Extracts were filtered through 0.45 µm microfilters (Chromafil PES-45/15, Macherey–Nagel) before injection. A reversed-phase HPLC column was used to separate OPA derivatives using an acetonitrile-sodium acetate gradient system and fluorimetrical detection. Samples (5 µL) were injected into a Shimadzu HPLC system (Shimadzu's-Hertogenbosch, The Netherlands): a Nucleodur C18 Pyramid column (125 × 4.6 mm internal diameter; 5 µm particle size) (Macherey–Nagel, Düren, Germany) was used. Elution of derivatives was performed using an acetonitril gradient in a 50 mM sodium acetate buffer pH 6.2 at a temperature of 30 °C at a flow rate of 0.7 mL min^{-1}. A spectra Shimadzu RF-20A fluorescence system (detector at 420 nm after excitation at 340 nm) was then used or detection. Glutathione was quantified using a nine-point calibration curve (standard solutions ranging from 0.0625 to 50 µM). A check standard solution was used to confirm the calibration of the system every ten injections. The recovery was determined using GSH as an internal standard.

The total non-protein thiols (NPT) concentration was assayed according to De Vos et al. (1992): 200 mg FW of tissue was ground in a pre-chilled mortar at 0 °C in 2 mL of sulfosalicylic acid (5% (*w/v*) containing 6.3 mM diethylenetriaminepentaacetic acid (pH < 1). After centrifugation during 10 min at 10,000 × *g* and 4 °C, collected supernatants were used for the determination of thiols using Ellman's reagent: 300 µL supernatant were added to 630 µL of 0.5 M KH_2PO_4 and 25 µL of 10 mM 5,5-dithiobis 2-nitrobenzoic acid (final pH 7.0) and incubated during 2 min at room temperature before reading the absorbance at 412 nm. The NPT concentration was estimated using an extinction coefficient of 13,600 $M^{-1}.cm^{-1}$. Phytochelatins (PC) content were evaluated as difference between NPT and GSH levels (Schäfer et al. 1997).

Malondialdehyde, Proline and Total Antioxidant Power

Malondialdehyde (MDA) was quantified according to Heath and Packer (1968) after homogenization of frozen samples (*c.a.* 200 mg FW) in the presence of 10 mL of ice-cold 5% (w/v) trichloroacetic acid. Centrifugation was performed at 12,000 × *g* for 10 min at 4 °C and 2 mL aliquot of supernatant were added to 2 mL of 0.67% (w/v) thiobarbituric acid. The mixture was heated during 30 min at 100 °C and then cooled to 4 °C. After centrifugation at 12,000 g for 1 min at 4 °C, the absorbance of the supernatant was read at 532 nm, and values corresponding to nonspecific absorption (600 nm) were subtracted. Malondialdehyde concentration was calculated using its molar extinction coefficient (155 $mM.cm^{-1}$).

Proline was extracted in 3% (w/v) sulfosalicylic acid. After incubation at 70 °C during 30 min, samples were filtered on Whatman 11 µm paper and 2 mL of ninhydrin solution (1.25 g of ninhydrin in 20 mL of H_3PO_4 6 M and 30 mL acetic acid) were added to 2 mL of supernatants. Samples were incubated at 100 °C for 1 h and reaction was stopped on ice. Absorbance was read at 520 nm in a quartz cuvette after addition of 2 mL toluene (Bates et al. 1973). Proline concentration was calculated based on a standard curve established with L-proline (Sigma Aldrich chemicals).

Total antioxidant activity was estimated by ferric reducing ability of plasma (FRAP) (Benzie and Strain 1996),

considering the ability of plant extract to reduce ferric to ferrous ion at low pH and to produce a colored ferrous-tripyridyltriazine complex detected spectrophotometrically at 593 nm. A second assay was performed using the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) discoloration procedure according to Pellegrini et al. (1999) assays. Results were expressed as µM Trolox equivalents (TE) g^{-1} FW.

Data Statistical Treatment

The obtained data were analyzed using one-way analysis of variance (ANOVA), and the means were compared utilizing Duncan's test at a 5% significance level. The statistical analysis was conducted using The XLSTAT statistical software, version 2014.

Results

Properties of Soil from the Native Areas and Soil Used for Phytoremediation Trials

Based on soil properties (Table 2), Tunisian clones of *A. halimus* investigated in the present study exhibit an affinity for presence in alkaline soils. Indeed, the pH in all five biotopes was higher than 7, with a maximum of 9.03 recorded in Sfax region. The xero-halophyte *A. halimus* clones collected in this study were spontaneously colonizing non-polluted soils in the areas of Tunis, Nabeul and Sfax with P_i (Cd) = 0, P_i (Zn, Cu, Cr, and Ni) < 0.7, and therefore P_n < 0.7 (Table 2). Soil sampled from Sousse appeared as slightly contaminated by Pb (P_i = 1.46 and P_n = 1.08). *A. halimus* was also found in the highly contaminated soils from the mining area of Gafsa, where P_n = 4.10 indicated a heavy polymetallic pollution by Zn (P_i = 1.20: slight pollution), Cr (P_i = 1.84: slight pollution), and Cd (P_i = 5.49; heavy pollution). Unusual high concentrations of Sr (776 ppm and 1309 ppm) were also detected respectively in soils sampled from Nabeul and Gafsa (Table 2). Pb was detected in small amounts (except in Sousse), and the lowest value was recorded for Gafsa. No arsenic was detected, whatever the considered site. The Fe content in Sousse was extremely high and almost 3 times higher than in Gafsa.

The soil from Gafsa mining area was heavily degraded at the initial state (t = 0), being alkaline and calcareous (pH: 7.23; Ci, inorganic carbon content as equivalent calcium carbonate: 193 $g.kg^{-1}$; soil EC: 3.43 dS m^{-1}). During the time course of the trial pH, Ci, and EC of the unvegetated soil increased up to 7.66, 207 g kg^{-1} inorganic carbon content, and 4.03 dS m^{-1} respectively, while the various clones had no significant impact on those properties. For each treatment, the total and the bioavailable concentrations of Cd,

Table 2 Soil heavy metal concentrations (ppm) and environmental indices in native habitats of *Atriplex halimus* (EC: electrical conductivity in dS. m^{-1})

Sites	pH	EC	Cation concentration (ppm)								Pi							Pn
			Cd	Sr	Zn	Cu	Cr	Ni	Pb	Fe		Cd	Zn	Cu	Cr	Ni	Pb	
Tunis	8.95	0.29	0.00	115	42	8	27	12	47	12,132		0.00	0.14	0.08	0.18	0.24	0.47	0.36
Nabeul	9.00	0.36	0.00	776	25	19	19	8	38	5103		0.00	0.08	0.19	0.13	0.17	0.38	0.29
Sousse	8.64	0.59	0.00	213	69	12	58	24	146	25,714		0.00	0.23	0.12	0.38	0.48	1.46	1.08
Sfax	9.03	0.48	0.00	227	23	8	24	10	36	9241		0.00	0.08	0.08	0.16	0.20	0.36	0.27
Gafsa	7.69	3.43	10.98	1309	359	27	276	25	18	8202		5.49	1.20	0.27	1.84	0.49	0.18	4.10

Sr, Zn, Cu, Cr, Ni and Fe in the soil are listed in Table 3. The soil at its initial state (t = 0) showed to be polymetallically-contaminated by Cd, Sr, Zn, Cr and Fe. Traces of Cu (19.80 ppm) and Ni (29.45 ppm) were also detected. For these elements 40%, 7%, 6%, 2%, 0.35%, 10% and 0.33% respectively of Cd, Sr, Zn, Cu, Cr, Ni and Fe were bioavailable for plant absorption. No bioavailable Pb was detected in our soil samples and Pb was therefore not included in Table 3. Compared to the initial level (t = 0), a significant decrease in the total concentration of Cd was recorded for soil cultivated with plants collected from Gafsa, whereas for Sr, cultivating all clones significantly reduced soil content comparatively to initial soil or to soil maintained without plants. Similarly, plants from Gafsa were able to reduce soil Cr content (*ca.* − 16 ppm) at the end of the phytoremediation trial, compared to the initial concentration at t = 0. No significant effect of *A. halimus* was recorded for Zn, Cu, Ni or Fe soil total concentration, irrespective of the considered population.

Compared to the initial soil, soil cultivated with the clones issued from Gafsa, Sousse and Sfax showed reduced Cd bioavailability in the substrate used in the present study (Table 3), whereas plants sampled from Sousse and Gafsa reduced Sr bioavailability. The clone from Gafsa also significantly reduced Zn bioavailability, while all clones significantly reduced Ni bioavailability. It has to be mentioned that unvegetated soil exhibited a slight increase in Cu bioavailability comparatively to initial soil and that all clones significantly decreased Cu bioavailability recorded at the end of the experiment. Similarly, the clones from Sousse, Sfax and Gafsa decreased Fe bioavailability comparatively to unvegetated control.

Growth Behavior of A. halimus Clones

All plants remained alive and able to grow during the time course of the experiment. The growth behavior of different *A. halimus* clones during the phytoremediation process was first assessed visually, through the morphological aspect of shoots and whole plants (Fig. 2b, c), and quantitatively through measurements of dry biomass, leaf number, stem and root lengths, and plant relative growth rate (RGR) (Fig. 3). Figure 2b and c revealed a higher leaf density in plants from Tunis, Sfax, Sousse, and Nabeul compared to those from Gafsa. A high leaf area was visually observed in plants from Nabeul and Sousse, while Gafsa plants were characterized by a lower leaf area.

The total weight and biomass distribution among plant parts of the five clones are shown in Fig. 3a. Plants from the Sousse clone were the most productive (total biomass: 3.16 g) compared to those from the Gafsa (2.19 g), Sfax (2.19 g), Nabeul (2.21 g), and Tunis (2.06 g) clones. For all clones, roots exhibited the lowest biomass comparatively to other organs. All plant organs had a higher biomass in the clone of Sousse comparatively to other clones, except in the case of Tunis which had similar total leaf biomass than Sousse. Stem biomass was the lowest in Tunis. Leaf numbers were the highest in Tunis and in Sousse, and the lowest in

Table 3 Total and bioavailable heavy metal concentrations in soil before and after plant cultivation

Conditions	Treatments	Cd	Sr	Zn	Cu	Cr	Ni	Fe
Total concentration (mg/kg DW)								
Before	(t = 0)	13.25 ± 0.31ab	1628 ± 5a	341 ± 16a	19.80 ± 0.46a	312 ± 5a	29.45 ± 1.70a	6931 ± 77a
After	No plants	12.96 ± 0.31ab	1613 ± 13a	344 ± 13a	20.88 ± 2.36a	311 ± 2a	29.20 ± 0.34a	6987 ± 48a
	Tunis	13.64 ± 0.70a	1571 ± 11b	335 ± 12a	18.72 ± 0.21a	305 ± 4ab	29.00 ± 1.10a	6801 ± 140a
	Nabeul	13.28 ± 0.06ab	1572 ± 7b	338 ± 9a	19.08 ± 0.24a	306 ± 2ab	28.98 ± 1.12a	6912 ± 31a
	Sousse	11.78 ± 0.91bc	1578 ± 16b	330 ± 8a	18.90 ± 0.14a	302 ± 3ab	27.50 ± 0.44a	6783 ± 91a
	Sfax	11.82 ± 0.56bc	1572 ± 7b	333 ± 5a	20.10 ± 0.46a	302 ± 2ab	26.39 ± 1.01a	6791 ± 139a
	Gafsa	10.34 ± 0.35c	1575 ± 14b	322 ± 8a	19.07 ± 0.20a	296 ± 6b	27.07 ± 0.38a	6747 ± 136a
Bioavailable concentration (mg/kg DW)								
Before	(t = 0)	5.27 ± 0.06a	113.41 ± 2.51a	20.18 ± 0.06ab	0.47 ± 0.00ab	1.09 ± 0.01a	2.81 ± 0.01a	22.85 ± 0.24ab
After	No plants	5.16 ± 0.06ab	107.64 ± 2.95ab	20.68 ± 0.26a	0.57 ± 0.11a	1.09 ± 0.01a	2.50 ± 0.04b	25.96 ± 1.06a
	Tunis	4.96 ± 0.03abc	108.02 ± 1.11ab	18.61 ± 0.57bc	0.40 ± 0.02b	1.07 ± 0.01a	2.26 ± 0.06c	20.80 ± 1.67ab
	Nabeul	5.10 ± 0.06ab	108.09 ± 0.80ab	18.88 ± 0.47abc	0.41 ± 0.01b	1.05 ± 0.01a	2.14 ± 0.00cd	22.95 ± 1.42ab
	Sousse	4.94 ± 0.01bc	105.11 ± 0.70b	18.23 ± 1.21bc	0.37 ± 0.02b	1.08 ± 0.00a	2.02 ± 0.05d	17.57 ± 2.06b
	Sfax	4.93 ± 0.15bc	107.70 ± 0.40ab	18.57 ± 0.38bc	0.41 ± 0.02b	1.11 ± 0.02a	2.12 ± 0.07cd	20.06 ± 1.55b
	Gafsa	4.78 ± 0.17c	101.12 ± 3.87b	17.90 ± 0.64c	0.37 ± 0.04b	1.06 ± 0.04a	2.03 ± 0.13d	17.75 ± 3.02b

Plants from 5 clones of *Atriplex halimus* were cultivated during 90 days on polymetallic-polluted soil of Gafsa. Pots with no plants were used as control. Each value is the mean of 5 replicates. For a given element and a given time, values sharing the same letter are not significantly different at $\alpha = 5\%$ according to Duncan's test

Fig. 2 Experimental design (**a**), shoot (**b**) and whole plant morphology (**c**) after a 90-d phytoremediation trial using clones from various origins of the xero-halophyte *Atriplex halimus*. Plants were cultivated for 90 days in polymetallic contaminated soil and cultivated as stated in Material and Methods. Pots without plants were used as control

Nabeul while Gafsa and Sfax presented intermediate values. Gafsa, Nabeul and Sousse had similar stem length, the highest and lowest value being recorded respectively in Sfax and Tunis. The longest roots were recorded for Gafsa and Sfax while Nabeul, Sousse and Tunis exhibited lower values. The daily biomass yield evaluated through Relative Growth Rate (RGR) was the highest for the clone of Sousse (0.026 d^{-1}), followed by the clone from Tunis (0.023 d^{-1}) and the one from Sfax (0.021 d^{-1}). The clones from Nabeul and Gafsa presented comparable low values (0.017 d^{-1}).

Heavy Metal Concentration in Plants and Phytoremediation Indices

Heavy metals concentrations in leaves, stems, and roots of the different clones of *A. halimus* revealed distinct distributions among organs of the various clones depending on the considered element (Fig. 4). In all clones, leaves exhibited higher concentrations of Cd, Sr, Zn, and Cu compared to stems and roots while roots always presented higher Cr, Ni and Fe concentrations than shoots. The highest leaf concentration of Cd, Sr, Zn and Cu was found in the clone from Gafsa and this was also the case for Cr and Fe root concentrations in roots. Root Cd and Sr concentrations were similar in all clones. Nabeul accumulated low levels of Cd, Sr and Zn in leaves and low concentrations of Cd, Zn and Ni at the stem level. Sfax presented the highest Ni retention at the root level. Neither lead nor arsenic was detected in the plants, whatever the considered organ or clone.

The AE, TF, BCF, and BAC values (Tables 4, 5) were computed to assess the capacity of *A. halimus* plants from various Tunisian clones to absorb, translocate, and accumulate HM in their shoots or roots. Considering BCF and BAC (Table 5), values were determined for each element on the basis of the total element concentration in the soil (upper Table) or on the basis of the bioavailable fraction (lower Table).

The absorption efficiency factor (AE) is a crucial parameter for characterizing plants' ability to extract HM. Our results (Table 4) indicate a higher value for this parameter in plants from the Gafsa clone for almost all analyzed elements. The clone from Tunis presented similar AE values for Sr and Zn than the clone of Gafsa while the clone from Sfax had similar AE than Gafsa for Ni. The root-to-shoot translocation of heavy metals (HMs) was quantified by the translocation factor (TF). Our findings (Table 4) indicate that for the five Tunisian considered clones of *A. halimus*, TF values were greater than one (> 1) for Cd, Sr, Zn, and Cu, indicating that shoots accumulated more of these metals than the roots (Table 5). In contrast, TF values for Cr, Ni, and Fe

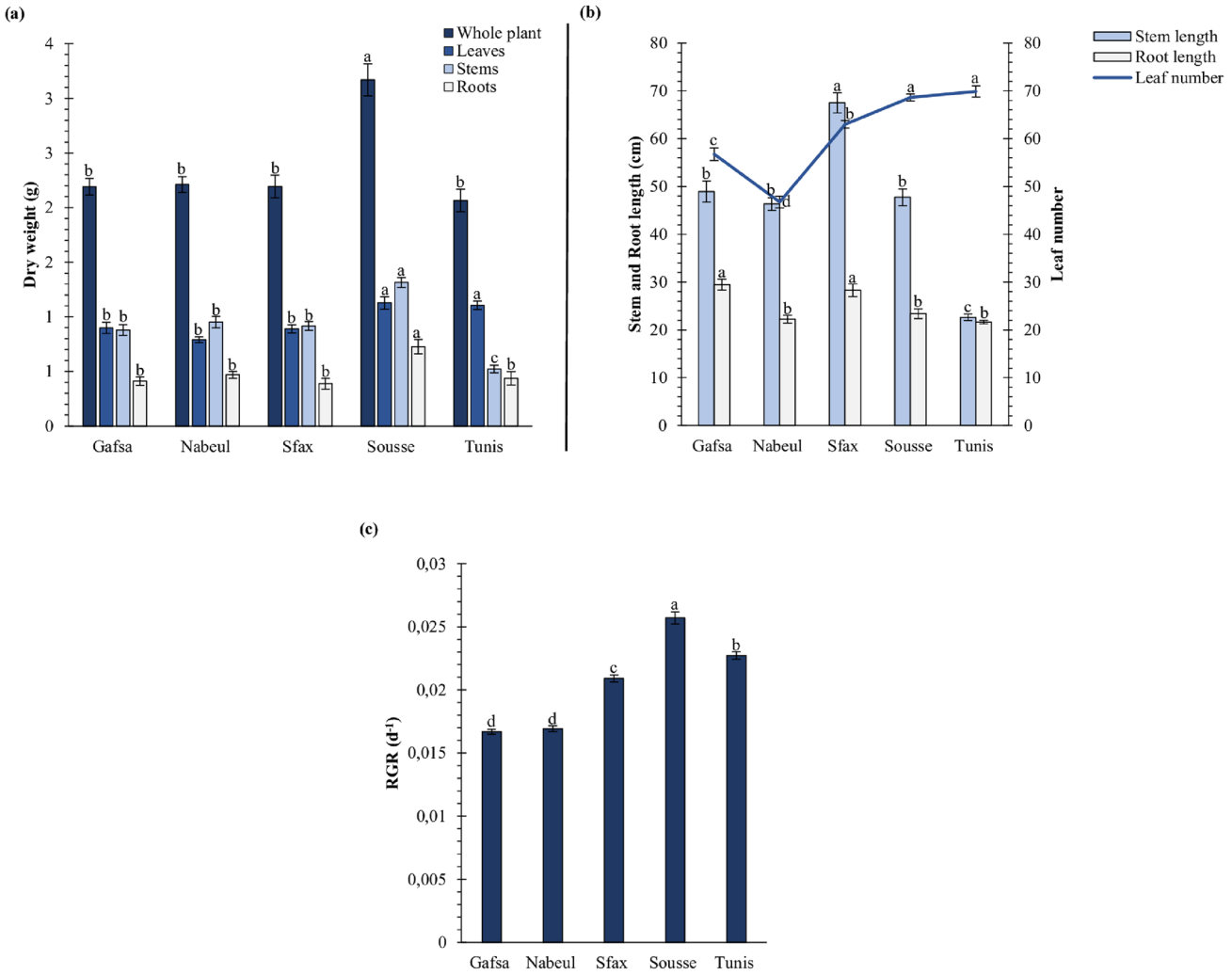

Fig. 3 Dry weight per organ (**a**), leaf number, stem length and root length (**b**) and relative growth rate (RGR) (**c**) of *Atriplex halimus* clones. Plants were cultivated for 90 days in polymetallic contaminated soil. The values are the means of 8 replicates and vertical bars are standard errors (S.E.). For a given organ, bars marked with the same letter are not significantly different at the level of 5% according to the Duncan test

were lower than or equal to one (≤ 1) for all plants, indicating root sequestration process for those elements. Gafsa exhibited higher TF for Cd and Ni than all other clones but no differences could be recorded for Sr translocation factor. Gafsa also exhibited higher TF values than Tunis for Zn, and higher TF values for Cu than Sousse and Sfax. The highest TF value was observed for Cd in the clone of Gafsa and the lowest value was recorded for Ni in the clone of Nabeul.

Bioconcentration factor (BCF) analysis in relation to total metal concentration (upper part of Table 5) revealed that for all metals and clones, the values consistently remained below 1, except for Cd (values higher than 1 for all plants except Nabeul). The same trend was observed for BAC values calculated based on the total HM concentration in the soil, although values higher than 1 were recorded for both Cd and Zn. The lowest values for both BCF and BCA were recorded for Fe, which can be explained by the significant difference between shoot and root Fe concentrations in *A. halimus* clones and the high Fe concentration in the soil. The highest BCF and BAC values were recorded for the clone of Gafsa. The only exception was the highest BCF value recorded for Ni in Sfax. Differences between Gafsa and the other clones were more marked for BAC than for BCF.

When BCF and BCA indices were calculated on the basis of bioavailable HM fraction rather than on the total HM content in the soil (lower part of Table 5), recorded values were always higher than 1. With this expression mode, BCF values for Cd was two-fold higher than for Sr, while the difference was much more marked when total soil HM content was considered. BCF estimated on the bioavailable fraction values were especially high for Cr and Fe. As far as clone from Gafsa was concerned, very high BAC values estimated

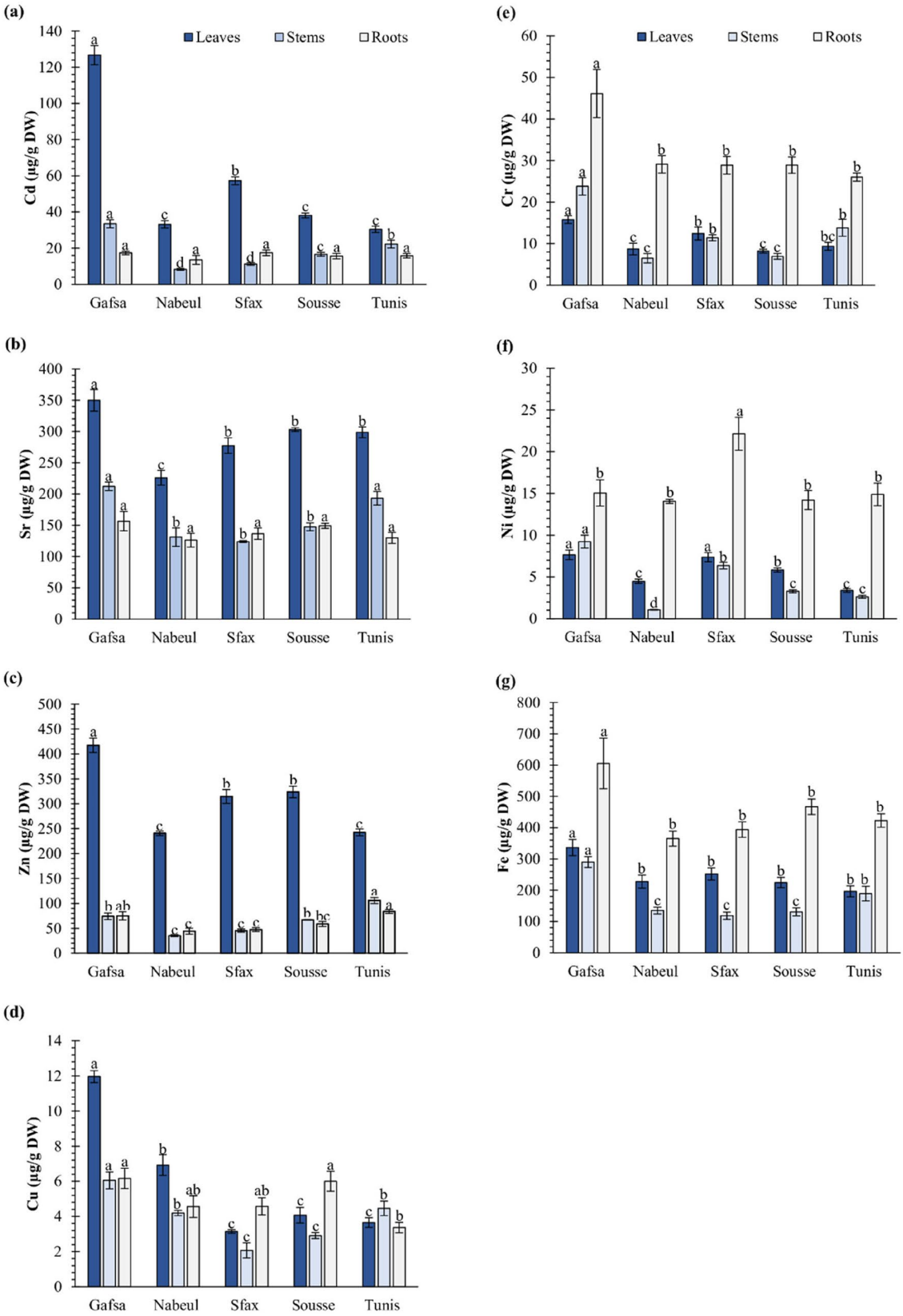

Fig. 4 Distribution of Cd, Sr, Zn, Cu, Cr, Ni, and Fe in different organs of the *Atriplex halimus* clones. Plants were cultivated for 90 days in polymetallic contaminated soil. The values are the means of 5 replicates and vertical bars are S.E. For a given organ, bars marked with the same letter are not significantly different at the level of 5% according to the Duncan test

Table 4 Absorption Efficiency (AE) and Translocation Factor (TF) of *Atriplex halimus* clones for heavy metals

Metal	AE					TF				
	Tunis	Nabeul	Sousse	Sfax	Gafsa	Tunis	Nabeul	Sousse	Sfax	Gafsa
Cd	134 ± 6b	88 ± 2c	99 ± 6c	154 ± 7b	307 ± 21a	3.42 ± 0.30b	3.42 ± 0.51b	3.64 ± 0.36b	4.14 ± 0.62b	9.38 ± 0.81a
Sr	1276 ± 109a	794 ± 62b	830 ± 35b	94 ± 78b	1194 ± 104a	3.89 ± 0.40a	2.93 ± 0.36b	3.03 ± 0.08ab	2.98 ± 0.18b	3.70 ± 0.28ab
Zn	942 ± 76a	534 ± 28c	640 ± 38bc	763 ± 31b	967 ± 77a	4.18 ± 0.16b	6.71 ± 0.91a	6.83 ± 0.55a	7.88 ± 0.89a	6.88 ± 0.81a
Cu	20 ± 2.37bc	25 ± 1.21b	17 ± 1.22c	15 ± 1.04c	39 ± 2.85a	2.43 ± 0.15a	2.71 ± 0.52a	1.19 ± 0.12b	1.21 ± 0.20b	3.04 ± 0.31a
Cr	71 ± 3.78bc	58 ± 6.35c	52 ± 2.78c	78 ± 9.35b	120 ± 6.71a	0.90 ± 0.12a	0.52 ± 0.05b	0.53 ± 0.04b	0.83 ± 0.06a	0.92 ± 0.13a
Ni	28 ± 1.27b	24 ± 0.41b	28 ± 0.96b	50 ± 5.64a	46 ± 1.81a	0.42 ± 0.04b	0.40 ± 0.02b	0.67 ± 0.08b	0.63 ± 0.05b	1.18 ± 0.16a
Fe	1241 ± 42b	1044 ± 87b	1004 ± 61b	1149 ± 117b	1763 ± 110a	0.92 ± 0.09ab	1.00 ± 0.08ab	0.77 ± 0.07b	0.94 ± 0.05ab	1.12 ± 0.16a

Plants issued from 5 clones of *Atriplex halimus* were cultivated during 90 days on polymetallic-polluted soil of Gafsa. Each value is the mean of 5 replicates ± S.E. For a given element, values sharing the same letter are not significantly different at $\alpha = 5\%$ according to the Duncan test

on the bioavailable fraction were reported for Cd, Zn, Cu and Cr. In all cases BAC values of Gafsa were by far higher than BAC values reported for the other clones.

Chlorophyll, Oxidative Stress-Related Parameters, Proline and Phytochelatin Estimation

Chlorophyll and carotenoid quantification (Table 6) indicated that Chl a, carotenoids and total chlorophyll concentration were the lowest in the clone of Sousse and Gafsa. Nabeul always presented the highest pigment concentration. Chl b concentrations were similar in Sousse, Sfax and Gafsa, and were lower than in Tunis and Nabeul.

Roots exhibited higher concentrations of MDA (Fig. 5a) than the other organs, except in the clone Tunis. The highest root MDA content was found in Sfax, followed by Gafsa. In leaves, MDA concentration was the highest in Gafsa and the lowest in Sousse, whereas in stems, plants from Gafsa and Tunis accumulated higher levels of MDA compared to those from Sfax, Sousse, and Nabeul. Proline was mainly accumulated in leaves (Fig. 5b): the highest leaf proline concentration was recorded in Sfax and the lowest one was observed in Sousse, while Gafsa, Nabeul and Tunis showed comparable intermediate values. Stem and root proline concentration hardly differed between the five studied clones.

The total antioxidant activity (Fig. 6) was assessed using the FRAP index, which differentiated between the hydrophilic AOAM (Fig. 6a) and hydrophobic AOAD (Fig. 6d) fractions. The FRAP index estimated on AOAM fraction was higher in leaves compared to other organs, the maximum and the minimum being found in Sousse and Tunis clones, respectively. In stems, the highest AOAM value was also reported for Sousse, whereas the other clones exhibited similar AOAM values. In roots, the lowest AOAM values were reported for Gafsa and Nabeul. The FRAP index was lower for the AOAD fraction (Fig. 6b) than for the AOAM fraction and showed a different pattern among organs depending on the considered clone. The lowest value of AOAD fraction was found in Gafsa, while the maximal values were recorded in leaves for Sfax and in roots for Nabeul and Tunis.

Total glutathione concentration (Fig. 7a) was maximal in leaves of Nabeul and Sfax comparatively to stems and roots, whereas in Sousse, high GSH concentrations were reported for both leaves and stems. The highest root GSH was observed in Gafsa, while Tunis had the lowest leaf GSH comparatively to other clones. The GSH/GSSG ratio was the lowest in Sousse and the highest in Tunis for all organs (Fig. 7b). Root phytochelatin concentration (Fig. 7c) was similar in all clones and stem phytochelatin concentration reached maximal values in Gafsa. Leaf phytochelatin concentration was the highest in Tunis, followed by Gafsa and Nabeul. The lowest leaf and stem phytochelatin concentrations were recorded in Sousse.

Table 5 Bioconcentration factor (BCF) and biological accumulation coefficient (BAC) of *Atriplex halimus* clones for heavy metals

Metal	BCF (*total metal*)					BAC (*total metal*)				
	Tunis	Nabeul	Sousse	Sfax	Gafsa	Tunis	Nabeul	Sousse	Sfax	Gafsa
Cd	1.04 ± 0.09bc	0.83 ± 0.02c	1.28 ± 0.15b	1.66 ± 0.07a	1.76 ± 0.15a	3.93 ± 0.33c	3.13 ± 0.07c	4.91 ± 0.49bc	5.83 ± 0.45b	16.04 ± 0.97a
Sr	0.08 ± 0.01b	0.08 ± 0.01b	0.09 ± 0.01ab	0.09 ± 0.0ab	0.10 ± 0.01a	0.32 ± 0.02b	0.25 ± 0.00c	0.28 ± 0.01c	0.26 ± 0.01c	0.37 ± 0.00a
Zn	0.24 ± 0.03a	0.15 ± 0.02b	0.18 ± 0.03ab	0.15 ± 0.0b	0.25 ± 0.04a	1.07 ± 0.08b	0.83 ± 0.04c	1.22 ± 0.04b	1.14 ± 0.00b	1.48 ± 0.05a
Cu	0.20 ± 0.02b	0.29 ± 0.02ab	0.34 ± 0.05a	0.23 ± 0.03b	0.34 ± 0.04a	0.44 ± 0.07bc	0.55 ± 0.05b	0.37 ± 0.03cd	0.24 ± 0.01d	0.94 ± 0.07a
Cr	0.09 ± 0.004b	0.10 ± 0.01b	0.11 ± 0.003b	0.11 ± 0.00b	0.18 ± 0.015a	0.07 ± 0.007c	0.06 ± 0.01bc	0.05 ± 0.005b	0.08 ± 0.005b	0.13 ± 0.006a
Ni	0.44 ± 0.03b	0.48 ± 0.03b	0.48 ± 0.06b	0.80 ± 0.05a	0.54 ± 0.01b	0.21 ± 0.00d	0.18 ± 0.01d	0.34 ± 0.01c	0.48 ± 0.03b	0.58 ± 0.01a
Fe	0.058 ± 0.00b	0.056 ± 0.00b	0.074 ± 0.0ab	0.059 ± 0.01b	0.090 ± 0.01a	0.056 ± 0.01b	0.057 ± 0.01b	0.053 ± 0.01b	0.051 ± 0.01b	0.097 ± 0.00a
Metal	BCF (*bioavailable metal*)					BAC (*bioavailable metal*)				
	Tunis	Nabeul	Sousse	Sfax	Gafsa	Tunis	Nabeul	Sousse	Sfax	Gafsa
Cd	2.86 ± 0.20b	2.18 ± 0.06c	3.03 ± 0.33b	3.96 ± 0.04a	3.80 ± 0.09a	10.72 ± 0.34c	8.15 ± 0.16d	11.55 ± 0.16c	13.92 ± 0.83b	34.66 ± 1.08a
Sr	1.10 ± 0.08b	1.15 ± 0.07b	1.42 ± 0.08ab	1.32 ± 0.06ab	1.56 ± 0.17a	4.68 ± 0.25b	3.62 ± 0.27c	4.24 ± 0.09bc	3.74 ± 0.18c	5.71 ± 0.28a
Zn	3.34 ± 0.21ab	2.73 ± 0.44b	3.32 ± 0.64ab	2.67 ± 0.12b	4.60 ± 0.85a	19.07 ± 0.25b	14.90 ± 0.13c	22.32 ± 1.72b	20.41 ± 0.49b	26.58 ± 1.30a
Cu	9.17 ± 0.97b	13.40 ± 0.96ab	17.66 ± 3.04ab	11.30 ± 1.80ab	18.53 ± 4.22a	20.63 ± 3.05bc	25.46 ± 2.86b	18.94 ± 1.74bc	12.10 ± 1.31c	48.99 ± 5.61a
Cr	24.82 ± 1.22b	29.50 ± 2.70b	29.55 ± 0.61b	29.28 ± 0.44b	49.81 ± 3.88a	20.75 ± 2.16b	16.88 ± 2.17bc	14.35 ± 1.58c	20.86 ± 1.23b	35.87 ± 2.19a
Ni	5.68 ± 0.45b	6.46 ± 0.16b	6.56 ± 0.79b	9.90 ± 0.70b	7.27 ± 0.56a	2.69 ± 0.04d	2.44 ± 0.11d	4.64 ± 0.22c	6.00 ± 0.35b	7.77 ± 0.35a
Fe	19.29 ± 2.06b	16.83 ± 0.37b	29.59 ± 4.40ab	20.15 ± 2.04b	36.83 ± 8.60a	18.18 ± 0.68b	17.09 ± 1.86b	20.94 ± 4.15b	17.48 ± 2.03b	38.55 ± 5.27a

Plants issued from 5 clones of *Atriplex halimus* were cultivated during 90 days on polymetallic-polluted soil of Gafsa Values were calculated on the basis of total metal concentration (upper part) or on the basis of bioavailable fraction (lower part). Each value is the mean of 5 replicates ± S.E. For a given element, values sharing the same letter are not significantly different at $\alpha = 5\%$ according to the Duncan test

Table 6 Chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll and carotenoids concentrations in leaves of *Atriplex halimus*

Clone	Chl a (mg/g FW)	Chl b (mg/g FW)	Total Chl (mg/g MF)	Carotenoid (mg/g FW)
Tunis	0.44 ± 0.00 b	0.19 ± 0.00 a	0.63 ± 0.01 b	0.14 ± 0.00 b
Nabeul	0.50 ± 0.03 a	0.20 ± 0.02 a	0.70 ± 0.05 a	0.16 ± 0.00 a
Sousse	0.26 ± 0.01 d	0.09 ± 0.01 b	0.34 ± 0.02 d	0.08 ± 0.00 d
Sfax	0.32 ± 0.01 c	0.11 ± 0.01 b	0.44 ± 0.01 c	0.10 ± 0.00 c
Gafsa	0.27 ± 0.01 d	0.09 ± 0.00 b	0.36 ± 0.01 d	0.08 ± 0.00 d

Plants issued from 5 clones of *Atriplex halimus* were cultivated during 90 days on polymetallic-polluted soil of Gafsa. Each value is the mean of 5 replicates ± S.E. For a given element, values sharing the same letter are not significantly different at α = 5% according to the Duncan test

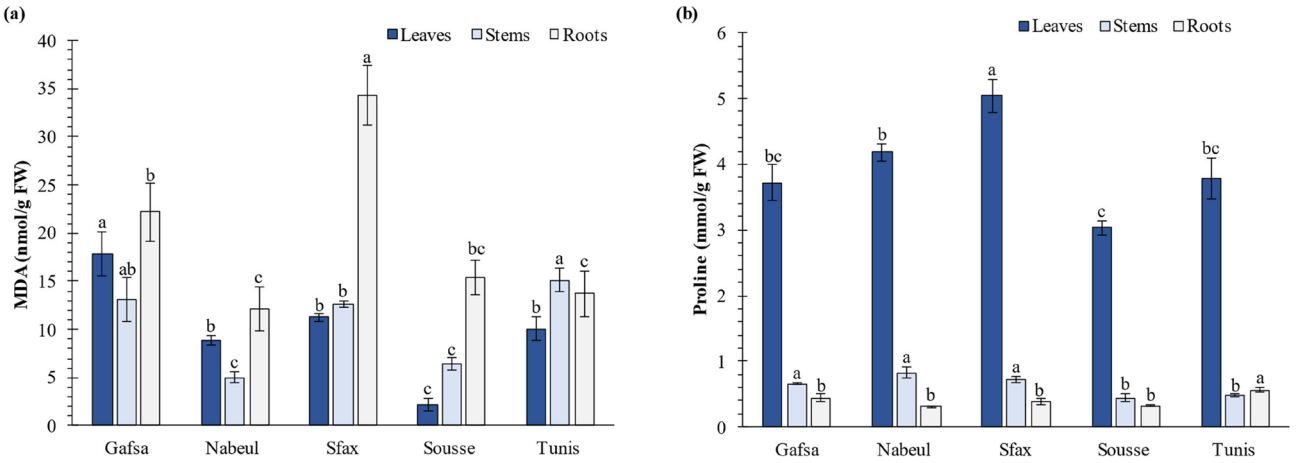

Fig. 5 Malondialdehyde (**a**) and proline (**b**) concentration in the different organs of the *Atriplex halimus* clones. Plants were cultivated for 90 days in polymetallic contaminated soil. values are the means of 4 replicates and vertical bars are S.E. For a given organ, bars sharing the same letter are not significantly different at the level of 5% according to the Duncan test

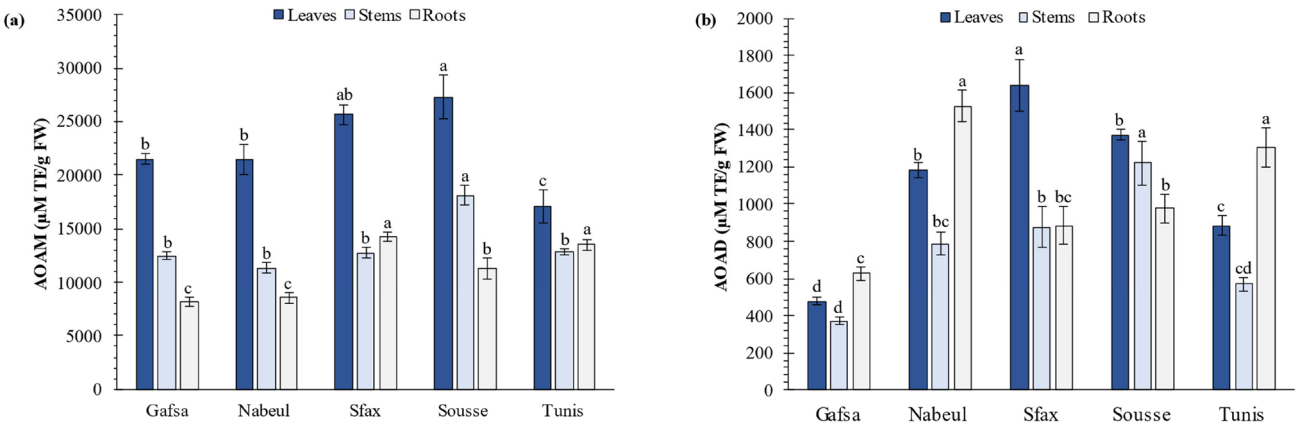

Fig. 6 Total antioxidant activity in the hydrophilic fraction (AOAM; **a**) and the hydrophobic fraction (AOAD; **b**) of different organs of the *Atriplex halimus* clones. Plants were cultivated for 90 days in polymetallic contaminated soil. Values are the means of 4 replicates and vertical bars are S.E. For a given organ, bars sharing the same letter are not significantly different at the level of 5% according to the Duncan test

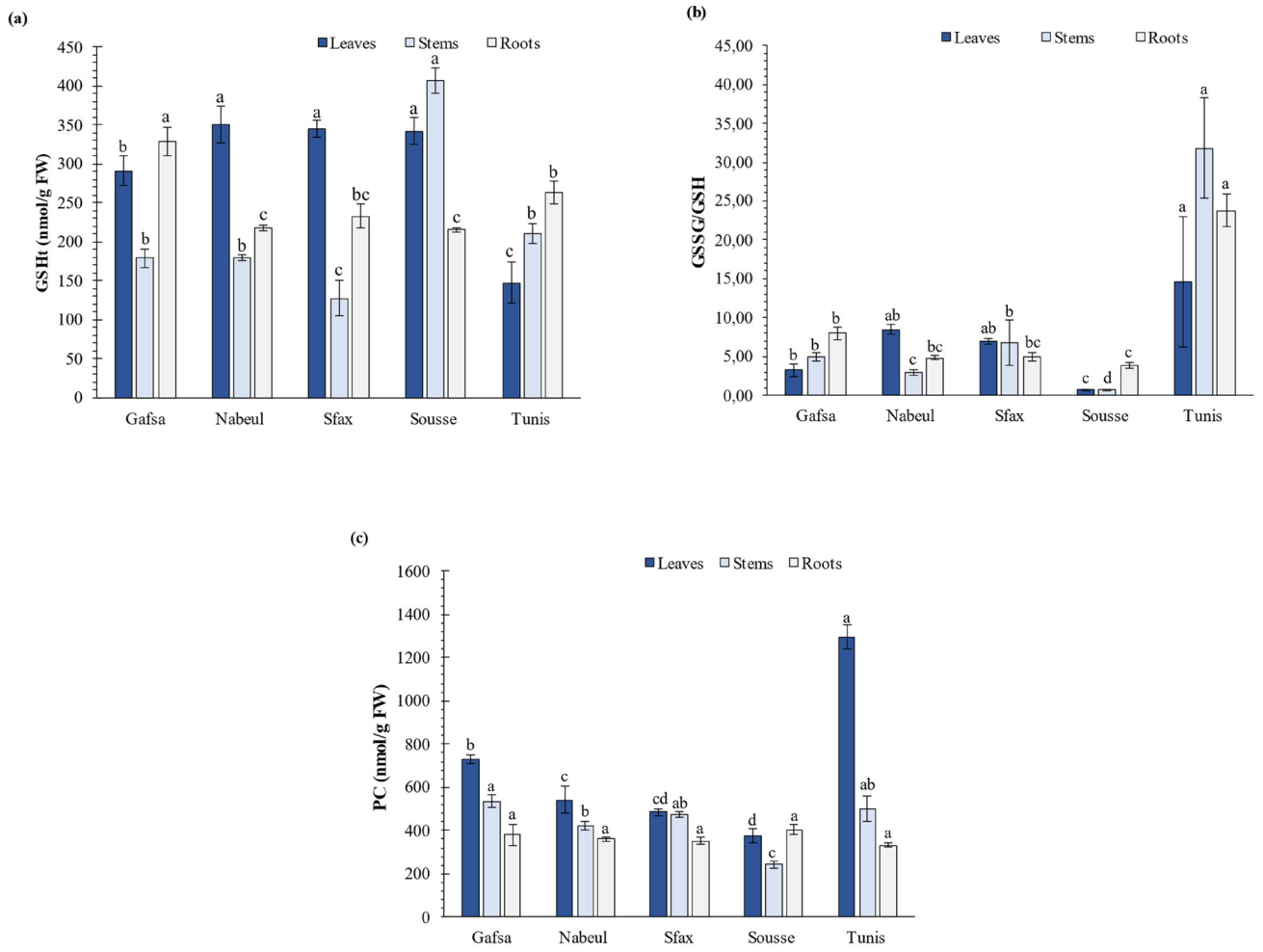

Fig. 7 Total glutathione (GSHt; **a**), oxidized (GSSG)/reduced (GSH) glutathione (**b**), and phytochelatine (PC; **c**) concentrations of the different organs of the *Atriplex halimus* clones. Plants were cultivated for 90 days in polymetallic contaminated soil. Values are the means of 4 replicates. For a given organ, bars marked with the same letter are not significantly different at the level of 5% according to the Duncan te

Discussion

Atriplex halimus has been recommended as a promising plant species for phytoremediation of polluted areas in semi-arid and arid conditions (Manousaki and Kalogerakis 2009; Lefèvre et al. 2009; Tapia et al. 2013; Orrego et al. 2020; Shahrokh et al. 2022). However, transferring the knowledge gained on this species from laboratory to field environments is particularly challenging due to the high level of intraspecific genetic variability characterizing *A. halimus* (Haddioui and Baaziz 2001; Ortíz-Dorda et al. 2005; Walker et al. 2005, 2014). According to Walker et al. (2014), variability within populations may even be sometimes higher than between populations. As an attempt to select a more uniform material that would be useful for field applications, we chose to compare five clones, each sampled from a specific site in Tunisia. Our data revealed that these clones strongly differed for their morphological and physiological properties but that, as expected, the phenotypic variability within clones was rather low and related to small environmental variability occurring under or semi-controlled environment, thereby confirming that the cuttings procedure was efficient. So, one may reasonably hypothesize that morphological differences between investigated clones may be, at least partly, related to the site of origin: this was especially the case for the clone from Gafsa, native to the most arid environment which exhibited longer roots (likely for more efficient water and nutrient uptake) and narrow leaves (to limit water losses by transpiration), which are common features of drought-tolerant plants (Dietz et al. 2021). However, since only of single plant per population was considered for cloning purposes, any attempt of generalization should be considered with caution.

The different sites exhibited different levels of contamination (Table 2): three sites were unpolluted sites, while the site of Sousse was slightly polluted with low doses of Pb only, and the site of Gafsa was strongly polluted with a typical polymetallic contamination, as frequently recorded in mining areas (Lindahl 2014; He et al. 2015; Karn et al. 2021; Zerizghi et al. 2022). Toxic elements such as Cd, Sr and Cr are present at high concentrations in Gafsa and high levels of Zn were also recorded. Strontium pollution is not frequently recorded in former industrial sites but has been found in some mining areas (Sasmaz and Sasmaz 2009, 2017; Sasmaz et al. 2021). This element is present in felsic magmatite and carbonate rocks and soluble strontianite ($SrCO_3$) may precipitate as celestite ($SrSO_4$) (Burger and Lichtsheidl 2019). The impact of Sr on plant physiology is still poorly documented. In our study, Sr concentration in Gafsa soil was as high as 1300 ppm and reached 776 ppm in Nabeul. Iron was also reported at high concentration but under oxidized conditions iron remains as Fe^{3+} and is not toxic for plants which possess efficient strategies to maintain iron homeostasis. Iron toxicity only occurs under flooded conditions when reducing environment leads to high concentration of fully available Fe^{2+} ions (Dufey et al. 2015).

Heavy metal bioavailability is an important and complex property determining the capacity of plants to thrive in polluted areas (Pérez-Esteban et al. 2013). In relative terms, Cd was much more bioavailable than Sr in Gafsa soil (40% versus 7%) but in absolute terms, considering the initial amount of each element in Gafsa soil, plants were still exposed to Sr concentrations 20 times higher than Cd concentrations (Table 3). At the opposite side, Pb bioavailability was close to zero; although Pb total concentration in Gafsa soil was rather low, it was higher in soil from Sfax but still remains unavailable (data not shown). It is well established that Pb is poorly available for plants, even at high external concentrations in the soil (Abdu et al. 2017; Aziz and Mujeeb 2022). However, when lead was applied in a free available form in nutrient solution, *A. halimus* was able to accumulate Pb and displayed efficient strategies of resistance as evidenced by Bankaji et al. (2019).

Bioavailability depends on numerous properties such soil mineral composition in relation to the presence of reacting surfaces and external or internal binding sites exhibiting different binding energy towards the considered elements (Bhat et al. 2022; He et al. 2015). Bioavailability is also strongly influenced by soil pH and heavy metal speciation (Karn et al. 2021; Mudgal et al. 2023). In some cases, root exudates and mucilages may prevent HM absorption (Morel et al. 1986; Javed et al. 2013; Montiel-Rozas et al. 2016). The fact that *A. halimus* reduced heavy metal bioavailabilty after 90 days may appear as a consequence of HM absorption by the plants during the time course of our experiment. However, it does not exclude that root physiological activity may influence bioavailability through biogeochemical processes such as pH modification, changes in redox conditions or exudation of organic substances like carboxylic acid, mucilages, phytosiderophore or non-proteic amino acids (Lambrechts et al. 2011; Lee et al. 2023; Shen et al. 2022; Lutts et al. 2024). However, the fact that our tested clones had contrasting effects on various elements bioavailability while plants were cultivated on the same soil suggests that their root physiological activity may differ between clones. It has also to be emphasized that element bioavailability slightly varied in unvegetated pots and this could be due to the fact that tap water containing low concentration of cations and chloride was used for plant irrigation rather than deionized water.

It is noteworthy that the clone of Gafsa accumulated higher concentrations of HM than other clones grown on the polluted soil. Despite accumulation of Cd, Sr, Zn and Cu at high levels in leaves, plants from Gafsa remained alive and did not exhibit toxicity symptoms such as yellowish, necrosis or senescence. This supports the hypothesis that this clone is tolerant to HM in that sense that it is able to cope with high amounts of toxic elements within photosynthetic tissues. Although the clone from Gafsa reduced Cd and Sr bioavailability in the soil, this did not preclude accumulation of these HM in the plant shoots. Hence, one hypothesis could be that natural selection acting in the Gafsa population selected includer shrubs rather than excluder individuals restricting HM uptake. The clone from Gafsa, however, exhibited a low RGR value, which could be explained by the fact that tolerance mechanisms have an energetic cost, so that metabolic energy used for this purpose could not be affected to biomass production (Feki et al. 2021; Thakur et al. 2022). Beside the presence of HM, plants from Gafsa have to thrive under very dry environment, high light intensities and high temperatures and are therefore exposed to multiple stresses which could explain constitutive low relative growth rates. In that sense, the recorded decrease in heavy metal bioavailability at the root/soil interface might be regarded as a successful attempt to adapt heavy metal uptake to the slow growth rate considering that an excess of toxic elements could not be diluted in a high volume of biomass.

High concentrations of HM such as Cd, Sr, Zn or Cu in leaves of most tested clones (Fig. 4) however remain puzzling. Indeed, with the noticeable exception of hyperaccumulating species, plants growing on polluted soils usually sequester toxic ions in the root system, preventing their translocation to the sensitive photosynthetic tissues (Riyazuddin et al. 2022; Noor et al. 2022). This was also documented in intact seedlings of *A. halimus* growing on Cd- or Zn-containing nutrient solution (Lutts et al. 2004; Lefèvre et al. 2009) and for adult plants spontaneously colonizing highly polluted mine tailings (Acosta et al. 2018), although Shahrokh et al. (2022) recently reported that specific

amendments may, to some extent, improve root-to-shoot heavy metal translocation in *A. halimus*. An opposite behavior was found in the present case and only Ni, Cr and Fe were sequestered in the roots of young cuttings (Fig. 4). In the halophyte *Sesuvium portulacastrum*, Mnasri et al. (2015) demonstrated that Cd and Ni translocations from the root to the shoot rely on similar mechanisms while those elements displayed contrasting distribution in our tested clones. This prompted us to hypothesize that adventitious roots developed in our young cuttings were not yet fully functional in terms of root retention as suggested by TF > 1 for several elements. Moreover, since plants were fully irrigated, stomatal opening in the absence of water stress may have contributed to the maintenance of high transpiration which, in turn, contributed to root-to-shoot HM translocation.

Root maturation issue has long been recognized as a serious impediment for clonal production of softwood species through cuttings. Rasmussen et al. (2009) demonstrated that vascular tissue development requires time to reach full maturation. More recently, Baba et al. (2018) reported heterorhizy in roots issued from softwood cuttings, with appearance of several types of roots (monarch, diarch, triach,…) exhibiting distinct morphological, anatomical and functional features. According to Lu et al. (2023), numerous steps are required to reach a full mature root stage and are highly dependent on environmental conditions in relation to phytohormonal profile. Hence, the hypothesis that the studied clone would present a different HM distribution when reaching maturity as a consequence of the root system development could not be ruled out. Nevertheless, all the tested clones remained alive and able to grow during the 90 days trial on polluted soil; this suggests i) that vascular tissues were operational for water transfer, even if selectivity for ions at the xylem loading sites is still incomplete and ii) that *A. halimus* (and especially the Gafsa clone) may trigger efficient mechanisms of heavy metal tolerance at the leaf level.

Accumulated HM commonly induce oxidative stress: generation of oxygen reactive species such as superoxide free radicals (O_2^{-}), hydroxyl radicals ($OH^{\cdot}$) or non-free radical species such as singlet oxygen ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2) is a major threat for stressed plants leading to cellular disorders and metabolic disturbances (Feki et al. 2021; Riyazuddin et al. 2022). Heavy metal tolerant plants can cope with oxidative stress through the coordinated involvement of enzymatic and non-enzymatic antioxidants (Noor et al. 2022; Shen et al. 2022). However, as far as Gafsa clone is concerned, antioxidant mechanisms did not appear as the main strategy for HM tolerance: this clone indeed exhibited the highest leaf MDA concentration suggesting that oxidative stress did occur (Fig. 5a). It also exhibited the lowest AOAD fraction comparatively to other clones while its AOAM fraction was lower than the value recorded for Sousse which accumulated lower amounts of heavy metals (Fig. 6). Besides its role as osmotic compatible solute, proline is also acting as a powerful antioxidant (Ghosh et al. 2022; Alvarez et al. 2022; Spormann et al. 2023) but, once again, proline accumulation in the leaves of Gafsa was close to values recorded for the non-accumulating clone from Nabeul and Tunis (Fig. 5b). The same observation was valid for GSH which was lower in Gafa than in Nabeul (Fig. 7a). Even if root GSH concentration was higher in Gafsa, roots were not the major site of heavy metal accumulation in this clone.

Glutathione is a precursor of phytochelatins (PC): these non-protein thiol compounds may bind to HM (mainly Cd, Cu and Pb) and sequester HM complexes in vacuole to avoid their deleterious impacts on cytoplasm metabolism (Thakur et al. 2022). In the present study, PC accumulated only in the clone from Tunis and to a higher extent in the leaves than in the roots while this clone accumulated quite lower amounts of HM than Gafsa. Phytochelatins may afford transient protection against HM in non-resistant plant species but their synthesis relies on sulfate reduction which is a highly expensive process from a metabolic point of view and PC synthesis is therefore not considered as an optimal strategy for plants permanently exposed to HM (Riyazuddin et al. 2022). Beside sequestration in the vacuole, HM may also bind to cell wall polymers, thus preventing heavy metal entrance in the symplasm. Binding mainly occurs on polymers containing negative charges and rich in galacturonic and glucuronic acids and the density of negative charge is partly controlled by pectine methylesterase activity (Noor et al. 2022; Riyazuddin et al. 2022). Cell wall binding of HM is frequently considered as an efficient first line of defense in heavy metal resistant plants (Shen et al. 2022). Leaves of numerous halophyte plant species, especially those belonging to the genus *Atriplex*, are covered by a high density of trichomes which also constitute putative site of accumulation for HM (Manousaki and Kalogerakis 2009; Lutts and Lefèvre 2015; Aziz and Mujeeb 2022). Although epidermal bladder cells and salt glands are mainly expected to accumulate Na and Cl in the presence of salt, the presence of Cd and Zn has been reported for mangrove halophyte species (Lutts and Lefèvre 2015) and HM excretion at the leaf surface might be regarded as a strategy explaining high amounts of HM at the whole leaf organ without mesophyll metabolism disruptions.

The putative interest to implement a phytoremediation strategy on a polluted area depends on the BCF and BAC factors (Bhat et al. 2022; Mudgal et al. 2023; Saxena et al. 2020). Although the reported values remained low when estimated on the total metal concentration in the soil (Table 5), both BCF and BAC presented quite high values when estimated on the bioavailable fraction. Keeping in mind that heavy metal bioavailability is a dynamic property and that soil desorption processes will replenish soil solution

after plant absorption of elements (Lambrechts et al. 2011), the use of *A. halimus* for phytoremediation appears as an attractive option. This is especially the case for the clone of Gafsa which always exhibited the highest BCF and BAC values. On another hand, AE and TF are also important parameters to consider for phytostabilization or phytoextraction projects (Mnasri et al. 2015; Yang et al. 2022; Sharma et al. 2023). Again, the clone from Gafsa exhibited the highest AE and TF values and should therefore be retained as a promising material. However, the above-mentioned phytoremediation indices (except AE) were based on HM concentrations and not on HM quantities, while the final purpose of phytoremediation is to maximize the amount of metal extracted from the polluted substrate. This implies that plant growth and biomass production need also to be considered. In this regard, the low RGR value exhibited by the clone from Gafsa might be disadvantageous. It is tempting to speculate that the low pigment concentrations recorded in Gafsa (Table 6) might be regarded as a consequence of HM accumulation, and that it could hamper photosynthesis, thus explaining the low RGR value recorded for this clone. However, this explanation is no more valid if we consider that Sousse had similar pigment concentration as Gafsa while it exhibited the highest RGR and the lowest leaf HM concentration.

When calculating the total amount of Sr and Zn removed by shoots, the clone from Sousse was in fact more efficient than the one from Gafsa (Sr: 566 mg in Sousse *versus* 490 mg in Gafa; Zn: 481 mg in Sousse *versus* 423 mg in Gafsa). This was also true at the root level for Sr (125 mg in Sousse *versus* 75 mg in Gafsa). Adequate combination of different clones exhibiting complementary properties could therefore be considered to improve the efficiency of phytoremediation using *A. halimus* clones.

Conclusion

Atriplex halimus is a promising species for HM phytoextraction from a polymetallic mining soil. The species however displays a high level of genetic variability and selection of a uniform material through cutting is thus require to improve homogeneity and phytoextraction efficiency. However, cuttings and young plants often constitute sensitive stage to environmental constraints. The present work demonstrates for the first time that cuttings obtained from plants growing in five polluted and non-polluted areas from Tunisia were able to survive and to grow on a soil containing high doses of Cd, Sr, Zn, Cu, Cr and Ni. The most tolerant clone was the clone obtained from the most polluted area (Gafsa) and it accumulated higher amounts of HM than other clones. It however encountered oxidative stress and a slight decrease in growth. Combination with other clones exhibiting lower shoot HM concentration but higher growth rates may thus be required to optimize the phytoextraction process.

Additional trials are now required to test this material in real field conditions which integrate all the component of the environment, including the presence of co-occurring stress factors and the use of fully mature plants. In concomitance, laboratory multidisciplinary approaches are needed to precisely identify the physiological and molecular basis of heavy metal tolerance in the clone of Gafsa.

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Data availability Data sets analysed during the study are available from authors upon personal request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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