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Physiological Responses and Adaptations of the Halophyte *Atriplex halimus* to Soil Contaminated with Cd, Ni, and NaCl

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Abstract: Soils contaminated with potentially toxic elements (PTEs) and salt manifest a large number of physical, chemical, and structural problems by various processes such as reduced water availability, water and air movement in soil space, water holding capacity of soil, as well as perilous effects on plant growth and physiology. Halophytes have the ability to grow in saline environments and are better adapted to accommodate environmental constraints including PTE ions. An experiment was designed to study the response of the halophyte *Atriplex halimus* to a range of salinities and different concentrations of Cd and Ni. Tolerance and soil remedial potential of the plant were quantified in terms of PTE uptake and portioning, plant biomass, root/shoot ratio, chlorophyll and anti-oxidative enzyme production, along with stress markers such as lipid peroxidation, proline, and glycine betaine. The plant was also evaluated for its potential to phytoremediate PTE contaminated soil. The results suggest that *A. halimus* can tolerate moderate concentrations of both the PTEs and salt. The species holds promise for bio-reclamation of saline and PTE-contaminated soil.

Keywords: halophytes; saline soil; phytoremediation; phytoextraction; osmoprotectants; ROS; antioxidants

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1. Introduction

Soil contamination by potentially toxic elements (PTEs) such as Pb, Cd, Hg, Zn, and Cu has been receiving increased attention worldwide in the past few decades. Their persistent nature is a severe threat to the environment, and affects the lives of plants, animals, and humans. Once they enter the body, they accumulate in vital organs for years causing severe health problems [1]. Effects of PTE poisoning can be toxic, carcinogenic, mutagenic, or teratogenic [2]. Unlike other contaminants, PTEs cannot be degraded by chemical or biological treatments, are eventually indestructible, and their toxic effects last longer.

Soil salinity is another major issue that adversely affects agricultural productivity and sustainability specially in arid and semi-arid areas. Like PTE pollution, salinity problems also result from both natural and mismanaged anthropogenic activities. Salinity and PTE toxicity in soil impact a considerable portion of agricultural areas globally by decreasing the growth and thus the yield of plants by affecting their metabolic activities such as respiration, carbon-dioxide assimilation, chlorophyll biosynthesis, cell membrane elasticity and permeability, water uptake, protein synthesis, reduction in root elongation and reduced stomatal openings with turgor loss, and inhibition of anti-oxidative enzyme activities.

Various clean-up technologies have been developed and used to remediate contaminated soils. Phytoremediation is counted among the safer, cleaner, cost-effective, and

environment-friendly technologies. It uses numerous biological organisms viz. bacteria, cyanobacteria, yeast, fungi, algae, and higher plants as tools for the restoration/remediation of soil and water contaminated within organic or organic contaminants. Ideal phytoremedial plants should exhibit a deep root system, high shoot biomass production, and perennial growing habit as well as be able to cope with low fertility and poor soil structure [3]. Most plants explored for phytoremediation are glycophytes, but these cannot survive in saline soils polluted by PTEs such as in semi-arid areas. Halophytes, however, are a group of plants that can tolerate saline soils and can even complete their life cycles in such conditions. They have evolved a range of morphological, physiological, and biochemical strategies to proliferate in high-salt environments [4] and may be ideal for phytoremedial applications in such cases. In fact, low to moderate levels of NaCl are believed to stimulate plant growth in halophytes and play an important role in protection against PTE toxicity by mediating ion absorption, osmotic adjustment, and induction of antioxidant systems [5,6].

Cd and Ni are among PTEs of great concern to environment and human health [7]. Cd is one of the most phytotoxic PTEs even at low concentrations causing impaired absorption of nutrients [8] and photosynthetic and growth inhibition [9,10]. Ni is a micronutrient, [11] but at higher concentrations it may have deleterious effects similar to Cd and Pb [12]. PTEs also increase the production of reactive oxygen species (ROS) in plant cells, which can cause oxidative damages such as the peroxidation of membrane lipids and carbonylation of proteins. Plants control such damage by protective ROS trapping systems including antioxidant enzymes such as superoxide dismutase (SOD), guaiacol peroxidase (GPX), and catalase (CAT), which control ROS levels [7,10,12].

Studies with halophytes have shown their tolerance to high concentrations of toxic PTE ions which most plants do not survive, in particular, under saline conditions [10,13]. To a certain extent, tolerance to salt and PTEs relies on common physiochemical mechanisms [3,14,15], and therefore, halophytes are being explored as candidates for phytomanagement of PTE-contaminated soils. Recent studies have demonstrated that halophytes such as *Mesembryanthemum crystallinum*, *Salicornia brachiata*, and *Atriplex nummularia* accumulate large amounts of Cd, Ni, and Pb [12,16–18].

Atriplex species are a very interesting group of halophytes that can survive and complete their life cycle at high salinity levels. They are equipped with salt bladders which accumulate salt. These glandular structures are not specific to Na and Cl but also accumulate and excrete PTEs such as Cd, Zn, and Pb on the leaf surface as a possible metal detoxification [19] mechanism as in *Atriplex halimus* and *Tamarix smyrnensis* [5,20]. They also have numerous layers of specialized balloon-like, vesiculated hairs called trichomes on the outer surface of the leaf [21]. These differ from salt glands and perform a range of different functions such as storage of water and its absorption from the atmosphere along with salt secretion. These plants show high potential to tolerate and concentrate PTE in their tissues by triggering detoxification mechanisms [12,22].

The present study aims to evaluate the response of *Atriplex halimus* to a range of salinities and concentrations of Cd and Ni in order to assess its potential to remediate soil contaminated with these two metals. Partitioning of the metals between the root and shoot, biochemical parameters that indicate stress, as well as enzymatic antioxidant activity will also be evaluated to obtain a comprehensive picture of Cd and Ni tolerance mechanism in the plant.

2. Materials and Methods

2.1. Plant Material and Soil

Seeds of *Atriplex halimus* (commercially available and certified) were procured from the United States Department of Agriculture–Agriculture Research Service (USDA-ARS), Washington State University, USA. These were allowed to germinate in the dark after sterilization in 0.1% $HgCl_2$ and soaking for 3 h in double distilled water. The experimental soil was collected from the botanical garden of St. John's College, Agra. The topsoil of the area is a sandy loam made of 60–80% sand, 10–24% silt, and 8–16% clay.

The soil has moderate water retaining capacity with pH 7.18 ± 0.51, electrical conductivity (EC) 1.04 ± 0.23 dSm^{-1}, moisture content 8.32 ± 0.59%. The organic carbon content was 1.10 ± 0.1%, available P_2O_5 8.7 ± 0.3 mg kg^{-1}, available nitrogen 143.14 ± 1.8 mg kg^{-1}, and available K_2O 271 ± 7.1 mg kg^{-1}. Sodium (Na), calcium–magnesium (Ca + Mg), and potassium (K) content of the soil was 730 ± 5, 4.4 ± 0.5, and 23 ± 4 meq L^{-1}, respectively. Non-perforated plastic pots were used for the experiment to avoid leaching of salt and PTEs, each pot was filled with 4 kg autoclaved and air-dried soil.

2.2. Experimental Design

A greenhouse pot experiment was set up in the botanical garden at St. John's College, Agra (27.18° N 78.02° E), India. The salinization of pot soil (4 kg) was carried out by adding 100 mL kg^{-1} of 1, 3, or 5% NaCl water solution to pots as per treatment plan. These were left for 15 days to stabilize. The temperature was maintained at 27–30 °C. Germinated seedlings were transplanted (one per pot) in pots having 4 kg soil. Plants were treated with different doses of cadmium (Cd) and nickel (Ni) as aqueous solutions using $CdCl_2 \cdot 5H_2O$ and $NiSO_4 \cdot 6H_2O$, respectively. The treatments selected were as follows: T0 (control [Cd0/Ni0/NaCl0]); T1 (1% NaCl); T2 (3% NaCl); T3 (5% NaCl); T4 (Cd 25 mg kg^{-1}); T5 (Cd 50 mg kg^{-1}); T6 (Cd 100 mg kg^{-1}); T7 (Cd 25 mg kg^{-1} + 1% NaCl); T8 (Cd 25 mg kg^{-1} + 3% NaCl); T9 (Cd 25 mg kg^{-1} + 5% NaCl); T10 (Ni 50 mg kg^{-1}); T11 (Ni 100 mg kg^{-1}); T12 (Ni 200 mg kg^{-1}); T13 (Ni 50 mg kg^{-1} + 1% NaCl); T14 (Ni 50 mg kg^{-1} + 3% NaCl); T15 (Ni 50 mg kg^{-1} + 5% NaCl). Three replicates of each treatment were maintained in a complete randomized block experimental design. The pots were watered with distilled water as and when required to minimize leaching of NaCl and/or PTEs. Each pot had a plastic tray under it. Any leachate collected was added to the pot at next watering. At the end of the study, i.e., 120 DAT (days after treatment), each plant was uprooted and washed in deionized water and divided into root and shoot parts and analyzed for metal content. Root length, shoot length, and dry biomass were also noted. The soil was tested to analyze the electrical conductivity (EC), sodium adsorption ratio (SAR), and residual metal content. In addition, leaves were plucked for testing chlorophyll, proline content, lipid peroxidation, glycine betaine, relative electrolyte leakage, and antioxidative enzyme activity of plants at 30, 60, and 120 DAT to monitor the adaptation and health of the test plants through the study period.

2.3. Growth and Physiochemical Analysis

2.3.1. Chlorophyll

Chlorophyll estimation was performed using acetone (80% v/v) [23]. Crushed fresh leaves (0.5 g) were dissolved in 10 mL acetone and incubated at 4 °C for 12 h in dark. The supernatant was collected after centrifugation (for 5 min at 4000 rpm and 4 °C) and its optical density was measured at two wavelengths (663, 645 nm) using a UV–Vis spectrophotometer (Perkin Elmer Lambda 25). The concentration of total chlorophyll was expressed in mg g^{-1} fresh weight.

2.3.2. Membrane Damage Rate (MDR)

Relative electrolytic leakage was estimated to determine the membrane damage rate [24]. One gram of fresh leaf tissue was suspended in 10 mL distilled water in a test tube which was then incubated at room temperature for 24 h. After 24 h, the initial electrical conductivity (EC1) was measured. The solution was then autoclaved at 121 °C for 15 min in order to release ions from the tissue and allowed to cool down before the final electrical conductivity (EC2) was measured.

$$REL = (EC1/EC2) \times 100.$$

2.3.3. Lipid Peroxidation

Lipid peroxidation was estimated from 1 g fresh leaf tissue and expressed as malondialdehyde (MDA) content [25]. A total of 5 mL of 10% trichloroacetic acid (TCA) was used to prepare a leaf extract crushed in liquid nitrogen. This was followed by centrifugation at 10,000 rpm and 4 °C for 10 min. An amount of 2 mL of supernatant was taken in a fresh test tube to which 2 mL of 0.67% thiobarbituric acid (TBA) was added. The test tube was placed in a water bath at 100 °C, followed by incubation in an ice bath to stop the reaction. MDA content (μg g^{-1} FW) was measured at 532 nm absorbance by using 0.025% TBA in 10% TCA as control.

2.3.4. Proline

The estimation of proline content as a stress marker was conducted according to Bates et al. [26]. Three percent sulfosalicylic acid (10 mL) was used to prepare an extract from fresh leaves (500 mg) followed by centrifugation at 2000 rpm for 15–20 min to separate the supernatant. A reaction mixture was prepared by adding 2 mL of ninhydrin and glacial acetic acid each to a test tube containing 2 mL of supernatant. This was then placed in a water bath for 30 min. The reaction was stopped by placing the test tube in an ice bath. After this, 4 mL toluene was added, followed by vigorous shaking to get a red chromophore (toluene layer) in the upper part. The optical density of the chromophore layer was measured at 520 nm. Toluene was used as a control (reference blank). Proline content was expressed in μmol g^{-1} FW and calculated with a standard curve of proline.

2.3.5. Glycine Betaine

Glycine betaine analysis was performed as per Grieve and Grattan [27]. A total of 500 mg fresh leaves were powdered in liquid nitrogen and suspended in deionized water (20 mL). This was followed by mechanical shaking at 25 °C for 16 h. The sample was strained. An amount of 500 μL filtrate and 2 N sulfuric acid (1:1) were mixed and cooled in an ice bath for 1 h. After this 200 μL of I_2-KI reagent (20% potassium iodide and 15.7% iodine) was added to the cooled samples and kept for incubation at 4 °C for 16 h. The samples were centrifuged for 15 min (at 10,000 rpm and 0 °C). In total, 9 mL of 1, 2-dichloroethane was used to dissolve the crystals (periodide). After 2 h, absorbance (365 nm) of the samples were measured. Glycine betaine content of the samples was expressed in g^{-1} fw.

2.3.6. Antioxidative Enzyme Assay

Fresh leaf samples were obtained for the analysis of antioxidative enzyme activity. Leaf extract was prepared in 10 mL of 50 mM phosphate buffer (pH 7.0) and 0.5 mM EDTA using 1 g crushed leaf sample (in liquid nitrogen). For ascorbate peroxidase (APX) analysis, 1 mM ascorbic acid was added in the extract at the time of crushing. The extract was filtered with cheesecloth (4 layered) and centrifuged at 16,000 rpm for 20 min at 4 °C. Supernatant was first used to analyze protein content of the extract and then processed further to measure the antioxidative enzymes of the plant. Bradford assay was used for protein estimation with bovine serum albumin (BSA) as standard.

Catalase (EC 1.11.1.6) activity was estimated according to Aebi [28]. An amount of 0.1 M phosphate buffer (pH adjusted to 7) was prepared and used to make reaction mixture with 50 μL enzyme extract and 0.5 mL 75 mM H_2O_2. Distilled water was used to make up the final volume to 3 mL. Decrease in absorbance (decomposed amount of H_2O_2) was calculated at 240 nm and used to measure the catalase activity with the help of a standard curve of H_2O_2.

The Nakano and Asada [29] method was used for ascorbate peroxidase (APX) estimation. Leaf extract (0.1 mL) + 1.5 mL phosphate buffer (0.1 M) + 0.5 mL ascorbic acid (3 mM) + 0.1 mL H_2O_2 (3 mM) + 0.8 mL distilled water (to make final volume 3 mL) was used to prepare a reaction mixture. The decrease in absorbance (290 nm) was measured to calculate the amount of ascorbic acid (μmol) oxidized min^{-1} mg $protein^{-1}$.

For superoxide dismutase (SOD) (EC 1.15.1.1), 560 nm wavelength was used to record the absorbance of the reaction mixture containing 0.2 mL 200 mM methionine, 0.1 mL 2.25 mM NBT, 0.1 mL 3 mM EDTA,1.5 mL 0.1 M phosphate buffer, 0.1 mL 1.5 M Na_2CO_3, 0.1 mL enzyme extract, and 0.9 mL of water [30]. A total of 2 M riboflavin (0.1 mL) was used to start the reaction under a 15 W fluorescent lamp, which was maintained for 15 min. Reaction mixture without enzyme extract served as control, and a non-irradiated complete reaction mixture served as blank. One unit of enzyme activity was taken as the amount of enzyme that decreased the absorbance by 50% in comparison to control.

The glutathione reductase (GR) (EC 1.6.4.2) assay was performed by using the Smith et al. method [31]. The reaction was started by adding 0.1 mL of 20.0 mM oxidized glutathione (GSSG) to 3 mL reaction mixture containing potassium phosphate buffer (pH 7.5), EDTA, DTNB, NADPH, enzyme extract, and distilled water. The activity was measured as total absorbance (increase in absorbance) at 412 nm and expressed as GSSG reduced min^{-1} mg $protein^{-1}$.

2.3.7. Sodium Adsorption Ratio (SAR), Cd and Ni Analysis

One gram soil (in 5 mL distilled water) was used as soil–water extract to estimate Na^+, Mg^{2+}, and Ca^{2+} using a flame atomic absorption spectrophotometer [32]. Sodium adsorption ratio (SAR) was calculated from the following formula:

$$SAR = Na+/[(Ca^{2+} + Mg^{2+})/2]^{1/2}$$

Soil, root, and shoot samples were digested using the wet method with HNO_3 (69%, Merck) in combination with HCl (30%, Merck) for soil and H_2O_2 (30%, *m*/*v*; Merck) for plant material. The samples were analyzed for Cd and Ni (mg kg^{-1}) using a flame atomic absorption spectrophotometer.

2.4. Statistical Analysis

The experiment and tests were performed in triplicate. Data were analyzed using one-way and two-way ANOVA (SigmaPlot 11.0).

3. Results

3.1. PTE Accumulation and Phytoremedial Potential of A. halimus

A. halimus showed greater accumulation of Cd in the roots (Table 1). At low concentration i.e., Cd25, an uptake of 6.41 mg kg^{-1} in roots and 2.61 mg kg^{-1} in shoots was observed at 120 DAT. Test plants accumulated Cd in a concentration-dependent manner. A further increase in Cd accumulation was obtained in NaCl treatments. Cd accumulation increased up to 6.89 and 7.41 mg kg^{-1} in roots and 4.75 and 6.97 mg kg^{-1} in shoots with 1% and 3% NaCl at 120 DAT. In correlation with the reduced physiology, 5% salt concentration was found to decrease Cd accumulation in both the root and shoot of the plant (6.02 and 3.85 mg kg^{-1}, respectively).

Ni distribution showed a different pattern; with greater accumulation in shoot tissues (Table 1). Ni uptake in shoots was almost twice that of roots. Maximum Ni uptake occurred in shoots of the plant (9.42 mg kg^{-1}) in 3% NaCl treatment. Ni uptake was found to increase with increasing concentration of the metal in soil i.e., 1.72, 2.52, and 4.5 mg kg^{-1} in roots and 5.56, 7.14, and 10.6 mg kg^{-1} in shoots for Ni50, Ni100, and Ni200 mg kg^{-1}, respectively. The addition of salt to soil at lower doses increased Ni uptake, but in 5% salt treatment, it decreased sharply. Results of one-way ANOVA indicate significant statistical difference ($p \leq 0.001$) in the mean Cd and Ni content in the root and shoot of *A. halimus* across the different PTE and salt treatments at the end of the study (120 DAT).

Table 1. PTE accumulation and growth parameters of *A. halimus* at the end of study.

Treatments		Cd/Ni Content (mg kg^{-1})			Growth Parameters	
		Soil	Root	Shoot	Dry Biomass (in g)	Root/Shoot Ratio
T0	Control	nd	Nd	nd	37.5 ± 1.7	0.25
T1	Salt1%	nd	Nd	nd	51.2 ± 0.6	0.16
T2	Salt3%	nd	Nd	nd	55.6 ± 1.0	0.16
T3	Salt5%	nd	Nd	nd	32.4 ± 1.1	0.26
		Cd content (mg kg^{-1})				
T4	Cd25	23.6 ± 0.3 ^{a}	6.41 ± 0.27 ^{a}	2.61± 0.13 ^{a}	38.6 ± 1.1	0.19
T5	Cd50	46.8 ± 0.7 ^{b}	10.5 ± 0.33 ^{b}	4.1 ± 0.18 ^{b}	37.4 ± 0.6	0.19
T6	Cd100	96.5 ± 0.7 ^{c}	16.7 ± 0.25 ^{c}	8.0 ± 0.22 ^{c}	36.8 ± 1.0	0.20
T7	Cd25 + Salt1%	22.5 ± 0.3 ^{d}	6.89 ± 0.24 ^{d}	4.75 ± 0.23 ^{d}	42.5 ± 0.3	0.15
T8	Cd25 + Salt3%	21.8 ± 0.5 ^{d}	7.41 ± 0.22 ^{e}	6.97 ± 0.22 ^{e}	49.8 ± 0.7	0.16
T9	Cd25 + Salt5%	22.9 ± 0.6 ^{a}	6.02 ± 0.14 ^{a}	3.85 ± 0.17 ^{b}	29.9 ± 2.6	0.26
		Ni content (mg kg^{-1})				
T10	Ni50	48.6 ± 0.4 ^{a}	1.72 ± 0.18 ^{a}	5.56 ± 0.33 ^{a}	37.7 ± 0.8	0.18
T11	Ni100	98.2 ± 0.1 ^{b}	2.52 ± 0.27 ^{b}	7.14 ± 0.27 ^{b}	36.4 ± 0.5	0.18
T12	Ni200	198.4 ± 0.5 ^{c}	4.5 ± 0.22 ^{c}	10.6 ± 0.22 ^{c}	36.1 ± 0.5	0.18
T13	Ni50 + Salt1%	46.8 ± 0.7 ^{d}	1.92 ± 0.17 ^{a}	7.12 ± 0.33 ^{b}	53.5 ± 1.3	0.15
T14	Ni50 + Salt3%	46.1 ± 0.8 ^{d}	3.45 ± 0.22 ^{d}	9.42 ± 0.24 ^{d}	56.2 ± 1.6	0.13
T15	Ni50 + Salt5%	49.2 ± 0.5 ^{a}	1.24 ± 0.19 ^{e}	2.42 ± 0.21 ^{e}	30.2 ± 1.3	0.29

(n = 3; readings indicate mean value ± SD; nd = not detected; Different letters in the same column denote significant statistical difference ($p \leq 0.001$) in mean PTE content).

Phytoremediation characteristics of *A. halimus* for Cd accumulation at 25 mg kg^{-1} dose indicate that the roots are capable of accumulating more Cd than shoots. This indicates the phytostabilizing nature of *A. halimus* for the metal. Similar pattern of accumulation was also observed at higher concentrations i.e., 50 and 100 mg kg^{-1}. Although the plant had low values of root uptake and transfer to shoot, the percentage of metal reduction from soil was satisfactory. The transportation of Cd from soil to root to shoot increased with increasing concentration of the metal. Transfer of Ni from root to shoot was more which indicates extraction and sequestration of the metal in above-ground parts. Clean-up % of Ni from soil by *A. halimus* was almost two times higher at low concentration. Around 2.8% reduction in soil Ni concentration was observed at Ni50 which increased up to 7.8% with 3% salt. Significant statistical difference ($p \leq 0.001$) in the mean Cd and Ni content in soil due to different treatments was obtained at 120 DAT.

3.2. Potential of A. halimus for Restoration of Saline Soil

The desalinization potential of *A. halimus* was measured by comparing the electrical conductivity (EC) and sodium adsorption ratio (SAR) of soil at initial and final day of the study. EC was 1.04 dSm^{-1} for control soil; and in the ranges of 4.5–4.9 dSm^{-1} for 1% NaCl treatments, 8.1–8.7 dSm^{-1} for 3% NaCl treatments, and 10.1–10.3% dSm^{-1} for 5% NaCl treatments initially. These were reduced to 2.5–3.7, 4.2–5.1, and 9.4–9.6 dSm^{-1} for 1, 3, and 5% NaCl treatments, respectively, after 120 days. Around 50% reduction in soil EC was obtained in 1% NaCl treatment (T1). Thus, 1% NaCl treatments (T1, T7, T13) were reduced from slightly saline to non-saline and 3% NaCl treatments (T2, T8, T14) were reduced from slightly saline to somewhere between non to slightly saline as per the limits suggested by FAO for saline soils. Reduction in soil EC indicates reduction in salt content of soil due to its uptake and sequestration by *A. halimus*. The highest reduction in soil SAR was observed in Cd25 + 1% NaCl (52.6%) followed by 3% NaCl (51.9%) treatment. The lowest reduction in soil SAR was obtained with 5% NaCl (2.5%). Thus, the optimum concentration of salt for optimum growth and development of *A. halimus* was 3%. The data indicate that metal ions competed with salt ions for translocation to shoots.

3.3. Growth and Physiochemical Analysis

3.3.1. Dry Biomass and Root/Shoot Ratio

Dry biomass and root/shoot ratio of the test plants were measured at the end of the study i.e., 120 DAT (Table 1). A slight increase in biomass was observed in response to Cd25 (38.6 g) and Ni50 (37.7 g) treatments. As the PTE concentration increased, biomass decreased gradually. As the concentration of NaCl increased from 1 to 3%, a significant increase in the biomass of the plants was observed. Plants treated with 3% salt were the healthiest as compared to other salt treatments. On the other hand, salt at 5% dose in soil inhibited growth individually and in combination with PTEs. Still, all the plants survived. Similar trends were observed for root/shoot ratios. Lower values in 1 and 3% salt treatments (with or without metals) can be explained by longer shoots. Higher values were obtained in 5% NaCl treatments because of reduced growth of shoots. Two-way ANOVA indicates significant statistical difference ($p \leq 0.001$) in the mean biomass, due to Cd and salt treatments (Table 2). The interaction between Cd and salt doses was also statistically significant ($p \leq 0.001$). In Ni treatments, significant difference was obtained for salt treatments as well as Ni and salt interaction ($p \leq 0.001$).

Table 2. Two-way ANOVA of PTE and NaCl treatments for biomass and antioxidative enzymes.

Parameters	Biomass	CAT	SOD	APX	POX	GR
Sources of Variations						
Cd	**	**	ns	ns	ns	ns
NaCl	**	**	**	**	**	ns
Cd × NaCl	**	**	ns	ns	ns	ns
Ni	*	**	ns	*	*	ns
NaCl	**	**	**	**	**	ns
Ni × NaCl	*	**	ns	**	**	ns

(** = difference of means significant at $p \leq 0.001$; * = difference of means significant at $p \leq 0.05$; ns = not significant).

3.3.2. Chlorophyll Content

Total chlorophyll content of *A. halimus* leaves was found to increase as the study progressed (Figure 1). No significant reduction in chlorophyll content of Cd treated plant was observed (except for Cd100 mg kg^{-1}). Surprisingly, plants treated with Cd25 mg kg^{-1} showed a slight increase in their chlorophyll content as compared to control, while plants treated with Ni showed significant increase in chlorophyll as compared to control at all testing days. Highest chlorophyll synthesis occurred in Ni50 followed by Ni100 mg kg^{-1}. *A. halimus* developed no visible symptoms of toxicity in NaCl treatments up to 3% concentration. An increase in the photosynthetic activity was seen in treatments of Ni and Cd with 1 and 3% salt. Two-way ANOVA indicates significant statistical difference ($p \leq 0.001$) in the mean chlorophyll content due to each PTE treatment and day of testing (Table 3). The interaction between treatments and day of testing, however, was not significant ($p \leq 0.001$).

3.3.3. Membrane Damage Rate

Initially, *A. halimus* leaves showed significantly higher membrane damage rate as compared to control in all treatments (Figure 2). Lesser electrolyte leakage (MDR) was observed in most treatments as the experiment progressed. This indicates that plants became more tolerant in due course of time. Two-way ANOVA indicates statistically significant difference in mean MDR ($p \leq 0.001$) due to different treatments and day of testing (Table 3). The interaction between treatment dose and testing day was also significant ($p \leq 0.001$). This can be understood as the effect of treatments changing over time. Lower dose of PTEs showed membrane damage comparable to control at 120 DAT. Cd and Ni treatments with 1–3% salt exhibited a reduction in MDR after 60 DAT. Higher combination of salt and metal showed increased membrane damage till the end of study. It is clear that salt was beneficial for the plant but only up to certain doses.

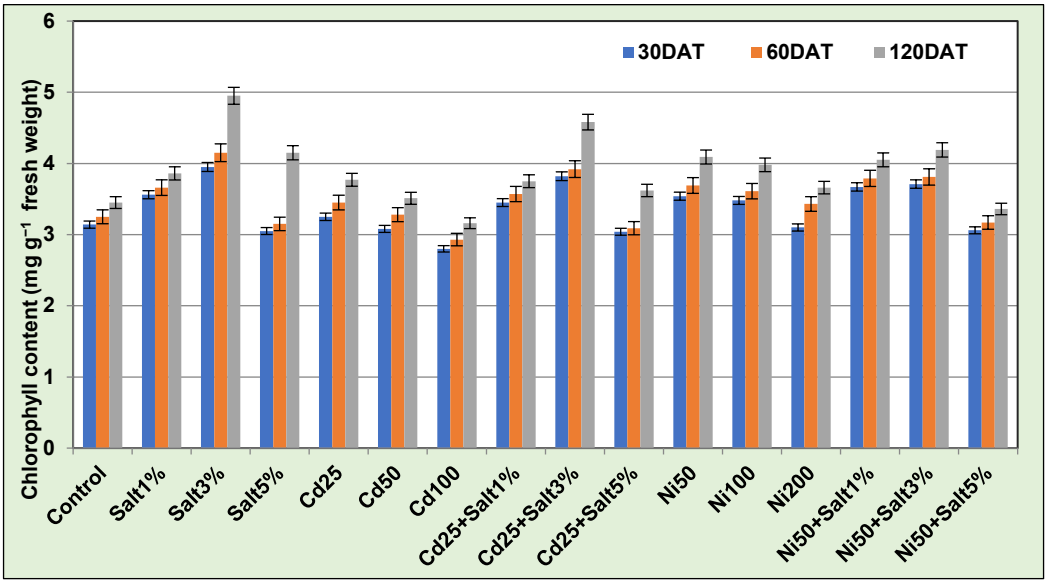

Figure 1. Total chlorophyll content (mg g^{-1} fresh weight) in *A. halimus* leaves at successive days after treatment. (Each bar indicates mean ± SD; n = 3).

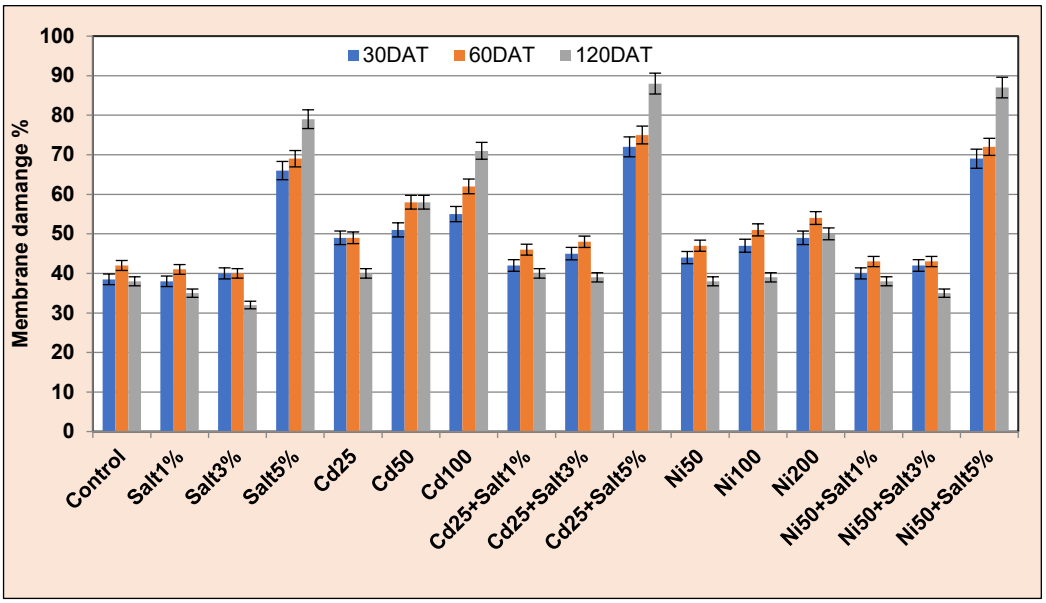

Figure 2. Membrane damage rate (as percentage) in *A. halimus* leaves at successive days after treatment. (Each bar indicates mean ± SD; n = 3).

3.3.4. Lipid Peroxidation

Plants treated with only metal treatments exhibited a significant increase in lipid peroxidation expressed as malondialdehyde (MDA) content (Figure 3). It was found to increase with increasing metal concentrations. Maximum MDA content was (8.24 µg g^{-1} fw) for Cd 100 ppm. Least MDA was recorded in plants treated with 3% salt. The amount of MDA was found to decrease as the study progressed showing the adaptability of the plant to salt and Cd/Ni contamination in soil. At 120 DAT, all treatments showed MDA levels comparable to control. The difference in mean values due to the PTE and salt treatments (Table 3) as well as testing date was statistically significant ($p \leq 0.001$).

Table 3. Two-way ANOVA of PTE and NaCl treatments for physiological parameters.

	Parameters	Chlorophyll	Membrane Damage Rate	Lipid Peroxidation	Proline	Glycine Betaine
Sources of Variations						
Cd	Treatments	**	**	**	**	**
	Day after treatment	**	**	**	**	**
	Treatment × DAT	Ns	**	Ns	**	**
Ni	Treatments	**	**	**	**	**
	Day after treatment	**	**	**	**	**
	Treatment × DAT	Ns	**	Ns	**	**

(** = difference of means significant at $p \leq 0.001$; ns = not significant).

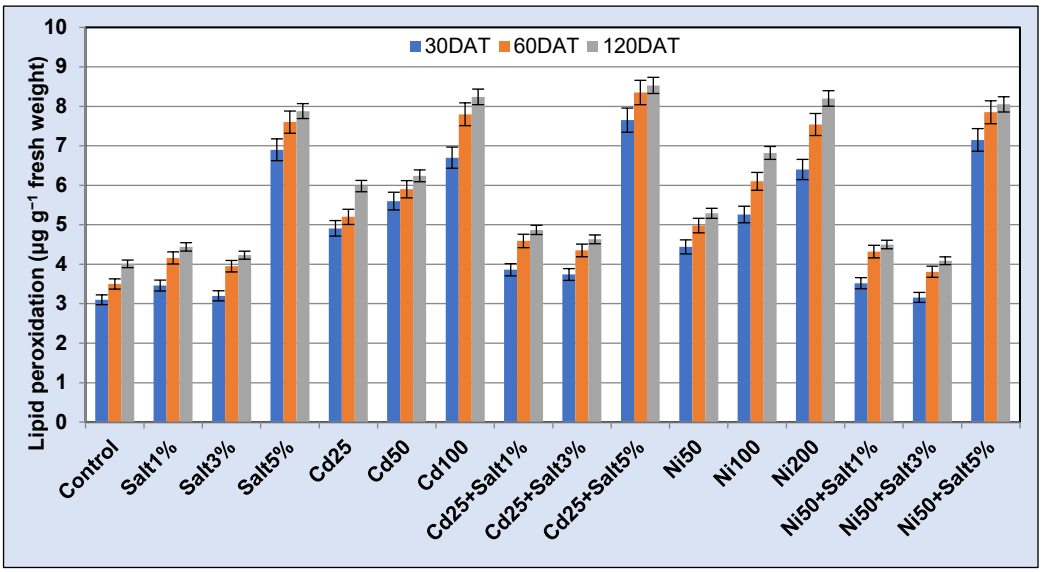

Figure 3. Lipid peroxidation (µg g^{-1} fresh weight) in *A. halimus* leaves at successive days after treatment. (Each bar indicates mean ± SD; $n = 3$).

3.3.5. Proline and Glycine Betaine Content

Proline accumulation in test plants was found to increase with increasing concentration of salt and/or PTEs (Figure 4). Maximum proline was accumulated in plants treated with 5% salt with or without PTEs. Treatments with Cd and Ni caused higher levels of proline in test plants at all testing days. Lowest range of proline (5.28–6.95 mg g^{-1} fw) was observed in Ni50 + 1% NaCl.

Glycine betaine content of *A. halimus* was significantly influenced by the PTE treatments (Figure 5). Increased values were noticed till 60 DAT. Cd 100 and Ni200 mg kg^{-1} had almost similar impact on GB content. Lower doses of salt and salt + metal combinations showed gradual decrease in GB content after 60 DAT, while plants treated with 5% salt individually and in PTE combinations showed continuous increase in GB content till 120 DAT. ANOVA results indicate that difference in mean proline and glycine betaine content in response to Cd/Ni and NaCl treatments as well as testing dates was statistically significant ($p \leq 0.001$) (Table 3). A statistically significant interaction between treatment dose and testing day ($p \leq 0.001$) in the mean values was also obtained for proline. This translates to effect of treatment changing as the study progressed.

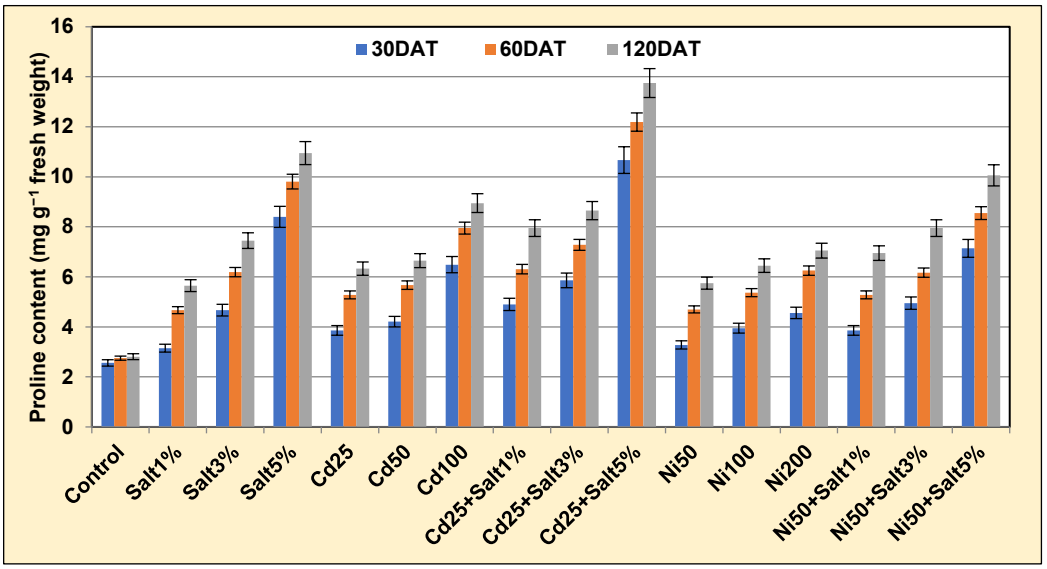

Figure 4. Proline content (mg g^{-1} fresh weight) in *A. halimus* leaves at successive days after treatment. (Each bar indicates mean ± SD; n = 3).

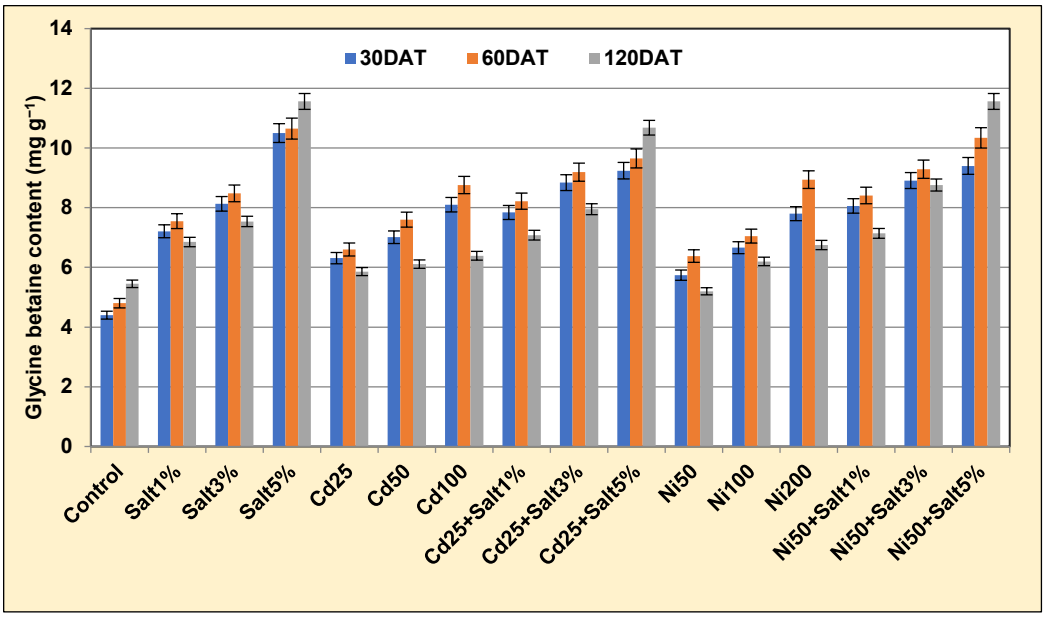

Figure 5. Glycine betaine content (mg g^{-1}) in *A. halimus* leaves at successive days after treatment. (Each bar indicates mean ± SD; n = 3).

3.3.6. Antioxidative Enzymes

Antioxidative defense of the plant was expressed by a pool of antioxidative enzymes (CAT, APX, SOD, POX, and GR) (Figures 6 and 7). Catalase activity was found to increase with increasing concentration of Cd and Ni. The order was Cd100 > Cd50 > Ni200 > Cd25 > Ni100 > Ni50 > control. Similar picture was obtained for SOD and POX as well. APX activity, however, showed slight variation. A minute increase was observed in Ni100 and Ni200 mg kg^{-1} treatments. The range of GR activity in plants under PTE treatments was 0.043–0.068 unit min^{-1} mg^{-1} protein for Cd.

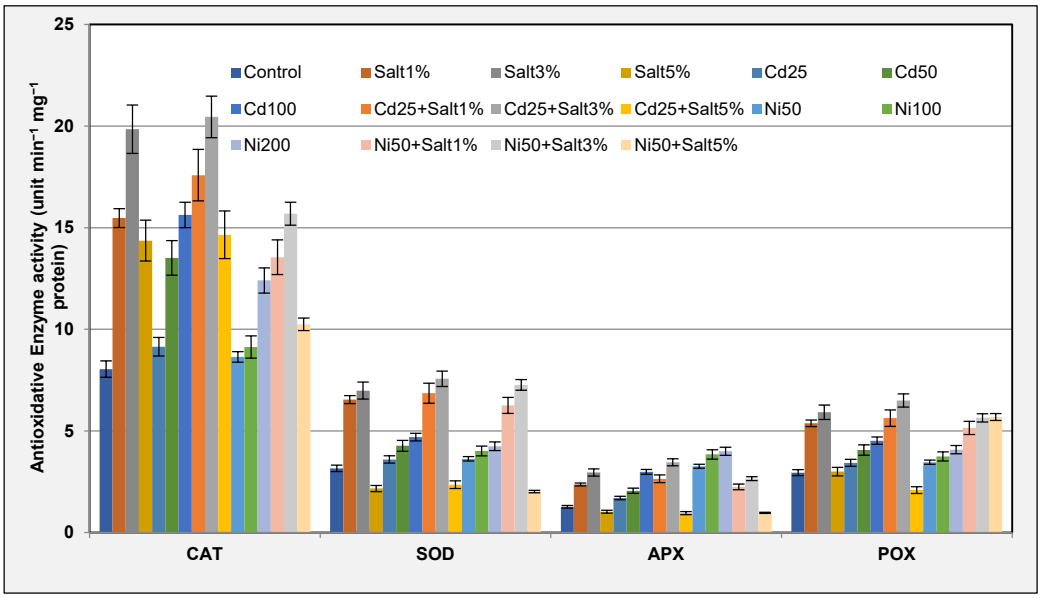

Figure 6. Antioxidative enzyme activity (unit min^{-1} mg^{-1} protein) in *A. halimus* at end of study (120 days). (Each bar indicates mean ± SD; $n = 3$).

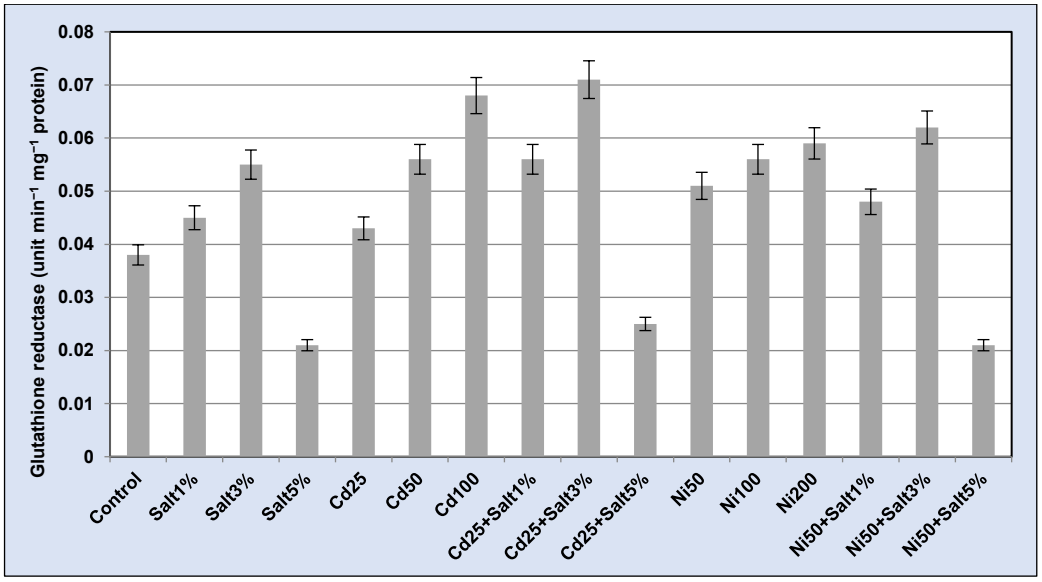

Figure 7. Glutathione reductase activity (unit min^{-1} mg^{-1} protein) in *A. halimus* at end of study (120 days). (Each bar indicates mean ± SD; $n = 3$).

Plants treated with 1 and 3% NaCl exhibited higher level of antioxidants as compared to control, but at 5% concentration of salt, a rapid decrease in the activity of all the enzymes was noticed. APX and SOD activity was noticed to be the most affected by 5% salt. Plants treated with both salt and PTEs showed further elevation in their antioxidative status. Highest CAT and SOD activity was found in plants treated with Cd 25 mg kg^{-1} + 3% salt. About 3.2-fold decrease in SOD was observed in plants treated with 5% salt as compared to the plants treated with 3% salt. GR activity showed positive correlation with APX. Mean values for enzyme activities (CAT, APX, SOD, and POX) were significantly different ($p \leq 0.001$) in response to Cd and salt treatments; however, the interaction of Cd and salt treatment was not (Table 2). In Ni treatments, significant difference in mean enzyme activity ($p \leq 0.001$) was obtained due to PTE and NaCl. The interaction Ni × NaCl was significant ($p \leq 0.001$) only for CAT, APX, and POX.

4. Discussion

The species of *Atriplex* have been reported to accumulate Pb, Zn, Cd, Ni, Cu, and As [10,33–35]. It has been demonstrated that Pb and Zn in *A. hortensis* and *A. rosea* accumulated largely in by root tissues; with accumulation increasing in accordance with soil PTE concentrations [34]. This is in line with this investigation as higher accumulation of Cd was observed in roots of *A. halimus*. Cd concentration is usually higher in roots compared to above-ground tissues in most plants [36]. *A. halimus* has been reported to accumulate Ni, Pb, Zn, and As in roots and translocate small fractions to above-ground biomass [9,35,37]. It is interesting to note that Kahli et al. [10] observed a similar preferential partitioning of Cd in *A. halimus* roots at 50 µM Cd but the reverse at 20 µM Cd. Additionally, in a five-year study, where *A. halimus* was grown in mile tailings amended with pig slurry, the authors reported better translocation of Zn, Pb, Cd, and Cu to leaves and/or stem over roots [38]. This indicates that environmental factors may affect plant physiology and cause different patterns of PTE sequestration and accumulation within plant biomass.

On the other hand, Ni content was greater in the shoots of test plants at 120 DAT. Studies indicate that plants accumulate Ni predominantly in their aerial parts [39,40]. In 2003, Ni was added to the list of essential plant nutrients by US Department of Agriculture [41] which also explains its higher concentration in above-ground parts and lesser phytotoxicity at low to moderate concentrations.

In Cd (25, 50, and 100 mg kg^{-1}) treatments, ~5.6, 6.4, and 3.5% clean-up of soil was obtained, while in Ni treatments (50, 100, and 200 mg kg^{-1}) the clean-up was ~2.8, 1.8, and 0.8%. Thus, *A. halimus* demonstrated maximum phytoremedial potential at 50 mg kg^{-1} doses for both the PTEs. Phytoremediation potential of halophytes including *Atriplex* species for PTEs has been recorded previously [17,35,42,43]. *Chenopodium botrys* an annual halophyte may remove up to 180 g Cd ha^{-1}, which is six times more than Cd removal by the hyperaccumulator *Noccaea caerulescens* [44]. Moderate level of Ni accumulation has been reported in *Sporobolus virginicus* shoots [17].

Halophytes have the ability to better adapt to many environmental constraints including toxic metal ions and could be more satisfactory for the extraction of PTEs from saline soils than glycophytes [5,45]. Milić et al. [46] found greater metal accumulation in aerial parts of *Sesuvium portulacastrum* (halophytes) in comparison to *Brassica juncea* (glycophyte) grown in a Pb amended growth matrix. Positive effect of the salinity on accumulation of Cd has been reported in *A. halimus*, *A. nummularia*, and *A. canescens*, especially at the low Cd doses [5,10]. Similar results were obtained in this study where the addition of salt to soil enhanced the uptake of both Cd and Ni in *Atriplex halimus*. Highest PTE uptake in root (Cd) and shoot (Cd & Ni) was observed with 3% salt. PTE uptake, however, seemed to take a toll on the desalinization ability of the test plant, which again strongly suggests that metal ions compete with Na^+ ions for sequestration within the plants.

Toxic effects of PTEs generally show common side-effects such as reduction in plant biomass, height, pigment, sugar, protein, and carbohydrate concentration; inhibition of photosynthesis; and production of stress markers such as proline, MDA, and glycine betaine. Reduction in shoot length, specific leaf area, dry biomass, relative growth rate, and net photosynthetic rate due to increasing Cd concentration has been previously recorded in *A. halimus* [9]. Results of this study indicate that *A. halimus* was quite tolerant to moderate concentrations of both PTEs (Cd and Ni) and salt. The tolerance of *A. halimus* to Cd may be due to its greater sequestration in roots to alleviate the inhibition of photosynthesis and simultaneous increase in antioxidant enzymatic activity in leaves.

Significant increase in proline and glycine betaine accumulation under PTE stress has been observed previously in *A. halimus* [19]. PTE Slama et al. [47] observed increased accumulation of many osmotically agile compounds such as proline, trehalose, sucrose, polyols, and glycine betaine in halophytes. These compounds have been reported to play key roles for the stabilization of many proteins and their complexes, maintenance of membrane integrity, ROS scavenging and osmotic adjustments. In the present study, significantly higher contents of proline and glycine betaine were recorded in plants grown

in PTE-contaminated soil. Plants showed highest production of proline and glycine betaine in response to Cd. Salt exposure also resulted in increased production of GB in *A. halimus*. Exposure to 5% salt caused the highest production of proline and glycine betaine. GB assists in salt tolerance by increasing the protection of thylakoid membranes of chloroplast, thereby strengthening the photosynthesis system of halophytes [48]. This could explain the increased production of chlorophyll in test plants treated with salt.

Cd, Ni, and Pb have been documented to exhibit adverse effects on membrane functionality of a cell and evoke a strong change in the compositions of lipids in cell membranes of plants [49]. These metals ultimately induce lipid peroxidation in plant cell which can be marked by excessive production of MDA content [50]. The test plant showed enhanced damage rate (MDR) and production of MDA under PTE exposure especially during the initial days of the study. Plants treated with NaCl in combination with PTEs exhibited a significant reduction in MDA content. since salt increased plant growth and improved their detoxification capability. Increased MDA content in plants after exposure to PTEs has been noted both in the absence and presence of salt in soil [10,35,51]. This suggests that halophytes plants have an effective antioxidant defence system which responds in proportion to stress [52].

Antioxidative mechanisms of plants are powered by many enzymatic scavengers (SOD, CAT, APX, etc.) and non-enzymatic compounds (ascorbic acid, proline, etc.). Fluctuations of their activity are generally used to forecast metal tolerance [53]. The results of the current investigation indicate that *A. halimus* showed increased production of antioxidative enzymes (CAT, SOD, APX, POX, and GR) in most PTE and salt treatments. Increased CAT and SOD activity in *A. halimus* leaves due to Cd and Pb has also been observed in previous studies [9,54]. El-Bakatoushi et al. studied the molecular and physiological mechanisms of Cd and Pb tolerance in *Atriplex halimus* from fourteen sites and found that among others, membrane transporter and ROS detoxification genes were consistently up-regulated in plants from polluted sites. They also detected overexpression of phytochelatin synthase responsible for producing phytochelatins, which bind to toxic PTEs forming complexes stored in cellular compartments [54]. This indicates the importance of using not just the right species of a plant but also the most suitable genotype.

5. Conclusions

Halophytic plant species are known to tolerate many abiotic stresses posed by their natural soil environment through efficient management of osmotic and oxidative disturbances. They are also able to take up appreciable amounts of potentially toxic elements from the soil and sequester them in salt glands, vacuoles, or trichomes to limit their toxic effects. In this context and taking into consideration the PTE accumulation patterns, biomass production and stress management strategies, *Atriplex halimus* holds promise for phytoremediation of Cd- and Ni-polluted sites with saline and non-saline environments. However, it would be prudent to note that every contaminated site is a unique microcosm where even a well-studied plant may behave differently due to environmental factors including soil chemistry and its own physiological responses. Molecular advances in the recent decades also indicate the importance of the correct genotype of the species being used for remedial purposes.

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