



Research article

Integrated physiological and molecular approaches to study salt-induced adaptation in halophytes collected from extremely saline lands

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Abstract

Diverse halophytes were collected across the extremely salt-affected lands of Haryana to explore their salt potential. Higher levels of Na⁺ and Cl⁻ were observed in leaves (3.20 and 4.55%) than in stem (0.78 and 1.09%) and root (0.81 and 0.80 %). Interestingly, these plants also accumulated higher concentrations of K⁺ for their survival. Similarly, Ca²⁺ accumulation was higher in leaves (1.57%) than in stems (1.15%) and roots (1.18%). The salt tolerance potential of these halophytes was assessed by examining the ratio of accumulation of toxic ions, namely Na/K, Na/Ca, and Na+Cl to K+Ca, resulting in their characterization and ranking from highly salt tolerant to least tolerant. The utilization of this ionic accumulation index has the potential to serve as a criterion for the elimination of surplus toxic ions from soil. The halophytes employed adaptive mechanisms by synthesizing osmolytes, such as proline, soluble sugars, and soluble proteins, in both leaves and roots. The upregulation of genes P5CS and NHX1, responsible for proline synthesis and membrane transportation, further confirmed the halophytes' ability to tolerate high salinity. The findings of these studies indicated the potential for domesticating salt-tolerant halophytes in salt-affected soils.

Keywords: Halophytes, Ion accumulation, Osmolytes, Salinity

Introduction

In the era of climate change, decreased precipitation and increased evapotranspiration, along with the soil salinity and poor groundwater resources, affect morphological, anatomical, and physiological traits/strategies, which ultimately reduce the yield and productivity of crops (Soni *et al.*, 2020; 2021; Dhansu *et al.*, 2022; Souguir *et al.*, 2019; Louhaichi and Hassan, 2023). Globally, around 1000 million hectares of land affected by salinity pose a risk to agricultural processes and restrict crop production. In India, the combination of salinity and water logging has resulted in the conversion of nearly 20% of irrigated lands into barren areas, rendering them uncultivable in extreme cases, particularly in arid and semi-arid regions. These twin problems of excess salts in soil and water logging are threatening the sustainability of agricultural production in large parts of Haryana state in the country. As per a report of the Haryana Kisan Aayog, more than 50,000 hectares are affected by soil salinity and water logging, having shallow water table depth of less than 1.5 meters. Salinity in waterlogged areas is 35 to 40 ds/m, although

the normal limit is 4 ds/m. Most of the water-logged saline soils are found in the central inland depression basin of the state, including Rohtak, Hisar, Jhajjar, Bhiwani and Palwal districts.

One of the sustainable goals for enhanced food security and livelihood includes efficient management of these salt-affected soils. Given the escalating issue of soil salinization and water scarcity, there is now a significant focus on investigating the tolerance mechanisms of halophytes, which are naturally salt-tolerant plants (Mangalassery *et al.*, 2017). Halophytes are a group of plants that have the ability to survive and flourish in challenging saline environments by undergoing various physiological, biochemical, and molecular adaptations. These alterations may include the biosynthesis of certain osmolytes or differentiation of toxic ions across various cellular compartments, thus maintaining ion homeostasis (Kumar *et al.*, 2016, 2018, 2019; Mann *et al.*, 2019a). The mechanism of salt tolerance is a complex and multigenic process mainly governed by ion homeostasis through transport and cellular location of Na⁺, K⁺, Ca²⁺ and Cl⁻ in

addition to the various other adaptive features. A lot of information is available for salt tolerance mechanism of model plant species *Arabidopsis thaliana* (Borsani et al., 2001) and *Thellungiella halophila* but these two plant species are short-lived and could survive at lower levels of salinity. Hence, the real *in situ* picture of true halophytes need to be elucidated for better salt adaptive strategies. Although many halophytes, including Suaeda (Guo et al., 2019), *Salicornia brachiata* (Jha et al., 2009), *Urochondra setulosa* and *Dichanthium annulatum* (Mann et al., 2021), *Sporobolus marginatus* (Mann et al., 2019b), Salvador, *Aegilops tauschii* (Mansouri et al., 2019), Amaranthus and Chenopodium species and *Atriplex* etc (Kumar et al., 2021) have been explored for their salt tolerance mechanisms but still a single adaptive strategy through physiological and biochemical interactions and the gene network analysis at high salt concentrations need to be explored. Transcriptomics studies on salt-related pathways/genes in halophytes are being explored now for the development of climate-smart crops through genetic engineering. Differentially expressed genes in *S. salsa* at 30% NaCl (Guo et al., 2019) and in *Beta vulgaris* at 200 and 400 mM NaCl (Lv et al., 2018) also identify the salt tolerance mechanism in leaves and roots. A major gene for Na⁺ ion exclusion, SOS2, was found to be involved in salt tolerance in mutated *Arabidopsis* plants (Ohta et al., 2003). *S. alterniflora* maintains the salt tolerance ability by regulating the uptake and accumulation of Na⁺, K⁺ and Cl⁻ through up-regulation of selective stress-related transporters (Bedre et al., 2016). In view of these facts, this study was planned to assess the salt tolerance potential of the collected halophytes based on the key traits for ion partitioning and osmolyte accumulation and the corresponding gene expression.

Materials and Methods

Experimental site and plant materials: A survey was carried out during 2017-18 in the saline lands of district Hisar, Jhajjar and Rohtak in Haryana state of India. Finally, after considering the salinity levels, four sites representing high salinity were selected for study- (1) Barren field of ICAR-CIRB, Hisar (2) Village Dhandlan, Jhajjar (3) Village Dighal, Jhajjar and (4) Village Chuliyana, Rohtak. These sites turned into wastelands due to the non-cultivation of crops in lieu of high salinity. Soil samples were collected from these sites at three different soil depths *viz.* 0 to 15, 15 to 30 and 30 to 45 cm in three replicates and further analysis was done at ICAR-CSSRI, Karnal. Soil samples were air-dried and passed through a 2 mm sieve in the laboratory for undertaking various analyses. Soil EC_e (EC of saturation extract) and pH_s (pH of saturation extract) were determined using EC-meter and pH meter. A total of 16 halophytic plant species were collected as *Heliotropium ramossimum*, *Withania somnifera*, *Aerva tomentosa*, *Atriplex amnicola*, *Desmostchya*

bipinnata, *Portulaca portucalustrum*, *Atriplex nummularia*, *Atriplex lentiformis*, *Tamarix aphylla*, *Arundo donax*, *Suaeda fruticosa*, *Suaeda nudiflora*, *Dysphania ambrosioides*, *Aelurolopus lagopoides*, and *Dichanthium annulatum* from different salinity sites across Rohtak, Jhajjar and Hisar district of Haryana. In addition to these, germplasm of four other halophytic plants *i.e.* *Urochondra setulosa* and *Sporobolus marginatus*, taken as highly salt tolerant check plants as per our previous reports which were originally collected from extreme saline-sodic Kachhch plains, Rann of Kachhch, Bhuj, Gujarat and *Leptachloa fusca*, from ICAR-CSSRI, Regional Research Station, Lucknow and *Chenopodium quinoa* from ICAR-CSSRI repository, Karnal were also included in the study. One set of collected halophytes were also established in porcelain pots under control conditions (without salinity) at ICAR-CSSRI, Karnal.

Ions and osmolytes analyses: The ionic contents of Na⁺, K⁺, and Ca²⁺ were determined using oven-dried and acid-digested samples of leaves, stems, and roots separately (Arora et al., 2018). The Na⁺ and K⁺ contents were measured with a flame photometer (Flame Photometer 128, Systronics) and Ca²⁺ with an atomic absorption spectrophotometer (Z Express 8000, Spectrum Technology) using a standard solution. The ionic contents, Na⁺, K⁺ and Ca²⁺ were expressed in percent (%) value after standard calculations. Chloride content (Cl⁻) of leaf, stem and root tissue was determined volumetrically by a modified method of Chhabra (1973). The major osmolytes involved in salt tolerance, like proline, total soluble sugars and total soluble proteins, were assessed in leaves as well as roots. Proline content (mg g⁻¹ fresh weight) was estimated by the method of Bates et al. (1973). Bradford reagent (Bradford, 1976) was used for the estimation of total soluble proteins (mg g⁻¹ FW) at 595 nm. Total soluble sugars (mg g⁻¹ FW) were measured at 625 nm using anthrone reagent by the method of Yemm and Willis (1954). The salt tolerance potential of these halophytes was calculated based on the ratio of accumulation of toxic ions, *i.e.*, Na+Cl, against beneficial ions K+Ca (Kumar et al., 2021) and were characterized as highly tolerant to moderately salt tolerant.

Real time PCR analysis: Fresh leaf tissue (~80 mg each) was used for the isolation of total RNA using RNA-Xpress™ Reagent (Hi-Media) according to manufacturer's instruction and treated with RNase-free DNase I (Thermo Scientific) to remove residual DNA. RNA was reverse transcribed using iScript™ cDNA Synthesis Kit (Bio-rad) according to the manufacturer's instructions. The reaction was carried out in 20 µL mixture by adding 1-µg of template RNA, 1-µL reverse transcriptase and 4 µL of reaction buffer (containing oligo-dT) in 0.2 mL centrifuge tubes. The volume was made up to 10 µL with DEPC-treated water and heated the mixture at 65°C for

5 minutes followed by quick chill on ice. The tubes were incubated at 25°C for 10 minutes and 46°C for 30 minutes and finally reaction was terminated by heating at 95°C for 1-minute. cDNA was synthesized in Thermal Cycler (Biometra TAdvanced, Analytik Jena) with initial priming at 25°C for 5 minutes, followed by reverse transcription at 46°C for 20 minutes. The reaction was terminated at 1°C for one minute and cDNA was stored in the refrigerator. The gene-specific primers (Table 1) were designed in-house using the Primer Quest Tool (<https://eu.idtdna.com/Primerquest/Home/Index>) from the available sequences of halophytes *D. annulatum* (Accession No. PRJNA665324) and *U. setulosa* (Accession No. PRJNA561259) for the major osmolyte proline (P5CR) and the membrane Na⁺/H⁺ transporter (NHX1).

Real-Time quantitative expression analysis was done as per the manufacturer's protocol of Sso Advanced Universal SYBR Green Supermix (Bio-Rad) in three technical replicates with three biological replicates. 100 ng cDNA was taken as a template, 1x Green Supermix was mixed with 500 mM forward and reversed gene-specific primers and total volume of 20 µL was made up with NFW (nuclease-free water). Quantitative reaction was carried out in real time-PCR (Bio-Rad CFX-96™) following polymerase activation and DNA denaturation at 95°C for 20 to 30 sec. Further amplification of 35 to 40 cycles included denaturation at 95°C for 5 seconds, annealing, extension and plate reading at 60°C for 15 to 30 seconds. The actin gene was used as a housekeeping gene for normalization. Quantitative gene expression was calculated by applying the 2^{-ΔΔCt} method (Livak and Schmittgen 2001). Out of 19 halophytic species collected, five contrasting grass halophytic species, i.e., *Arundo donax*, *Aeluropus lagopoides*, *Urochondra setulosa*, *Sporobolus marginatus* and *Leptachloa fusca* were selected based on the salt tolerance potential for real time PCR analysis.

Statistical analysis: All the data were subjected to variance analysis using the SAS (Version 9.3, SAS Institute Inc., Cary, NC, USA). TUKEY's Honestly significant difference was applied at 5% probability level to compare the mean differences.

Results and Discussion

Ion (Na⁺, K⁺, Ca²⁺ and Cl⁻) concentrations: In the majority of salt-affected soils, the dominating cation was

Na⁺ with Cl⁻ as the dominating anion. Salt tolerance is mainly associated with the reduced Na⁺ and Cl⁻ loading into the xylem, as both these ions are metabolically toxic to the plants and present in high concentrations in saline soils (Tavakkoli *et al.*, 2011). In addition to this, higher Na⁺ alters the concentration of other ions, particularly K⁺, Ca²⁺ and NO₃⁻ which results in ion imbalance (Kumar *et al.*, 2016). Keeping in mind the behavior of Na⁺ and Cl⁻, partitioning of Na⁺, K⁺ and Cl⁻ ions was estimated in leaves, stems and roots (Table 2). Na⁺ was the most notorious cation which significantly varied among different halophytic species and different plant parts. It was observed that leaves had a higher mean accumulation of Na⁺ (3.20% of dry weight (DW)) in comparison to stem (0.78% DW) and root (0.81% DW). In leaves, highest Na⁺ was accumulated in *S. nudiflora* (5.44% DW) than the check *U. setulosa*, *D. ambrosioides*, *A. nummularia*, and *S. fruticosa*, whereas *H. ramosimum*, *W. somnifera* and *A. tomentosa* accumulated the lowest (1.07% - 1.17% DW). All the three *Atriplex* species, i.e. *A. amnicola*, *A. nummularia* and *A. lentiformis* also showed good Na⁺ hyper-accumulator abilities and accumulated 3.48, 4.93, and 3.07% Na⁺, respectively (Table 2). In the stem portion, *T. aphylla* accumulated the highest Na⁺ (1.92% DW) followed by *S. fruticosa*, *A. lentiformis* and *L. Fusca*, while *A. tomentosa* accumulated the lowest (0.16% DW). In the case of roots, *A. nummularia* accumulated the highest Na⁺ (1.73% DW) followed by *A. lentiformis* and *S. fruticosa*, whereas the lowest accumulation was noted in *A. donax* (0.29% DW) and *D. ambrosioides* (0.31% DW), which were statistically at par followed by *A. tomentosa* (0.34% DW). The mature leaves of *Atriplex* species accumulated more Na ions than roots and shoot and roots tended to maintain the lowest Na⁺ concentration (Devi *et al.*, 2016). Sodium metabolism in plants is very tricky since, on the one hand, Na⁺ is required for maintaining turgor and osmotic potential, and on the other hand, excess Na⁺ is harmful (Kumar *et al.*, 2018; 2019). Overall, it was found that most of these halophytic plants translocated Na⁺ from the root to the leaf, which is an important strategy under salt stress (Maathuis *et al.*, 2014; Mann *et al.*, 2019b). This showed that these plants, particularly hyper-accumulator plants, could achieve the balance between intake of inorganic osmoticum and efficient sequestration of potentially harmful ions by effective cellular Na⁺ partitioning (Maathuis, 2014). The quantitative expression of the Na⁺/H⁺ transporter (Fig 1) also showed the highest expression in *S. marginatus*

Table 1. List of primer sequences for real time quantification

Gene	Forward primer	Reverse primer	Amplicon length
<i>Actin</i>	CGTACAACTCCATCATGAAG	GAGCCACCAATCCAAACACT	237 bp
P5CR	ATTGGTCTGGTCAACGACG	ATCTCTCCCAAGCACATGAC	91 bp
<i>NHX1</i>	GGCATAATGTGACAGAGAGCTC	GGCATCCATCCCAACATATAGG	104 bp

Table 2. Na⁺, K⁺ and Ca²⁺ ion partitioning in different plant parts of halophytes collected from saline lands of Haryana

Halophytes/traits	Na ⁺ content (% DW)			Cl ⁻ content (% DW)			K ⁺ content (% DW)			Ca ²⁺ content (% DW)		
	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root
<i>Heliotropium ramosissimum</i>	1.08 ^k	0.98 ^{cd}	0.67 ⁱ	6.10 ^{cd}	0.82 ^j	0.73 ^{fg}	1.34 ^{ij}	0.55 ^h	0.95 ^{efg}	1.12 ^{gh}	0.35 ^{ijkl}	0.51 ^g
<i>Withania somnifera</i>	1.07 ^k	0.63 ^f	0.74 ^{gh}	3.02 ^h	0.74 ^{ij}	0.18 ^m	1.68 ⁱ	0.37 ^{ij}	0.70 ^{fg}	1.05 ^{gh}	0.21 ^m	0.48 ^g
<i>Suaeda nudiflora</i>	5.44 ^a	1.01 ^{cd}	0.99 ^d	6.59 ^{abc}	0.69 ^{jk}	1.01 ^e	8.74 ^b	2.14 ^d	4.17 ^a	1.56 ^a	0.68 ^{de}	1.04 ^b
<i>Aeluropus logopoides</i>	2.39 ^h	0.18 ^h	0.80 ^{fg}	5.72 ^{de}	1.06 ^{gh}	1.13 ^d	3.11 ^h	1.94 ^e	2.20 ^{bcd}	1.48 ^{ab}	0.42 ^{hijk}	0.91 ^{de}
<i>Suaeda fruticosa</i>	4.69 ^c	1.32 ^b	1.37 ^c	6.39 ^{bc}	1.91 ^a	1.39 ^a	10.59 ^a	3.49 ^a	2.38 ^{bc}	1.32 ^{cde}	0.81 ^{bc}	0.86 ^{de}
<i>Aerva tomentosa</i>	1.17 ^k	0.16 ^h	0.34 ^l	5.30 ^{ef}	0.98 ^h	0.43 ^k	1.08 ^j	0.33 ^j	0.41 ^g	1.17 ^{efg}	0.38 ^{ijkl}	0.67 ^f
<i>Atriplex amnicola</i>	3.48 ^f	0.98 ^{cd}	0.82 ^f	5.36 ^{ef}	1.78 ^b	1.26 ^b	6.56 ^{cd}	3.40 ^a	1.68 ^{cde}	1.35 ^{bcd}	0.57 ^{ef}	0.94 ^{cd}
<i>Atriplex nummularia</i>	4.93 ^b	0.76 ^e	1.73 ^a	4.49 ^g	0.82 ^j	1.15 ^d	5.92 ^e	2.50 ^c	2.49 ^{bc}	1.21 ^{def}	0.49 ^{fgh}	1.03 ^{bc}
<i>Atriplex lentiformis</i>	3.07 ^g	1.04 ^c	1.66 ^b	5.29 ^{ef}	1.09 ^{fg}	1.22 ^{bc}	6.93 ^c	2.94 ^b	1.07 ^{efg}	1.49 ^{ab}	0.54 ^{fg}	0.84 ^{de}
<i>Arundo donax</i>	4.47 ^d	0.56 ^f	0.29 ^l	3.30 ^h	0.49 ^j	0.24 ^l	6.40 ^d	1.28 ^f	2.55 ^b	0.98 ^{hi}	0.87 ^b	0.81 ^e
<i>Tamarix aphylla</i>	3.82 ^e	1.92 ^a	0.89 ^e	7.13 ^a	1.61 ^c	1.20 ^c	8.58 ^b	2.31 ^d	1.35 ^{def}	1.13 ^{fg}	0.76 ^{cd}	0.91 ^{de}
<i>Dysphania ambrosioides</i>	5.07 ^b	0.42 ^g	0.31 ^l	6.83 ^{ab}	1.50 ^d	0.51 ^j	6.48 ^{cd}	1.26 ^f	2.50 ^{bc}	0.89 ^j	0.48 ^{fghi}	0.68 ^f
<i>Portulaca portulacastrum</i>	1.62 ^j	0.33 ^g	0.53 ^j	1.76 ^k	0.19 ⁿ	0.47 ^{jk}	1.24 ^{ij}	0.29 ^j	1.00 ^{efg}	0.37 ^j	0.29 ^{lm}	0.34 ^h
<i>Desmostachya bipinnata</i>	1.76 ^j	0.57 ^f	0.69 ^{hi}	1.79 ^{jn}	0.40 ^{mn}	0.28 ^l	1.49 ^{ij}	0.54 ^{hi}	1.04 ^{efg}	1.08 ^{fgh}	0.42 ^{hijk}	0.43 ^{gh}
<i>Sporobolus marginatus</i>	2.30 ^h	0.79 ^e	0.69 ^{hi}	2.12 ^{jk}	1.88 ^a	0.55 ⁱ	4.10 ^g	0.31 ^j	1.13 ^{efg}	1.47 ^{ab}	0.45 ^{fghi}	1.05 ^{ab}
<i>Leptochloa fusca</i>	3.93 ^e	1.04 ^c	0.83 ^f	2.37 ^{ij}	0.61 ^k	0.63 ^h	3.90 ^g	0.92 ^g	1.04 ^{efg}	1.39 ^{bc}	0.72 ^{cd}	1.15 ^a
<i>Urochondra setulosa</i>	5.42 ^a	0.62 ^f	0.78 ^{fg}	4.81 ^{fg}	1.61 ^c	0.71 ^g	8.41 ^b	0.64 ^h	2.23 ^{bc}	1.45 ^{abc}	0.68 ^{de}	1.09 ^{ab}
<i>Dicanthium annulatum</i>	3.10 ^g	0.92 ^d	0.42 ^k	2.74 ^{hi}	1.16 ^f	0.76 ^f	1.14 ^j	0.35 ^j	0.68 ^{fg}	1.20 ^{ef}	0.31 ^{klm}	0.85 ^{de}
<i>Chenopodium quinoa</i>	2.03 ⁱ	0.62 ^f	0.94 ^{de}	5.28 ^{ef}	1.32 ^e	1.39 ^a	5.43 ^f	1.25 ^f	1.02 ^{efg}	1.57 ^a	1.18 ^a	0.62 ^f
General mean	3.20	0.78	0.81	4.55	1.09	0.80	4.90	1.41	1.61	1.23	0.56	0.80
CV (%)	1.61	4.20	2.27	4.21	2.64	1.87	3.00	4.01	17.46	7.34	12.18	7.64
SE(d)	0.042	0.027	0.015	0.156	0.023	0.012	0.120	0.046	0.229	0.073	0.056	0.050
HSD (P<0.05)	0.1594	0.1013	0.0571	0.592	0.0886	0.0462	0.4548	0.1748	0.868	0.149	0.2102	0.1013

Means with at least one letter common are not statistically significant (P≤0.05) using Tukey's HSD Test

followed by *U. setulosa*, *Aeluropus* and *L. fusca* with the lowest expression in *A. donax*. The fold change values of *NHX1* represent higher activity of membrane transporter Na^+/H^+ which controls the accumulation of Na. Higher *NHX* activity lowers the Na/K ratio. *NHX* proteins are ubiquitous membrane transporters for the sequestration of additional Na^+ in vacuoles or the removal of excess Na^+ from the cells (Himabindu *et al.*, 2016). The expression of the gene encoding Na^+/H^+ exchanger was up-regulated along with the salinity stress in *D. annulatum* and *U. setulosa* (Mann *et al.*, 2019; 2021). Similarly, the Arabidopsis ABC transporter, AtABCG36, was involved in adaptation to salinity stress by reducing the shoot sodium content (Kim *et al.*, 2010). Na^+ pumping into the vacuole against the electrochemical gradient for ion homeostasis through Na^+/H^+ transporter has also been reported in halophytic plant *Mesembryanthemum crystallinum* L (Barkla *et al.*, 1995).

Besides Na^+ toxicity, Cl^- is another important micronutrient that helps in cell turgor and pH homeostasis act as a co-factor in photosynthesis, maintaining potential across the membranes and catalysts for some enzymes (Tavakkoli *et al.*, 2011, Kumar *et al.*, 2019). In our studies, it was observed that Cl^- accumulation was higher in leaves (4.55% DW) as compared to stem (1.09% DW) and roots (0.80% DW), although few reports depicted more Cl^- accumulation in shoot parts as in *Atriplex* species. The highest amount of Cl^- was seen in leaves of *T. aphylla* (7.13% DW) followed by *D. ambrosioides* (6.83% DW) and *S. nudiflora* (6.59% DW), whereas *P. portucalustrum* restricted Cl^- accumulation (1.76% DW) to the minimum level in comparison to other halophytic plants (Table 2). This showed that the halophytic plants developed adaptive strategies to compartmentalize Na^+ and Cl^- in leaves and their proportionate accumulation in vacuoles protected the cytosol from harmful effects. The range of Cl^- accumulation varied significantly from 0.19 to 1.91% in the stem, whereas roots of these halophytic plants restrict Cl^- accumulation or might transport Cl^- to the leaves portion via transpiration stream. The highest accumulation of 1.39% on dry weight basis was recorded

in *S. fruticosa* and *C. quinoa* followed by *A. amnicola* (1.26%) and *A. lentiformis* (1.22%). *W. somnifera*, *A. donax*, *D. bippinnata*, *A. tomentosa* and *P. portucalustrum* showed less than 0.5% Cl^- accumulation in their root tissues. K^+ is an essential macronutrient (4–6% of its dry matter) that is involved in most of the regulatory processes such as protein synthesis, stomata opening-closing, phloem sugar loading and also acts as organic osmolyte, along with its inhibitory uptake by excess Na^+ leading to growth impairment in plants under salt stress (Kumar *et al.*, 2018; Wu *et al.*, 2018).

Results depicted that these halophytic plants had accumulated a sufficient amount of K^+ in their plant parts, i.e., 4.90% in leaves, 1.41% in stems and 1.61% in roots on a dry weight basis. Significantly higher K^+ (2.9 times) was found in leaves as compared to stems and roots. In leaves, maximum accumulation was noted in *S. fruticosa* (10.59% DW) followed by *T. aphylla* (8.58% DW) even more than *U. setulosa* (8.41% DW). The lowest leaf K^+ was seen in *D. annulatum* (1.14% DW) and *P. portucalustrum* (1.24% DW). Interestingly, it was also observed that halophytes that had higher leaf Na^+ also retained higher K^+ as well. Stems of *S. fruticosa* (3.49% DW), *A. amnicola* (3.40% DW) and *A. lentiformis* (2.94% DW) had higher K^+ in comparison to other studied halophytic plants. Root K^+ ranged from 0.41 to 4.17% in these halophytes and maximum root K^+ was recorded in *S. nudiflora* while minimum in *A. tomentosa*. Interestingly, it was also noted that besides accumulating higher concentrations of Na^+ , these plants also accumulated higher K^+ and *A. amnicola*, *Suaeda* species, *A. donax*, *A. logopoides*, *C. quinoa* and *U. setulosa* had the lowest Na^+/K^+ in all the plants parts. Our findings also supported the long-distance transport of K in these halophytes, as suggested earlier by Wu *et al.* (2018), where the lowest K levels were observed in roots and highest in young leaves of halophytes. It was observed that these halophytes developed the salt tolerance strategy through the retention of K^+ in root, stem and leaves. Calcium (Ca^{2+}) is the most important secondary messenger in many signal transduction pathways and also maintains membrane integrity. Significant variations were noted for Ca^{2+} accumulation in our results and it was observed that *C. quinoa* had the highest Ca^{2+} in its leaf and root tissue (1.57% DW and 1.18% DW), whereas *L. fusca* (1.15% DW) in stem tissue. Among different plant parts, Ca^{2+} content varied from 0.37 to 1.57% in leaves, 0.34 to 1.15% in stem and 0.21 to 1.18% in roots. Increasing water salinity increases Ca^{2+} ions in the shoots and leaves of *Atriplex* compared to roots. The Ca^{2+} ameliorates the inhibition of cell wall binding of Na^+ and plasma membrane, leading to reduced leakiness of membranes as well as enhanced cell division (Al-Khateeb, 2006).

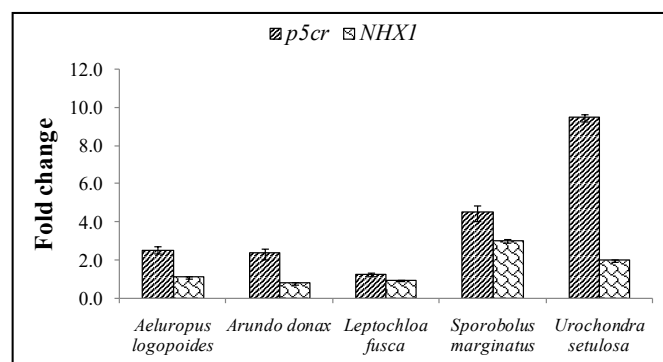


Fig 1. Quantitative gene expression under salinity in different halophytes

Osmolytes (proline, total soluble sugars and total soluble proteins): Osmolytes are the metabolites that maintain the osmotic potential, protein folding, detoxify

the free radicals and hence, protect various enzymes and macromolecules (Kumar *et al.*, 2018b; Mann *et al.*, 2019a). Among these, proline is a widely known osmolyte that could serve as a source of C and N and might act as a signaling molecule that could act as a sink for excess reductants, providing the NAD^+ and NADP^+ necessary for photosynthesis and respiration processes (Mann *et al.*, 2015). A significant accumulation of proline in leaves and root tissues was observed in these halophytes. The amount of proline accumulated in leaves ranged from 1.69 (*A. nummularia*) to 5.67 mg g^{-1} (*C. quinoa*), with a mean value of 2.63 mg g^{-1} (Table 3). Only two halophytes, *Chenopodium quinoa* and *Aerva tomentosa* had higher proline in leaves than the check plants of *U. setulosa*. In the case of roots, the highest proline was accumulated in *A. tomentosa* (2.68 mg g^{-1}), followed by *A. lagopoides* (2.32 mg g^{-1}), whereas *L. fusca*, *D. annulatum* and *D. bippinnata* showed the lowest accumulation. Similar to Na^+ and Cl^- , proline is also accumulated more in leaves than roots to protect the plant from the injurious effect of these ions by

shielding membrane integrity and increasing structural stability of ion transporters and these variations in proline accumulation might be due to various factors, such as the environmental factors, habitats, stress intensity and duration, which in turn enables the survival of these halophytic plants in their natural saline habitats (Al Hassan *et al.*, 2016). Accumulated proline also played an adaptive role in regulating osmotic balance in the cytoplasm and reduction of K^+ loss by up-regulating and down-regulating genes responsible for proline synthesis and degradation simultaneously (Al Hassan *et al.*, 2016; Lata *et al.*, 2017; 2019).

The quantitative expression analysis also showed that the gene responsible for the synthesis of proline *i.e.* *P5CR* (Pyrroline-5-carboxylate reductase) in leaf tissue, was found to be up-regulated in all the halophytes. Among 5 halophytic grasses, the maximum expression of *P5CR* was found in case of *U. setulosa* and the least expression was found in the case of *L. fusca* (Fig 1). Pyrroline-5-carboxylate (P5C) is an intermediate product

Table 3. Assessment of compatible osmolytes (mg g^{-1} FW) in leaves and roots of halophytic plant

Halophytes/Traits	Proline content		Total soluble sugars		Total soluble proteins	
	Leaves	Roots	Leaves	Roots	Leaves	Roots
<i>Heliotropium ramosissimum</i>	1.91 ^{gh}	1.99 ^e	3.29 ⁱ	2.12 ^k	8.53 ^a	1.48 ^k
<i>Withania somnifera</i>	2.24 ^{ef}	2.17 ^c	2.50 ^k	2.90 ^g	7.08 ^f	1.63 ^j
<i>Suaeda nudiflora</i>	1.89 ^{ghi}	1.11 ⁱ	7.41 ^a	3.84 ^d	7.84 ^c	3.00 ^f
<i>Aelurolopus lagopoides</i>	4.22 ^c	2.32 ^b	4.23 ^g	5.12 ^a	7.44 ^d	3.42 ^e
<i>Suaeda fruticosa</i>	1.73 ^{kl}	1.30 ^g	6.19 ^d	3.44 ^e	7.26 ^e	3.07 ^f
<i>Aerva tomentosa</i>	5.51 ^b	2.68 ^a	2.30 ^l	1.29 ^l	4.43 ^k	4.72 ^b
<i>Atriplex amnicola</i>	1.96 ^g	1.39 ^f	3.25 ^{ij}	4.55 ^b	6.72 ^g	5.55 ^a
<i>Atriplex nummularia</i>	1.69 ^l	1.20 ^h	3.36 ⁱ	3.20 ^f	8.02 ^b	4.40 ^c
<i>Atriplex lentiformis</i>	1.96 ^{gh}	1.14 ⁱ	3.57 ^h	4.13 ^c	6.16 ^h	2.03 ^{hi}
<i>Arundo donax</i>	1.90 ^{gh}	1.14 ⁱ	2.27 ^l	1.30 ^l	5.17 ^j	1.47 ^k
<i>Tamarix aphylla</i>	2.27 ^e	1.29 ^g	4.48 ^f	3.44 ^e	6.07 ^h	3.10 ^f
<i>Dysphania ambrosioides</i>	1.80 ^{ijk}	1.98 ^e	7.07 ^b	2.19 ^k	7.55 ^d	2.99 ^f
<i>Portulaca portucalustrum</i>	3.33 ^d	1.42 ^f	6.40 ^c	2.43 ⁱ	7.60 ^d	2.72 ^g
<i>Desmostachya bippinnata</i>	1.91 ^{gh}	1.03 ^j	6.35 ^c	3.23 ^f	6.91 ^f	2.09 ^h
<i>Sporobolus marginatus</i>	2.15 ^f	1.13 ⁱ	3.13 ^j	3.30 ^f	4.17 ^l	1.93 ⁱ
<i>Leptochloa fusca</i>	1.79 ^{jk}	1.01 ^j	5.17 ^e	2.30 ^j	6.97 ^f	3.48 ^e
<i>Urochondra setulosa</i>	4.21 ^c	2.09 ^d	3.35 ⁱ	2.92 ^g	5.19 ^j	1.15 ^l
<i>Dicanthium annulatum</i>	1.87 ^{hij}	1.02 ^j	2.53 ^k	3.23 ^f	5.41 ⁱ	1.39 ^k
<i>Chenopodium quinoa</i>	5.67 ^a	2.18 ^c	3.12 ^j	2.67 ^h	5.31 ^{ij}	3.73 ^d
General mean	2.63	1.56	4.21	3.03	6.52	2.81
CV (%)	2.15	1.83	1.95	2.00	1.62	2.62
SE(d)	0.046	0.023	0.067	0.050	0.086	0.060
LSD ($p < 0.05$)	0.0938	0.0471	0.136	0.1005	0.1752	0.1219

Means with at least one letter common are not statistically significant ($p \leq 0.05$) using Tukey's HSD Test

of both proline biosynthesis and catabolism. Thus, higher expression of this gene with increasing salt levels showed the increased synthesis of proline. Mann *et al.* (2021) also reported the upregulation of transcripts for Δ -1-pyrroline-5-carboxylate synthase, Betaine aldehyde dehydrogenase and Trehalose 6-phosphate phosphatase, indicating the protective role of proline, glycine betaine and trehalose with increasing salt concentration in halophytes. In *Lycium humile*, proline accumulation and antioxidant capacity, together with the development of a large leaf water-storage parenchyma that allowed Na^+ accumulation acted as an efficient osmotic adjustment system at pulsed salt treatments of 750 and 1000 mM NaCl (Palchetti *et al.*, 2021). Transgenic lines of rice constitutively overexpressing ornithine- δ -aminotransferase (OAT) produced higher levels of proline, suggesting its role in salt tolerance (You *et al.*, 2012).

Soluble sugars are the major carbon source of energy that also maintain homeostasis and act as regulatory messengers for gene expression (Peng *et al.*, 2016). Total soluble sugar (TSS) content varied significantly ($p \leq 0.01$) in leaves and roots of studied halophytic species. The soluble sugars accumulated more in leaves (4.21 mg g^{-1}) than in roots (3.03 mg g^{-1}). In leaves, TSS ranged from 2.27 to 7.41 mg g^{-1} , whereas in roots 1.29 to 5.12 mg g^{-1} (Table 3), helped in buffering the redox potential of cell, maintaining storage reserves and protecting the cellular structures, which were driven by the up-regulation of genes of sugar synthesis and transport (Theerawitaya *et al.*, 2015). Soluble proteins play a crucial role in osmotic adjustment by acting as a storage form of nitrogen that could be used during recovery from stress conditions and also involved in redox metabolism, osmolyte metabolism, defense-related proteins and gene expression (Kosová *et al.*, 2013; Pooja *et al.*, 2017; 2019). Significant variability was noted for protein accumulation among different halophytes in leaves and roots. Leaves accumulated 6.52 mg g^{-1} proteins on a mean basis, with maximum accumulation in *H. ramosissimum* followed by *A. nummularia*. Roots showed lesser protein accumulation than leaves and found that *A. amnicola* had the highest protein (5.55 mg g^{-1}) and *A. donax* had the lowest (1.47 mg g^{-1}). This differential accumulation of proteins in leaves and roots under higher salinity showed adaptive defense mechanisms, which were triggered to re-establish homeostasis through synthesis or degradation of some important metabolites and cellular proteins with a differential status of appearance or disappearance due to salt stress and ultimately enable the plants to survive. Such changes in the metabolism under stress conditions might lead to stress adaptation, which was ensured by the alterations in enzyme activities, and changes in protein synthesis machinery that resulted in the restructuring of the relevant signaling pathways (Shinozaki and Yamaguchi-Shinozaki 2007; Pooja *et al.*, 2019).

Salt tolerance potential based on ionic ratios:

Sodium, generally, is not considered a major element but plants accumulate Na^+ in exchange for Ca^{2+} and K^+ under salt stress and hence, Na^+/K^+ and $\text{Na}^+/\text{Ca}^{2+}$ ratios are considered the most important indicators to study the response of plants grown under salt stress (Koksal *et al.*, 2016). In the present study, it was observed that most of the halophytic plants maintained low Na^+/K^+ in leaves, stem and root tissues (Fig 2). This showed that besides accumulating a higher concentration of Na^+ , these plants also accumulated a higher concentration of K^+ as well for their survival. *A. amnicola*, *Suaeda* species, *A. donax*, *A. lagopoides*, *C. quinoa* and *U. setulosa* had the lowest Na^+/K^+ in all the plant parts and maximum Na^+/K^+ was observed in the leaf and stem of *D. annulatum* (2.73 and 2.58). To cope with the detrimental effects of salt-induced ionic stress, these halophytic plants maintained a low tissue Na^+/K^+ ratio, which could be considered their key characteristic feature (Kumar *et al.*, 2016). *L. fusca*, *A. tomentosa* and *C. quinoa* had the lowest $\text{Na}^+/\text{Ca}^{2+}$ ratio in leaf, stem and root tissues (Fig 3). It is interesting to note that leaves had relatively higher $\text{Na}^+/\text{Ca}^{2+}$ ratio in all the studied halophytes except *H. ramosissimum*, *W. somnifera* and *D. annulatum*, where stem and root tissue had higher $\text{Na}^+/\text{Ca}^{2+}$ than leaf tissue (Fig 3). $\text{Na}^+/\text{Ca}^{2+}$ ratio, other important trait that characterized salt tolerance potential because Ca^{2+} restricted the excessive uptake of the other salt ions, especially Na^+ and enhanced the membrane stability (Villora *et al.*, 2000).

Salt-induced ionic stress leads to such variability for $\text{Na}^+/\text{Ca}^{2+}$ ratio in different halophytes because Ca^{2+} significantly generates ion selective movement for Na^+ , K^+ and Cl^- respectively and hence negotiates the toxicity of salinity (Al-Khateeb, 2006). In the present study, differential salt tolerance potential was calculated based on the accumulation of Na^+ and Cl^- in the whole plant over the accumulation of $\text{K}^+ + \text{Ca}^{2+}$ and characterized the halophytic plants as highly tolerant having the lowest ratio (Fig 4). The studied halophytes were ranked in the following order from highest to moderate salt tolerance

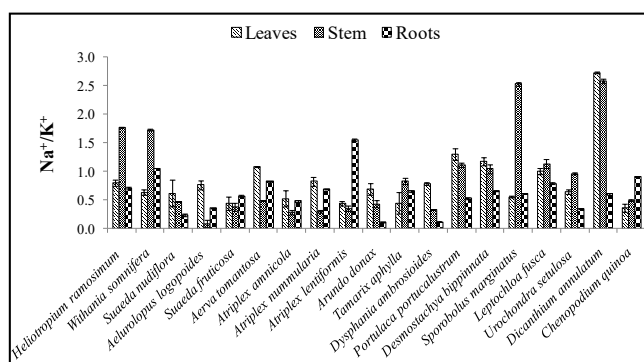


Fig 2. Na^+/K^+ in different plant parts of halophytes collected from waste saline lands of Haryana

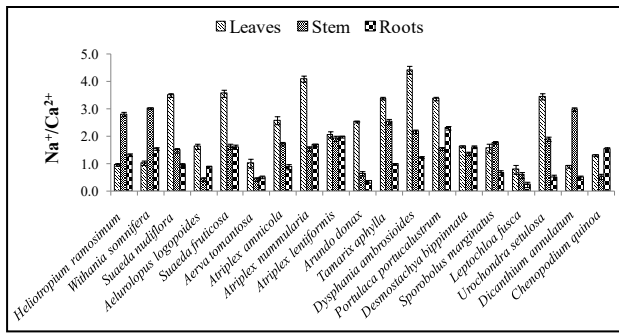


Fig 3. $\text{Na}^+/\text{Ca}^{2+}$ in different plant parts of halophytes collected from waste saline lands of Haryana

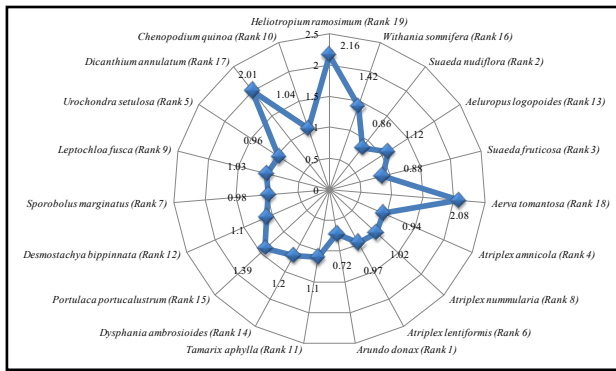


Fig 4. Characterization of halophytes based on salt tolerance potential $[(\text{Na}+\text{Cl})/(\text{K}+\text{Ca})]$

as *A. donax* $\geq$ *S. nudiflora* $\geq$ *S. frutescens* $\geq$ *A. amnicola* $\geq$ *U. setulosa* $\geq$ *A. lentiformis* $\geq$ *S. marginatus* $\geq$ *A. nummularia* $\geq$ *L. fusca* $\geq$ *C. quinoa* $\geq$ *T. aphylla* $\geq$ *D. ambrosioides* $>$ *A. lagopoides* $>$ *D. bipinnata* $>$ *P. portulacastrum* $>$ *W. somnifera* $>$ *D. annulatum* $=$ *A. tomentosa* $=$ *H. ramosissimum*. Owing to the complexities of assessing plant performance under natural conditions, halophytic plants maintained turgor by sequestering Na^+ and Cl^- and accumulated compatible solutes for osmotic adjustment because soil salinity is spatially heterogeneous even within the root zone (Shabala and Mackay, 2011).

Conclusion

This study helped to identify the salt tolerance potential of selected halophytes through their salt-loading capacity to survive in extreme saline lands. Hence, firstly it is recommended that salt-affected soils and poor-quality irrigation water resources be judiciously utilized for growing highly salt-tolerant halophytic species as an ideal option for rehabilitation of the existing degraded agricultural lands. Secondly, owing to the adaptability of these plants to saline habitats, further understanding of adaptive mechanisms is essential for their possible utilization in advanced crop development programs through modern tools to impart better resources for salt-affected regions.

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