

# Cd-phytoextraction potential in halophyte *Salicornia fruticosa*: Salinity impact

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**Abstract:** Cadmium (Cd) deposition and salinity are combined environmental stresses in coastal or dry areas irrigated with non-treated wastewater. In those areas, halophytes have been found to be more effective in the phytoextraction of metals rather than Cd-hyperaccumulating glycophytes that are unqualified for growing in saline soil. Nevertheless, the impact of salt on the accumulation properties of Cd in a variety of halophytic species remains undetermined. The hydroponic culture was used to investigate the impact of salinity on Cd tolerance as well as accumulation in distinct halophyte *Salicornia fruticosa*. The plant was subjected to 0, 25, and 50  $\mu\text{g l}^{-1}$  Cd (0-Cd, L-Cd, and H-Cd, respectively) in combination with or without 50, 100, and 200 mM NaCl in the nutrient solution. Data demonstrated that Cd individually induced depletion in biomass accumulation. The NaCl-amplified Cd tolerance induced by enhanced biomass gaining and root length was associated with adequate transpiration, leaf succulence, elevated levels of ascorbic acid (ASA), reduced glutathione, and phytochelatins (PCs) and proline as well as antioxidant enzymatic capacity via upregulation of peroxidases (PO), glutathione peroxidase, ascorbate peroxidase, and superoxide dismutase. All Cd treatments decreased the uptake of calcium (Ca) as well as potassium (K) and transit to the shoots; however, sodium (Na) accumulation in the shoots was not influenced by Cd. Consequently, *S. fruticosa* retained its halophytic properties. Based on the low transfer efficiency and high enrichment coefficient at 0-50 mM, an examination of Cd accumulation characteristics revealed that phytostabilization was the selected phytoremediation strategy. At 100-200 mM, the high ground parts Cd-translocation and high absorption efficiency encourage phytoremediation via phytoextraction. The results revealed that *S. fruticosa* could be potentially utilized to renovate saline soils tainted with heavy metals (HMs) because of its maximized capacity for Cd tolerance as well as enrichment magnified by NaCl. Cd accumulation in *S. fruticosa* is affected differently depending on the NaCl concentration. Future studies may be conducted to detect other heavy metal pollutants screening that could be extracted and stabilized by the *S. fruticosa* plant. Furthermore, other substrates presenting a high electrical conductivity should be identified for reclamation.

**Keywords:** Antioxidants; Halophyte; Phytochelatines; *Salicornia fruticosa*

## 1. Introduction

Soil salinization is the most damaging environmental stress limiting agricultural crops and land usage [1]. There is evidence that by 2050, 50% of cropland, as well as 20% of agricultural land, will be influenced by salinity [2]. Furthermore, when fresh water supply for agricultural purposes decreases, utilizing saline and semi-saline streams serves as an alternative to water, although salinization of soil is still a possible problem, especially in semiarid as well as arid locations. Surprisingly, irrigation has led to elevated salt concentration beyond normal in the arable land's rooting zone since elevated transpiration as well as evaporation drain soluble salts from deep soil profile layers. Simultaneously, because of industrial pollution and irrigation practice, several areas, especially the coastal as well as semiarid and arid areas, are influenced by the deposition of HMs and salinity [3], inducing HM pollution to the saline soil, arising as a global environmental issue.

Since HMs are not degradable by biological or chemical processes, they remain in the environment [4]. It was proven that Cd is the most deleterious element for plants owing to its propensity, high mobility, and quickly take up before being transmitted to the aerial, thus entering the food chain. This process suppresses plant development and growth and poses a significant environmental threat as well as human health [5]. Many anthropogenic activities such as fertilizer impurities as well as utilizing sewage sludge and refuge-derived composts [4], as well as the output of waste products of the ship scrapping industry transported by tidal flooding and subsequent deposition into the sediments, are the most participants in the prevalent addition of significant amounts of Cd to soils and water resources. Environmental Protection Agency (USEPA) has set a 3 mg Cd kg<sup>-1</sup> maximum value in agricultural soils that receive sludges [6].

Researchers and government agencies have recently paid particular attention to the elevated Cd concentration in soil. Multiple physicochemical methods were examined to remove these pollutants from the soil. Nevertheless, they may cause damage to the soil and are costly [7]. Due to the inherent potential of some species to collect certain HMs, interest in utilizing plants for soil rehabilitation has lately grown. Even though phytoremediation is considered an alternative strategy due to its low cost, high safety, and relatively low cost [8], the majority of plants utilized for metal accumulation like pea (*Pisum sativum*), corn (*Zea mays*), sunflower (*Helianthus annuus*), mustard (*Brassica juncea*) [9] utilized for Cd phytoextraction, e.g., *Arabidopsis halleri* and *Thlaspi caerulescens*, are glyco-phytes and cannot be utilized for HM phytoextraction in regions with elevated levels of salinity. Halophytes account for around 1% of the world flora and can live and reproduce in habitats with salt concentrations of 0.8-4.2 percent (dry or based soil) or more, and they can endure concentrations of salt that can destroy up to 99 percent of other species [1].

Additionally, multiple investigations have shown that some halophytes may be resistant to HMs and accumulate significant bioavailable HMs concentrations in their tissues [7]. Because of processes that impart tolerance to ions other than chloride and sodium. Evidence shows that halophytes' evolutionary adaptations may potentially offer resistance to other harmful substances [10]. Therefore, halophytes are the optimum species of plants for remediating HM-contaminated salty soils. Halophytes' high tolerance of metals substantially correlates with salt tolerance traits such as antioxidant systems [11]. Osmoprotectant production includes proline to scavenge free radicals as well as retrain the balance of water [11, 12] and salt gland excretion onto the surface of the leaf. In addition to NaCl, this mechanism involves inorganic contaminants [12]. Several plant species depend on salinity for Cd absorption and transfer from roots to shoots [9]. Prior investigations revealed that adding salt (NaCl) to the medium increases Cd phytoextraction and phytoavailability [13]. Nevertheless, Sepehr et al. [14] illustrated that salinity alleviated Cd accumulation in maize plants. Therefore, salinity impact on the uptake of Cd is plant-specific. In addition, moderate NaCl doses are hypothesized to enhance the growth

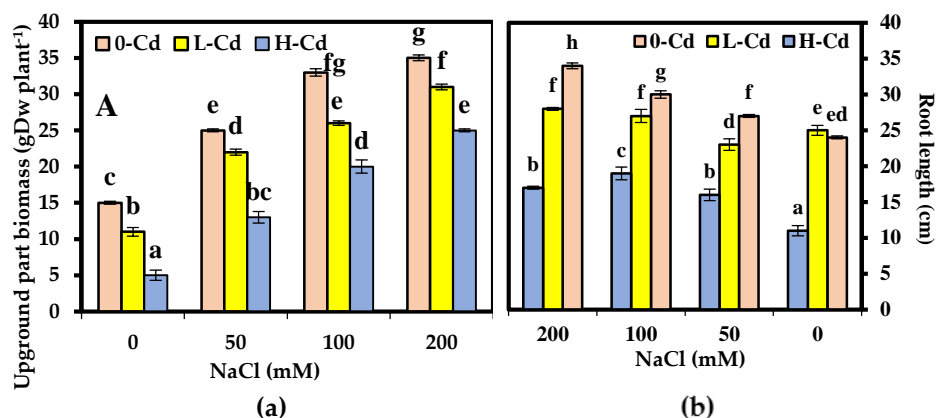
of plants besides protecting against HM poisoning via modulating osmotic adjustment, ion uptake, and stimulating antioxidant mechanisms [15].

*Salicornia fruticosa*, termed glasswort, is an annual succulent halophyte belonging to the Chenopodiaceae family. Globally, halophyte plants account for 44% of all genera in this family (312 genera) [16]. In Egypt, this family includes 25 genera and about 300 species. It thrives on muddy seashores as well as in saline marshes. The utilization of *S. fruticosa* herb for folk medicine deprived its pharmaceutical importance in these regions. Although the plant exhibits a great tendency to grow well across various concentrations of soil salt, reaching 8% [17], its HM remediation capability is still not well documented, and there is a paucity of data on the metabolic responses to the combined stress of Cd and NaCl. In addition, there is currently no evidence on the effect of NaCl on plant development and metabolites in *S. fruticosa* that can be linked to Cd translocation, absorption, and therefore deposition under Cd stress. The current research could provide a fundamental foundation for interpreting the NaCl impact on the uptake of Cd, accumulation, and translocation in recently screened *S. fruticosa*, Cd and NaCl metabolic response, as well as the connection between Cd desposition and metabolites in plants under Cd stress, in the absence and presence of NaCl, suggesting the possibility of using HMs as phytoremediators.

## 2. Results

### 2.1. Growth indices affected by interaction between Cd stress and salinity in *Salicornia fruticosa*

Morphologically, plants treated with L-Cd showed initial chlorosis that worsened with raising the concentration of Cd before developing necrosis with abscission as well as leaf senescence. In the absence of Cd, 100 and 200 mM NaCl did not impact *Salicornia fruticosa* morphology. The difference was remarkable, particularly between high salt and low salt-treated plants receiving 50  $\mu\text{g l}^{-1}$  of Cd. These observations demonstrated that high NaCl concentrations (100 and 200 mM) decreased the symptoms of Cd toxicity in this halophyte. In the absence of Cd, all plants showed highly growth tendency in terms of upground parts biomass and root length throughout the experimental period up to 200 mM NaCl, indicating that salt is characterized by impacts on *Salicornia fruticosa* halophyte growth features (Fig. 1 A and B). The existence of H-Cd alone without NaCl negatively impacts plant biomass and root length; however, L-Cd applied imposed unchanged biomass and slightly stimulated root length, indicating that *Salicornia fruticosa* is resistant to Cd at a diminished concentration. NaCl addition substantially enhanced root length and the acquisition of plant biomass and restored normal plant growth; however, plant growth response to the Cd and NaCl combination depends on the concentration of NaCl. High salt-treated plants had better growth than low salt-affected plants.



**Figure 1.** Upground part biomass and root length (A and B) of *Salicornia fruticosa* exposed to nutrient solution containing 0, 25, and 50  $\mu\text{g l}^{-1}$  Cd (0-Cd, L-Cd and H-Cd, respectively) without or with 50, 100 and 200 mM NaCl. Each value is the average of four replicates  $\pm$  SE. Values bearing different letters are significantly different at  $P < 0.05$  based on Tukey's test.

## 2.2. Water relation indices affected by Cd stress and salinity co-occurrence in *Salicornia fruticosa*

The transpiration rate of different salt concentrations treated plants remained unaffected compared with non-salinized plants (Table. 1). Only, L-Cd treatment exhibited an insignificant reduction in *S. fruticosa* transpiration rate while L-Cd severely inhibited this trait. Elevating salt concentration in the medium from 50 to 200 mM significantly restored the transpiration rate of the H-Cd treated plant, whereas no substantial difference was detected in plants grown in L-Cd compared with only H-Cd and L-Cd, respectively. Shoots from different salt concentrations treated plants did not differ in succulence, confirming this species's halophytic character (Table. 1). Only L-Cd treatment displayed an unchanged succulence degree of shoots in comparison with leaves of 0-Cd non-salinized plants, whereas H-Cd severely reduced this trait (Tab 1). NaCl co-occurrence markedly restored shoot succulence degree. The sustained TOP value of plants grown along all NaCl concentrations denotes that these plants are less suffering from osmotic stress as they are grown in the preferable concentrations of salt (Table. 1). Increasing doses of Cd alone may induce consequent osmotic stress that elicits the importance of increasing the TOP. Co-occurrence of salt effectively reduced the TOP value to that of the corresponding salt-treated plant.

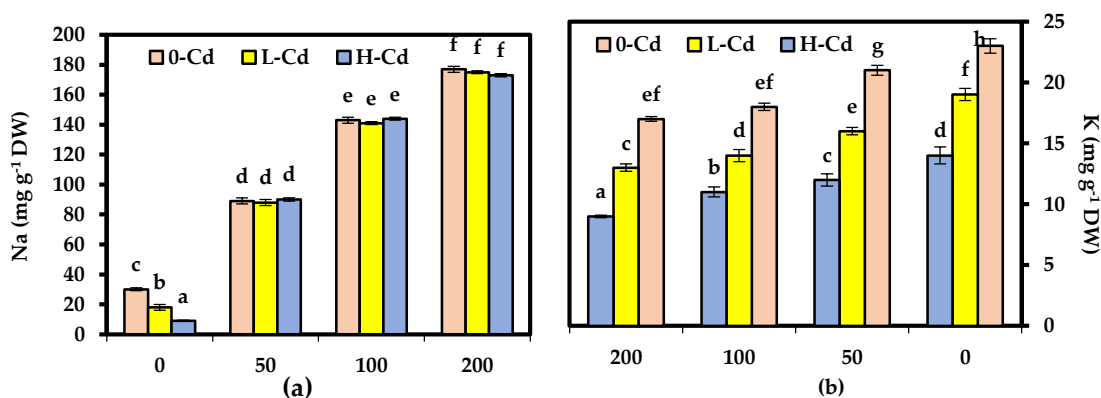
**Table 1.** Transpiration rate, shoot succulence degree and total osmotic potential (TOP) in leaf of *Salicornia fruticosa* exposed to nutrient solution containing 0, 25, and 50  $\mu\text{g l}^{-1}$  Cd (0-Cd, L.-Cd and H.-Cd, respectively) without or with 50, 100 and 200 mM NaCl. Each value is the average of four replicates  $\pm$  SE. Values bearing different letters are significantly different at  $P < 0.05$  based on Tukey's test.

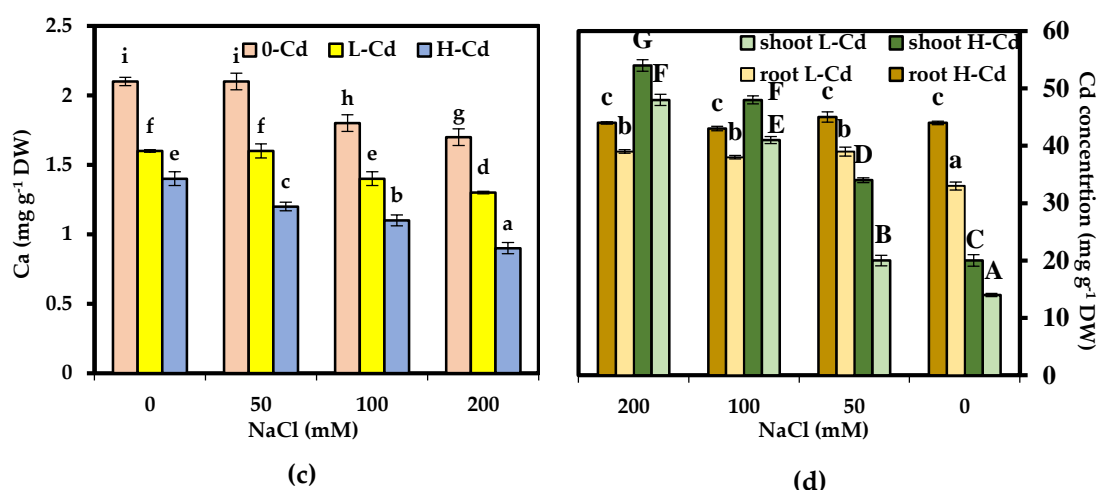
Treatments		Transpiration rate (ml transpired. g- 1 FW)	Shoot succulence degree (g.g-1)	TOP (-MPa)
0 mM NaCl	0-Cd	30d ± 0.5	5.02c ± 0.04	24.7a ± 0.32
	L-Cd	22c ± 0.4	5.00c ± 0.05	25.5b ± 0.22
	H-Cd	6a ± 0.08	3.45a ± 0.06	28.3f ± 0.15
50 mM NaCl	0-Cd	28d ± 0.6	5.41c ± 0.04	24.7a ± 0.25
	L-Cd	20c ± 0.9	5.11c ± 0.03	25.1b ± 0.13
	H-Cd	11b ± 0.4	3.99b ± 0.05	27.8e ± 0.14
100 mM NaCl	0-Cd	30d ± 0.9	5.33c ± 0.04	24.9a ± 0.44
	L-Cd	21c ± 0.6	4.99c ± 0.03	24.8a ± 0.24
	H-Cd	19c ± 0.7	4.11b ± 0.03	26.4d ± 0.13
200 mM NaCl	0-Cd	29d ± 0.7	5.34c ± 0.02	24.8a ± 0.12
	L-Cd	20c ± 0.6	5.11c ± 0.02	24.7a ± 0.25
	H-Cd	22c ± 0.6	4.28b ± 0.01	25.8c ± 0.44

0-Cd: no cadmium added; L-Cd: low cadmium concentration; H-Cd: high cadmium concentration; FW: fresh weight; g: gram; ml: milliliter; TOP: total osmotic potential; MPa: mega pascal

### 2.3. Mineral composition affected by Cd stress and salinity co-occurrence in *Salicornia fruticosa*

All Cd alone treatments reduced Na, K, and Ca shoot concentrations (Fig. 2 A, B, C and D). The accumulation of Ca and K decreased as the external NaCl supply elevated, consistent with the larger Na accumulation in the former, but elevating the Cd amount from 25 to 50 µg l<sup>-1</sup> had no effect on Na accumulation in the shoots. Ca and K concentrations in the shoots were lowered more when Cd and NaCl were applied concurrently compared to when Cd was applied individually. In non-saline conditions, the concentrations of Cd in roots and *S. fruticosa* shoots elevated with the elevation in Cd supply and were substantially elevated in the roots compared to shoots (Fig. 2 D). Compared to Cd alone, NaCl addition substantially enhanced Cd concentration. Plants of *S. fruticosa* grown in saline soils acquired higher amounts of Cd compared with plants grown in non-saline soils (Fig. 2 D).





**Figure 2.** Concentrations of leaf sodium (Na), potassium (K), calcium (Ca), and shoot and root cadmium (Cd) (A,B, C and D) in *Salicornia fruticosa* exposed to nutrient solution containing 0, 25, and 50  $\mu\text{g l}^{-1}$  Cd (0-Cd, L-Cd and H-Cd, respectively) without or with 50, 100 and 200 mM NaCl. Each value is the average of four replicates  $\pm$  SE. Values bearing different letters are significantly different at  $P < 0.05$  based on Tukey's test.

#### 2.4. Phytoremediation parameters affected by Cd stress and salinity co-occurrence in *Salicornia fruticosa*

In non-saline conditions, BCF and TF values were unaffected (Table 2). For both Cd external doses (25 or 50  $\mu\text{g l}^{-1}$ ), increasing the salt content in the medium from 100 to 200 mM increased Cd translocated in the shoots and decreased Cd maintained in roots (Table 2), but at 50 mM NaCl, the majority of Cd was apportioned in the root rather than the shoot. Increasing the salt concentration substantially improved the quantity of Cd accumulated in the shoots. This elevation occurred due to increased biomass secretion in plants exposed to the Cd and NaCl mixture.

Furthermore, as evidenced by the elevation in TFs as well as BCFs, exogenous NaCl substantially enhanced Cd translocation and absorption. TF was elevated in plants receiving the Cd and NaCl mixture than in those receiving only Cd (Table 2). Elevating the concentration of salt in the medium from 100 to 200 mM resulted in more Cd transmitted from roots to shoots. Therefore, factors of translocation were highest in plants receiving 200 NaCl. The Cd absorption efficiency of this halophyte was measured further to assess the potential and efficacy of root Cd absorption. Under non-saline conditions, the AE of *S. fruticosa* was substantially improved with raising Cd stress ( $P < 0.05$ ). NaCl application further elevated the AE of *S. fruticosa* in L-Cd, and H-Cd combined with 200 mM compared with that in L-Cd and H-Cd separately.

**Table 2.** Effect of NaCl on some Cd-phytoremediation parameters in *Salicornia fruticosa*: cadmium root and shoot concentration; bioaccumulation factor (BCF); translocation factor (TF) and absorption efficiency (AE).

Treatments		Accumulated		BCF	TF	AE ( $\mu\text{g g}^{-1}$ )	Phytoremediation strategy
		Cd ( $\mu\text{g plant}^{-1}$ DW)					
		Root	Shoot				
0 mM NaCl	L-Cd	12.3d $\pm$ 0.1	4.4a $\pm$ 0.2	1.1a $\pm$ 0.07	0.42a $\pm$ 0.01	221a $\pm$ 1.5	Phytostabilization
	H-Cd	15f $\pm$ 0.2	6.1b $\pm$ 0.2	1.4a $\pm$ 0.08	0.45a $\pm$ 0.02	404c $\pm$ 2.0	
50 mM NaCl	L-Cd	11c $\pm$ 0.1	8.9c $\pm$ 0.3	5.3b $\pm$ 0.1	0.51b $\pm$ 0.03	340b $\pm$ 1.8	Phytostabilization
	H-Cd	13e $\pm$ 0.2	16d $\pm$ 0.4	6.8c $\pm$ 0.2	0.76c $\pm$ 0.05	611e $\pm$ 2.2	
100 mM NaCl	L-Cd	2.5b $\pm$ 0.05	23e $\pm$ 0.5	7.4d $\pm$ 0.1	1.08d $\pm$ 0.01	552d $\pm$ 2.0	phytoextraction
	H-Cd	2.9b $\pm$ 0.04	43f $\pm$ 0.6	9.9e $\pm$ 0.2	1.12e $\pm$ 0.06	907f $\pm$ 2.1	
200 mM NaCl	L-Cd	1.4a $\pm$ 0.07	55g $\pm$ 0.9	11.3f $\pm$ 0.3	1.23f $\pm$ 0.07	690e $\pm$ 1.4	phytoextraction
	H-Cd	1.9a $\pm$ 0.05	62h $\pm$ 0.8	12.1f $\pm$ 0.3	1.23f $\pm$ 0.07	1303g $\pm$ 1.6	

No Cd was detected in 0-Cd treatment so they were not included in the table; L.-Cd: low cadmium concentration; H.-Cd: high cadmium concentration; DW: dry weight

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#### 2.4. Non-enzymatic antioxidant indices as affected by Cd stress and salinity co-occurrence in *Salicornia fruticosa*

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Plants treated with L-Cd only had a higher content of low molecular weight antioxidant ASA, whereas this trait was depleted by H-Cd (Table 3). NaCl imposition demonstrated no change in ASA content regardless of the dose of NaCl. ASA was exacerbated by salinity and L-Cd co-occurrence and restored to be comparable to 0-Cd salinized plant by combined H-Cd and salinization.

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All the study treatments significantly enhanced GSH content (Table 3). The highest GSH content was recorded for combined NaCl and Cd treatments, then for non-salinized-Cd treated plants, whereas Cd salinized plants demonstrated the lowest GSH elevation. In contrast to GSH (despite their co-regulation), PCs showed a slight reduction under salinity and Cd co-occurrence. PCs were triggered due to alone Cd treatment, whereas no substantial change was recorded for 0-Cd salinized plants (Table 3).

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Cd treatment triggered proline accumulation. This response was Cd-dependent, with H-Cd inducing more proline than L-Cd (Table 3). However, all NaCl-treated plants exhibited a slight non-significant increase in proline accumulation compared to non-salinized plants. NaCl occurrence efficiently ameliorated Cd impact on proline accumulation where salinized Cd treated plants had a reduced proline content compared to plants treated only Cd.

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**Table 3.** Concentrations of ascorbic acid (ASA), phytochelatin (PCs), reduced glutathione (GSH), and proline in leaf of *Salicornia fruticosa* exposed to nutrient solution containing 0, 25, and 50  $\mu\text{g l}^{-1}$  Cd (0-Cd, L.-Cd and H.-Cd, respectively) without or with NaCl 50, 100 and 200 mM. Each value is the average of four replicates  $\pm$  SE. Values bearing different letters are significantly different at  $P < 0.05$  based on Tukey's test.

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Treatments		ASA	GSH	PCs	Proline
		( $\mu\text{mol g}^{-1}$ FW)	( $\text{nmol g}^{-1}$ FW)	( $\mu\text{mol g}^{-1}$ DW)	( $\mu\text{g g}^{-1}$ FW)
0	NaCl mM				
	0-Cd	2.60d $\pm$ 0.05	110a $\pm$ 2.1	10.9a $\pm$ 0.9	5.2a $\pm$ 0.07
	L-Cd	3.5e $\pm$ 0.04	170d $\pm$ 1.5	28.6b $\pm$ 0.4	11.6e $\pm$ 0.4
	H-Cd	1.4a $\pm$ 0.01	201e $\pm$ 1.0	37.3g $\pm$ 0.3	21.0i $\pm$ 0.5
50	NaCl mM				
	0-Cd	2.51 $\pm$ 0.01	113b $\pm$ 2.0	10.5a $\pm$ 0.5	5.1a $\pm$ 0.01
	L-Cd	3.9f $\pm$ 0.02	210f $\pm$ 1.4	27.0b $\pm$ 0.4	9.8d $\pm$ 0.2
	H-Cd	1.6b $\pm$ 0.01	260g $\pm$ 2.3	35.1f $\pm$ 0.2	19.3h $\pm$ 0.3
100	NaCl mM				
	0-Cd	2.61 $\pm$ 0.03	116c $\pm$ 1.1	11.01a $\pm$ 0.5	4.9a $\pm$ 0.01
	L-Cd	4.5g $\pm$ 0.03	269h $\pm$ 2.3	22.2c $\pm$ 0.4	8.5c $\pm$ 0.1
	H-Cd	2.01c $\pm$ 0.01	304j $\pm$ 3.0	31.8e $\pm$ 0.8	16.6g $\pm$ 0.2
200	NaCl mM				
	0-Cd	2.54d $\pm$ 0.01	118c $\pm$ 1.1	10.7a $\pm$ 0.2	5.0a $\pm$ 0.09
	L-Cd	5.1h $\pm$ 0.03	297i $\pm$ 1.6	20.5d $\pm$ 0.2	7.2b $\pm$ 0.07
	H-Cd	2.55d $\pm$ 0.01	366k $\pm$ 2.2	30.2e $\pm$ 0.7	15.4f $\pm$ 0.1

cadmium added; L-Cd: low cadmium concentration; H-Cd: high cadmium concentration; FW: fresh weight; DW: dry weight; g: gram

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### 2.5. Alternations in the capacities of enzymatic antioxidant of *Salicornia frutescens* as affected by Cd stress and salinity co-occurrence

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SOD, APX, and GPX activities, along with an elevation in external Cd concentration (Fig. 3 A, B, and C), were substantially improved. However, elevated supplementation of NaCl showed no change in their pattern compared to non-salinized plants during the 17 days of experimentation. Further enzyme activity was recorded for combined salinity and Cd stressed plants. In contrast to SOD, APX, and GPX, PO activity was reduced significantly by NaCl and Cd co-occurrence following the highest PO activity for alone Cd treatments and minimized PO activity in salinized plants in the former (Fig 3 D).

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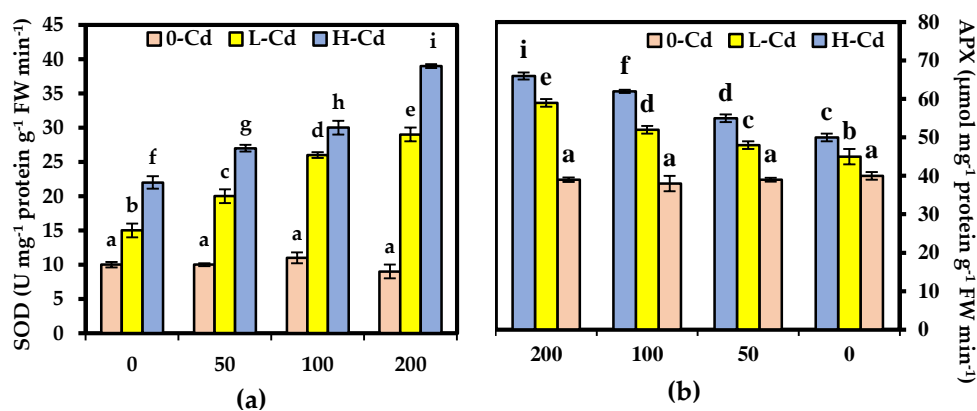
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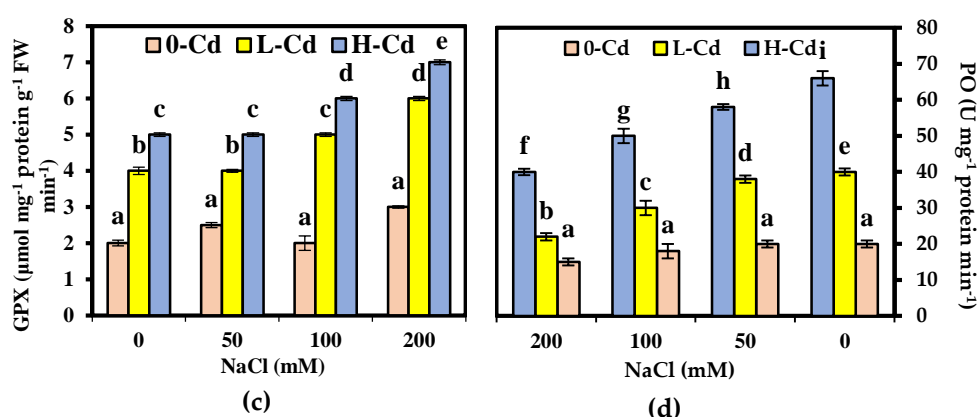
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**Figure 3.** Activities of superoxide dismutase (SOD), Glutathione peroxidase (GPX), ascorbate peroxidase (APX), and peroxidases (POs) (A,B,C and D) in leaf of *Salicornia fruticosa* exposed to nutrient solution containing 0, 25, and 50  $\mu\text{g l}^{-1}$  Cd (0-Cd, L-Cd and H-Cd, respectively) without or with 50, 100 and 200 mM NaCl. Each value is the average of four replicates  $\pm$  SE. Values bearing different letters are significantly different at  $P < 0.05$  based on Tukey's test.

### 3. Discussion

Several halophytes accumulate a high amount of HMs, and exogenous NaCl may have a direct impact on the absorption rate as well as speciation of HMs. Some halophytes are potential candidates for future management of HMs-polluted regions in both saline and non-saline settings due to their ability to maintain plant water status as well as biomass output. To our knowledge, it is the first time to screen *Salicornia fruticosa* for its Cd-phytoremediation potential under the NaCl effect and highlight its operation of multiple mechanisms of essential biochemical tolerance that may provide an advantage to this halophyte concerning HMs as eco-environmental factors.

Several dicotyledonous halophytes demonstrate optimum concentration at an NaCl concentration of 50-250 mM [10]. *Salicornia fruticosa* demonstrated optimal development when exposed to an NaCl of 200 mM, demonstrated maximized tolerance of salt, and could yield an elevated quantity of extraction portion (plant tissue for harvest) even in the h-Cd. Furthermore, accumulation, and Cd concentration in *S. fruticosa* shoots, were significantly improved by the addition of NaCl, indicating its potential for Cd-contaminated saline soils' phytoextraction. Ghnaya et al. [9] demonstrated that salinity substantially boosted *Sesuvium portulacastrum* growth under Cd stress.

Because of the dilution impact, Marco et al. [17] and Lutts and Lefèvre [18] hypothesized that growth stimulation induced by salt in some halophytes might lead to a decreased content of HMs. The saline condition can offer more optimum conditions for the *S. fruticosa* growth, in addition to improving its Cd accumulation and resistance by increasing biomass and assigning more energy to shoot growth. Furthermore, salt enhanced the *S. fruticosa* shoot succulence degree, which is normal for a halophyte. In this experiment, we found a comparable impact since high salt-treated plants (200 mM NaCl) produced more biomass than low salt-treated plants. In this work, NaCl protected *Salicornia fruticosa* against Cd stress and significantly increased biomass at various concentrations of Cd. *Salicornia fruticosa*, a typical halophyte, can tolerate Cd at 25  $\mu\text{g l}^{-1}$ . Plant biomass was not substantially affected by a 50  $\mu\text{g l}^{-1}$  Cd concentration.

Its large aboveground canopy regarding the accumulation of aboveground biomass and vast root system (root length) qualified it for Cd polluted soil phytoremediation. According to Eissa and Abeed [4], plants with strong deep roots may be employed in the phytoremediation of polluted soils. HMs lowered the dry weight of stem and leaf, demonstrating that accumulated components may relatively impair plant development. It is worth noting that whereas NaCl enhanced Cd accumulation, it strangely decreased the growth inhibition induced by HMs.

HMs influence plant roots in the growth medium by alleviating the primary root elongation, hindering secondary growth, and thus adversely impacting the primary organ's absorbing capacity and inorganic nutrients and storing food and nutrients at elevated Cd concentrations. Reducing water absorption reduces the plant's water content [18]. Smýkalová and Zámečnicková [19] revealed that L-Cd modestly promoted root elongation, which was attributed to the fast reaction to Cd and metal ion required for improved root development.

Salinized *S. fruticosa* plants demonstrated shoot succulence equivalent to control plants reaching 200 mM in the current study. Compared to the control group, this capacity to retain water content demonstrates that salt tolerance in this species is partially attributable to its capacity to accomplish an osmotic adjustment, rendering it less prone to stress. Succulence tends to lower transpiration requirements by decreasing leaf heating. Salinity considerably elevated *S. fruticosa* shoot succulence degree, which is typical for a halophyte even in the presence of Cd. In fact, plants cultivated in 100 and 200 mM demonstrated high shoot succulence degrees than plants grown in 50 mM, which may be explained in part by the substantial Na and K accumulation in leaves of plants treated with high elevated salt, which was around double that of plants treated with diminished salt.

Transpiration is a significant factor of the ion transport necessary for salt tolerance since it permits xylem ions to enter from the root's cortical cells and then leave the stem cells. In this approach, the stomatal function may drastically impact salt overturning to the stem, which may enable a stronger succulence of the leaf to drive root extension at decreased osmotic potentials, as in the adaptive response to water stress caused by HMs and salinity. Similarly, previous research has linked Cd translocation, as well as uptake to the transpiration stream, which is also a major driver of nutrient transport and regulated by mass flow [13-20]. Consequently, increased shoot succulence caused by NaCl could be an essential route for the translocation of Cd in *S. fruticosa*, as Cd can quickly be transferred down the water pathways to the shoot. The suppression of transpiration in h-Cd-treated plants alters the accumulation as well as mobility of Cd in shoots, resulting in Cd retention in roots. Hence, significant Cd deposition in the root may result in Cd toxicity, which slows root apex development and causes water absorption dysfunction, which is detected in minimized water content [13]. Increased mass flow and transpiration, and photosynthesis, supply oxygen and energy for active salt mobilization. According to Sruthi et al. [21], HMs may have better mobility in saline conditions owing to enhanced transpiration, resulting in a larger flow of metals into the plant. Fitzgerald et al. [22] detected elevated Pb translocation to the shoots of another halophyte species, *A. tripolium*, when salinity elevated throughout the Suir Estuary, Ireland. Similarly, metal absorption in *A. alba* leaves is enhanced with salinity; therefore, it is plausible to assume that salinity aids HM buildup in *A. alba* leaves [23].

The maintained TOP value of plants cultivated in all NaCl concentrations may imply that these plants are less prone to osmotic stress since they are grown in the preferable salt. Increasing the dosage of Cd alone may cause osmotic stress, emphasizing the significance of increasing the TOP. The presence of salt significantly lowered the TOP value, which was supported by increased HMs-induced proline in alone h-Cd stressed plants. The

changes in TOP value induced by the presence or absence of HMs in saline soil demonstrated that the high adaptation capacity of *S. fruticosa* and NaCl significantly contributes to the modification of plant responses to Cd. According to Hamed et al. [11], a putative *Sesuvium portulacastrum* resistance mechanism to the combination of salinity and Cd indirectly contributes Na to osmotic adjustment. Because of the significant contribution of anions and cations (Na, K, Ca, and Cl had 67 percent of the solute concentration), all halophytes must overcome the problem of osmotic adjustment to water stress (molar in shoot water). As evidenced by the impact of Cd stress detected in elevated TOP, a decrease in these inorganic fractions lead to failed effective cellular osmotic adjustment. Given the elevated Cd concentration in the medium, *S. fruticosa* maintained its halophytic activity and delivered massive Na amounts to the shoots. Furthermore, this species may substitute K with Na for certain activities such as vacuolar osmotic adjustment [9]. As a result, the decreased deposition of K in the shoots of plants exposed to the combined impacts of NaCl and Cd had no negative impact on growth. We further propose that in this halophytic species, salt could defend the xylem vessel against the harmful impacts of Cd, ensuring adequate transfer of water and other soluble materials toward the shoots. Leaves have been observed to collect significant levels of Na and Cl, which are compartmentalized to the vacuole and reduce the osmotic potential of cells in saline circumstances. Na, rather than K, was thus the ion implicated in cell expansion, leaf succulence, as well as shoot growth. Na uptake was antagonistic to the uptake of K uptake in non-Cd salinized plants. It has also been found that NaCl exposure reduces K uptake in *Salicornia europaea* [24]. Following that, decreases in K and Ca levels were detected in our plants at every salinity level. As a result, it appears that *S. fruticosa* halophytic properties are sustained in the presence of salt, and Cd decreased K concentrations amplified this. Cd is more deposited in the roots compared to shoots, suggesting that *S. fruticosa* root system is the principal organ for Cd deposition.

The present research found that, besides reducing the impact of Cd on plant development, NaCl altered Cd absorption as well as transportation from roots to shoots. Interestingly, increasing the NaCl content had no impact on Cd concentration in the root of *S. fruticosa*. Consequently, we can conclude that under saline conditions, the *S. fruticosa* plant tends to transmit Cd to the shoot since both increased water content and biomass may confer this species the ability to withstand and conserve more Cd in the shoot. Hence, Cd levels in the root may be kept low in order to minimize toxicity while maintaining normal root system functioning. Zhang et al. [13] found similar findings for *Suaeda glauca*. The total quantity of Cd deposited in shoots is an essential measure for assessing the potential of Cd extraction in plants, and it is the product of shoot biomass by the concentration of Cd. The capacity of plants to translocate Cd from roots to shoots was assessed utilizing the translocation factor, computed as the ratio of Cd levels in roots and shoots. Elevated TFs and shoot Cd deposition (measured per plant) also indicate that more Cd was transferred to the shoot in *S. fruticosa*, which might be because rising salinity improves Cd mobility by creating Cd-Cl complexes. Cd-Cl complexes are generally thought to be phytoavailable and readily absorbed by plants. Lopez-Chuken and Young [25] discovered a strong link between Cd and Cl and Cd chloride complexes in salt-resistant plants shoots grown in the presence of Cd and NaCl (100 mmol L<sup>-1</sup>). Furthermore, salinity's unequivocal transpiration rate enhances the drag of additional Cd elements into the plants. These different methods may propel *S. fruticosa* to the forefront of Cd phytoextraction, especially at high salt concentrations. Additionally, plants grown in the presence of NaCl and Cd grew normally despite the elevated Cd concentrations in the shoots (>100 g g<sup>-1</sup> DW). Cd hyperaccumulator organisms have this characteristic (shoot Cd >100 g g<sup>-1</sup> DW without growth decrease) [9]. The *S. fruticosa* Cd absorption efficacy was much greater, demonstrating strong capacity and efficient Cd absorption in roots. Salinity promoted Cd root-to-shoot translocation in this halophyte, as shown by a significant rise in TF levels. Under saline circumstances, *S. fruticosa* may acquire strong Cd absorption and translocation capabilities

at the expense of experiencing elevated Cd phytotoxicity. Nevertheless, the findings of this research imply that *S. fruticosa* may also be resistant to Cd, and salinity may boost this halophyte's resistance. The intensity of Cd stress tolerance is quite low at diminished concentrations of salt. Hence, HM immobilization was detected in the root system, which may be regarded as a technique for counteracting HM toxicity in photosynthesizing organs [26, 27], and the selected phytoremediation approach might be phytostabilization. Proline and many proline analogues as well as methylated proline compounds, are the major organic solutes found in halophytes. There is a positive association between treatment and proline, which may be related to these halophytes' ability to accumulate proline, serving as an intracellular osmotic solute. Nevertheless, the small and insignificant proline production in reaction to salt suggests that *S. fruticosa* has the potential to deal with salinity with no expansion of energy or damaging plant organs. Samiei et al. [16] reported similar findings for *Climacoptera crassa*. In addition, Parida and Jha [28] found that 200 mM NaCl did not cause an elevation in proline in *Salicornia brachiata* and that proline generated under elevated salt treatments (400 mM) may be more important in maintaining the enzymatic system in the cytoplasm but not in altering osmotic homeostasis. Previous research has shown a strong relationship between the level of Cd stress in the plant and the quantity of proline produced in halophytes, such as *Climacoptera crassa*, *Sesuvium portulacastrum*, and *Juncus Gerardi* [15, 16, 29]. Consequently, the current work revealed high proline content is one of the key methods of *S. fruticosa* to cope with HM stress rather than salinity, with HMs stress triggering more production of proline than salinity. Nevertheless, in our investigation, the increase in proline at alone h-Cd was associated with a decrease in plant biomass. This apparent proline buildup was not guaranteed to be beneficial; instead, it may negatively influence Cd. As a result, it was possible to determine that proline buildup was a response to excessive Cd exposure rather than a plant response related to imparting metal resistance. Clemens [30] proposed that HMs-induced proline buildup in plants is not directly caused by HMs stress but rather by a disruption in water balance caused by the accumulation of metals. Water stress caused by HMs needs proline generation and biosynthesis, both of which use energy at the expense of cell development. In our investigation, salinity co-occurrence effectively saves energy by lowering the level of proline level. This reduction might be linked to the increased use of carbon skeletons to support development in a hazardous environment.

Consequently, an osmotic adjustment mechanism under Cd stress was developed that primarily depended on the buildup of inorganic cation fractions. We may conclude that proline serves as an osmoticum rather than a ROS scavenger in our study, which is consistent with Wiszniewska et al. [27]. Lefèvre et al. [3], on the contrary, discovered more significant proline contents in the leaves of Cd + NaCl-treated *Atriplex halimus* plants compared to plants treated with NaCl.

Glutathione, as well as ascorbate, are essential non-enzymatic antioxidants in plants primarily via their contribution to the ascorbate-glutathione cycle. Non-enzymatic antioxidants are also vital since they have the potential to scavenge ROS that enzymatic systems cannot detoxify; ROS like  $^1\text{O}_2$ ,  $\text{HO}^*$ . The basal level of non-enzymatic antioxidants in halophytes was twice that in glycophytes. Salinity reduced oxidative stress in *S. fruticosa* by enhancing glutathione, vitamins E and C, and glutathione reductase activity, consistent with Han et al. [31] for the halophyte *Kosteletzkya virginica*.

In HM-stressed plants, glutathione has two functions: it is a primary antioxidant and a precursor of PCs implicated in HM complexation and vacuolar sequestration. The abundance in phytochelatin production in the current research may be explained by abundant glutathione since glutathione is the substrate for phytochelatin biosynthesis. This assumption, nevertheless, contradicts the current results in the case of combined Cd and NaCl

therapy. The NaCl-induced elevation in Cd deposition in the shoot, however, did not result in elevated PCs content in plants treated with Cd + NaCl, and PCs content was more diminished in plants exposed to Cd in the absence of NaCl (Table 2), implying that the plant can adopt other strategies to cope with high Cd content. The first proposed strategy, recently reported by Lutts and Lefe'vere1 [18], was that salinity could upregulate proteins/enzymes genes that contribute to sequestration to non-active compartments as well as metal chelation by binding organic acids, amino acids, and low molecular proteins such as metallothioneins MTs or peptide-like PCs. According to recent research, the expressed sequence database of *Salicornia brachiata* indicates MTs genes' abundance that is mediated mainly by salt, which might be one of the causes of metal tolerance in this plant.

Moreover, Lutts and Lefe'vere1 [18] illustrated that, however, the potential of synthesis PCs and functional PC presence contribute to coping with high HMs doses, metallophones seldom utilize this expensive strategy to detoxify HMs, but they instead overproduce organic acids. Another suggested mechanism that may be integrated with avoiding toxic cellular Cd in *Salicornia fruticosa* under combined Cd and salinity is through binding by inorganic anion, chloride. High NaCl concentration increases Cd's ratio to the mineral fraction (chlorides). Complexation of Cd with Cl results in Cd-Cl formation. Cd-Cl is widely known in halophytes under combined Cd and salinity [15]. Hence *Salicornia fruticosa* with high salt concentration treatment had higher chloride concentration than low salt-treated plant. Thus, reduction in PC content was more for the high salinized plant as herein chloride binding mechanism is the substitute for chelating by organic compound PCs.

On the contrary, Hamed et al. [11] and Ghnaya et al. [9] proposed that treatment with NaCl could alleviate the most toxic form of Cd ( $Cd^{2+}$ ) in favor of another form bound to chloride anions. Accordingly, all prior findings have demonstrated that improved binding of Cd chloride anion caused by NaCl could play an important role in ameliorating Cd tolerance by salinity and is also considered a protective strategy against Cd. More extensive investigation on this topic is consequently needed to be asserted.

Against different stresses, plants secrete various scavenging enzymes like SOD, APX, and GPX. The PO activity consumed any hydrogen peroxide formed from SOD activity or other pathways. Our study exhibited a similar ROS quenching activity pattern, such as SOD and  $H_2O_2$ -metabolizing enzymes (APX and GPX). The similar exhibited pattern was probably due to their co-regulation of underdeveloped NaCl concentrations. Moreover, displaying non-noticeable responses in scavenging enzymes along the experiment period and up to 200 mM NaCl compared with control non-salinized plants indicated no excess accumulation of ROS and that plants were not suffering from oxidative stress. For halophytes, it was reported that the concentration of NaCl, which first causes a significant oxidative injury and increased lipid peroxidation, thus inducing antioxidant activity, was 400 mM in *Salicornia brachiata* species and *Suaeda salsa* after 14 and 7 days of salinity exposure, respectively [32]. Accordingly, this study's salt concentration (200 mM) was relatively low to trigger severe oxidative stress for this halophytic species, where ROS accumulation in halophytes arises above a certain threshold that vastly differs from glycophytes. The ROS generated from HM stress were trapped with the co-function of antioxidant enzymes. Therefore, *S. fruticosa* induced enhancement in their activities. Salt co-occurrence impose further elevated levels of ROS scavenger enzymes activities SOD, APX, and GPX and their substrates (parallel to the increase of their substrates ASA and GSH), revealing a powerful antioxidant system that constrained the exacerbation of the toxic ROS. The high salt-treated plants (100 - 200 mM) had much more enzyme activity and less Cd toxicity than low salt-treated ones (50 mM) correlated with better plant growth.

Unlike the other studied, PO in the present study showed heterogeneous activities, whereas PO (involved in lignin biosynthesis in the plant cells) activity was activated in response to HM stressors because it suppresses HMs in plants via lignin production [16]. Lignification in the cell wall induced by PO activity may involve the destruction of the photosynthetic apparatus due to aging and senescence, revealing restriction of the growth of stressed Cd-impacted cells, resulting in aged leaves, not adequate cells displaying suitable lignin content, and diminished PO that may be in case of 0-Cd salinized plant leaves, implying that salinity may ameliorate this effect by sufficiently reducing PO activity in Cd affected plant. According to Zhou et al. [33], concurrently applied Cd + NaCl enhanced plant all-senescence-related metrics, as shown by a considerable reduction in IPO and SPO. The significant increase in PO in the presence of HMs may be a valuable diagnostic of soils polluted with HM, which aligns with the results of Nimptsch et al. [34], who recommended this enzyme as a feasible perspective biomarker for identifying HMs contamination.

#### 4. Materials and Methods

##### 4.1. Plant material

*Salicornia fruticosa* dried plants were collected from Miami island, Alexandria (31°16'04"N 29°59'43"E) at the coastline's salt marshes of the Mediterranean in winter 2021. Seeds were collected from dried stems before being preserved to be germinated during summer 2022 in pots filled with the collected soil and irrigated on alternate days for experimental purposes.

##### 4.2. Hydroponic culturing

The hydroponic medium was utilized to decrease the soil's confounding factors, including alternations in soil water potential, soil pH, and soil Cd chemistry induced by adding salt. It was prepared using ¼ Hoagland's [36] and supplemented with 0, 50, 100, or 200 mM NaCl. According to Marco et al. [17], the plant exhibits a great tendency to show optimal growth at 200 mM NaCl, but at 300 mM, it was noticeably decreased, so the applied NaCl doses herein never exceed this limit (200 mM). The 2-week-old healthy plants were grown in the nutrient solution for seven days for acclimation under 20–30 °C temperature, 16 h light and eight-hour dark photoperiodic cycle at room temperature with a fifty percent relative humidity as well as light intensity of 350  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Four sets of ten plants with identical size, fresh weight (0.9 ~ 1.2 g per plant), and uniform health were grown in 250 ml nutrient solution for each treatment. An HM (stock solution) was added to obtain the final concentrations of 25  $\mu\text{g l}^{-1}$  CdCl<sub>2</sub> .H<sub>2</sub>O (L-Cd) and 50  $\mu\text{g l}^{-1}$  CdCl<sub>2</sub> .H<sub>2</sub>O (H-Cd) in the nutrient solutions. The Cd-untreated plants received 0  $\mu\text{g l}^{-1}$  CdCl<sub>2</sub> .H<sub>2</sub>O was mentioned as 0-Cd. After ten days of treatment, the roots emerged for 15 min in 25 mM EDTA-Na<sub>2</sub> solution for removing Cd from the root surface. Root length was recorded and expressed in cm. Afterward, plants were divided into roots (belowground) as well as shoots (up-ground). The samples were immediately dried at 70 °C in an oven to constant weight before being grounded and utilized to evaluate the biomass. The treated plants' green leaves were rinsed with sterile distilled water, harvested, and frozen in liquid nitrogen before being stored at -80 °C.

##### 4.3. Shoot succulence degree (SSD)

SSD was calculated by measuring shoot fresh weight and its dry weight from four independent plants for each treatment [37].

SSD (g.g-1) = Shoot fresh weight/ Shoot dry weight

- 4.4. *Transpiration rate* 539
- The transpiration rate was assessed using the method of Llanes et al. [38]. Plants were maintained for 24 hours under the same conditions as indicated for hydroponic cultures, and the amount of solution used was measured. The amount of solution absorbed was determined after 24 hours under the same photoperiod circumstances as the hydroponic culture to assess the volume of transpired water. The ml of transpired water per leaf weight was estimated, and the result was reported ml. g<sup>-1</sup> of FW (leaves).
- 4.5. *Total osmotic potential (TOP) determination* 546
- TOP was estimated. The leaf sap was prepared using the technique proposed by Abeed and Dawood [39] by crushing fresh leaves, followed by centrifugation for 15 minutes at 10,000g, and the resulting extract was utilized to measure the osmotic potential (s) utilizing TridentMed's 800 CL Osmometer. The osmotic potentials (bar) were then calculated using Walter's tables [40].
- 4.6. *Proline* 552
- As illustrated by Bates et al. [41], free proline was assessed in dry leaves. Homogenization of leaf samples was done in 3% sulfosalicylic acid (6 ml) before centrifugation at 10,000×g. The supernatant (2ml) was blended with glacial acetic acid (2ml) as well as ninhydrin. Samples heating was done for one hour at 100 °C before cooling to room temperature. Extraction of the reaction mixture was performed with toluene (4 ml), and the content of free toluene was estimated to be 520 nm as well as expressed as milligrams per gram (dry weight).
- 4.7. *Enzymatic as well as non-enzymatic antioxidant capacities* 560
- Non-enzymatic antioxidants like ASA as well as reduced glutathione (GSH): The supernatant of freshly ground leaves in trichloroacetic acid was used to quantify ASA and GSH using procedures developed by Jagota and Dani [42] and Ellman [43], respectively. According to Nahar et al. [44], PCs were calculated by subtracting the quantity of GSH from non-protein thiols, which were produced by combining the supernatant of leaves crushed sulfosalicylic acid with Ellman's reaction mixture [43].
- The homogenization of each treatment's fresh leaves was done in a mortar and pestle with sodium phosphate buffer 0.05 M (pH 7.5). The centrifugation of homogenate was done for 20 minutes at 10,000 r/min, and the supernatant was used to analyze leaf enzymatic potential as identified by scanning Glutathione peroxidase (GPX/EC.1.11.1.9, μmol mg<sup>-1</sup> protein g<sup>-1</sup> FW min<sup>-1</sup>), ascorbate peroxidase (APX; EC1.11.1.11, μmol mg<sup>-1</sup> protein g<sup>-1</sup> FW min<sup>-1</sup>), and (SOD/EC.1.15.1.1, μmol mg<sup>-1</sup> protein g<sup>-1</sup> FW min<sup>-1</sup>) by the method of Flohé and Günzler [45], Abeed et al. [46], and Abeed et al. [47], respectively. The peroxidase activity (PO, U mg<sup>-1</sup> protein min<sup>-1</sup>) was quantified following enzyme extraction from leaves, as described by Ghanati et al. [48]. The PO activity was assessed according to the absorbance increase at 470 nm utilizing 168 mM guaiacol in H<sub>2</sub>O<sub>2</sub> (30 mM) and phosphate buffer (100 mM). The absorbance change was altered to units (U) using 26.6 mM<sup>-1</sup> cm<sup>-1</sup> extinction coefficient.
- 4.8. *Cation assay* 579
- Grounding desiccated samples was performed until obtaining a fine powder with a pestle as well as porcelain mortar before being digested in a 4:1 (v/v) solution of HNO<sub>3</sub>-HClO<sub>4</sub>. An atomic absorption/flame emission spectrophotometer was used to measure the

amounts of Ca and Cd (Shimadzu- model AA-630-02). The K and Na content in the same homogenate was measured following the flame emission method (Carl-Zeiss DR LANGE M7D flame photometer) [39]

#### 4.9. Cd accumulation characteristics

The halophyte *Salicornia fruticosa* phytoremediation potential was according to [49, 50] via calculation of the following indicators:

- 1- Bioconcentration factor (BCF), enrichment factor = Cd concentration in the plant/ Cd concentration in external medium
- 2- Translocation factor (TF) = Cd concentration in the shoot/ Cd concentration in the root
- 3- Cd absorption efficiency (AE) = Cd accumulation in the whole plant/ Root biomass

#### 4.10. Statistical analysis

The obtained data were evaluated utilizing the 21st of SPSS software. The one-way evaluation of variance was followed by a post hoc test (Tukey's multiple range tests). The level of statistical significance was set at ( $p < 0.05$ ).

## 5. Conclusions

Identifying the mechanisms of halophyte salinity tolerance in conjunction with other co-occurring limitations like HMs, drought, flooding, nutrient deficiencies, and HMs, would assist in utilizing halophytes for saline land revegetation, in addition to providing new interpretations that could be considered in future plant breeding for salt-affected agricultural lands. Since *S. fruticosa* is a halophyte, salinity tolerance capabilities may indirectly lead to HM tolerance. Without Cd, the variations in most of the investigated parameters between low salt and high salt-affected plants were relatively minor, owing primarily to the positive osmotic potential of salt. Cd toxicity was substantially more severe in low salt-treated plants than in high salt-treated plants. Cd toxicity mechanisms in *S. fruticosa* include significant disruption of plant water interactions as well as the activation of aging and senescence-mediated enzymes. *S. fruticosa* demonstrated adequate transpiration rate and shoot succulent degree, which may aid in the maintenance of plant water status, as well as a large amount of upground biomass production and deep-rooting, and efficient management of oxidative stress via elevated levels of Asc + GSH and enzyme activity modulation. Furthermore, *S. fruticosa* sequesters heavy metals intercellularly rather than in the vacuole, as shown by decreased PCs by salinity, indicating a positive role in phytoextraction. The salt-induced increase in Cd tolerance refers to the possibility of utilizing *S. fruticosa* for Cd phytoextraction. In the current investigation, the efficacy of employing *S. fruticosa* to remediate and enhance HM-contaminated saline soils is restricted to the kind of HM and the dosages used. More research should be done on the effectiveness of *S. fruticosa* in removing additional HMs from salty soils and other surfaces with high electrical conductivity.

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**Data Availability Statement:** In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section “MDPI Research Data Policies” at <https://www.mdpi.com/ethics>. If the study did not report any data, you might add “Not applicable” here.

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