

Physiological and differential gene expression reveals a trade-off between antioxidant capacity and salt tolerance in halophytes *Urochondra setulosa* and *Dichanthium annulatum*

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Abstract

Background

Among abiotic stresses, soil salinity is one of the major global constraints to growth and productivity in most of the crop plants, limiting current and future agricultural sustainability. One of the strategies to dissect the salinity tolerance phenomenon can be the study of plants growing naturally in saline environments and halophytes can serve as another model plants for salt tolerance studies.

Methods and Results

Here, we studied two un-explored halophytes, moderately salt tolerant, *Dichanthium annulatum* and extremely salt tolerant, *Urochondra setulosa* for investigating the contributory role of antioxidative system, the first line of defence, in salinity tolerance mechanism at salinity levels of E_{Ce} ~ 30, 40 and 50 dSm⁻¹ (~ 300,400,500 mM NaCl). H₂O₂ content, SOD and ascorbate peroxidase activities were higher in *U. setulosa* at all saline treatments whereas MDA content and catalase activity was high in *D. annulatum* although the specific enzyme activities of ROS system increased with increasing levels of salinity in both the halophytes. This differential physiological expression was in parallel with the transcriptomic data generated through High throughput sequencing on Illumina platform depicting 276 and 66 differentially expressed genes coding for various components of ROS system like antioxidant activity, cell redox and glutathione metabolism in response to salinity in *U. setulosa* and *D. annulatum* respectively. In *D. annulatum*, H₂O₂ is detoxified by increased activities of SOD, APX and catalase where as in halophyte *U. setulosa*, peroxidase takes over catalase to remove H₂O₂ along with DHAR and MDHAR which significantly correlates with the differentially expressed transcripts.

Conclusions

The salinity responsive gene expression for ROS enzymes and antioxidants clearly differentiate between these two halophytes supporting the detoxification of H₂O₂ and survival at different salinity levels. This study provides reference information on the key genes responsible for salt tolerance which can be used for related plant species for genetic improvement.

Introduction

Salt stress causes toxicity in the plant cell and thus disturbs important pathways within plants. There are various factors responsible for creating salinization of soils including poor irrigation water quality, non-uniform irrigation management, limited water supply in urban areas and climate changes etc. All these factors contribute to desertification, soil degradation and ultimately pose a threat to future food production. Globally approximately 1000 million hectares land affected by salinity has put on risk the agricultural processes in addition to limited crop production. In this view, the tolerance range of agricultural crops for salinity has a critical importance. Plant responses to salt stress and its tolerance mechanism is always a

major topic of plant research. Soil salinity adversely affects plants through restricted water and nutrients uptake due to accumulation of reactive oxygen species (ROS) and also disturbed hormonal levels. Generally, under normal plant growth conditions, at low cellular concentrations these ROS act as signaling molecules in various physiological mechanisms [1]. These ROS can stimulate various Na^+ and K^+ membrane ion channels required for maintaining optimum cytosolic K^+/Na^+ ratios imparting salt tolerance [2]. ROS are produced consistently by chloroplast, mitochondria and peroxisomes during the normal growth, affecting the composition and integrity of cell membrane, protein denaturation, breakdown of photosynthetic pigment and activities of several enzymes [3]. For scavenging the excess ROS/toxic radicals, plants produce various enzymatic and non-enzymatic antioxidants to counteract the negative effects of high salt concentrations and help the plant in performing regular processes [4]. Generation and sensing of reactive oxygen species (ROS) and their distribution in plant cell is maintained by the cellular redox homeostasis which is the fundamental property to readjust the redox state under salinity. These redox-related processes have been identified as critical for acclimation and adaptation under salinity.

ROS generation and toxicity mechanisms are found in all plants where halophytes also have similar mechanisms. Plants' antioxidative ability, on the other hand, is capable of balancing these ROS and keeping them at non-toxic levels. Halophytes are the plants that exist and grow successfully in stressful saline environments by relying on various physiological or biochemical adaptations through osmolyte accumulation, ion homeostasis, tissue compartmentalization of ions and scavenging ROS systems or through gene regulation at molecular level [3, 5–7]. Here, we have selected two halophytes; *Urochondra setulosa* and *Dichanthium annulatum*. *Urochondra setulosa* is a perennial halophytic grass showing optimum growth up to 500 mM NaCl as reported earlier [8–10] and has also been reported to tolerate sea water salinity. It has wide distribution from Northern Africa to Arabia through Southern coast of Pakistan (Sindh) and upto Northwestern part of India. This grass halophyte overcomes the salinity effects by adapting several morphological, physiological and biochemical strategies. This plant finds a productive potential as a donor candidate for salt tolerant genes along with remediation of salt affected soils and use as forage and fodder in salt affected soils. *Dichanthium annulatum* is usually used in livestock for forage, habitat to parts of Africa, tropical Asia and the Middle East. It creates a turf that can withstand grazing pressure, hence, it is also known as miracle grass or Hindi grass. The plant root system is just one metre deep showing tolerance against alkaline and salty soils or sandy and clay soils with poor drainage. In our earlier reports [7, 11], we have observed that it can survive upto salinity of $\text{EC } 30 \text{ dSm}^{-1}$ and hence is less tolerant in comparison to *U. setulosa*. Although various physiological mechanisms and molecular reports are available for salt tolerance in halophytes including *Suaeda* [12], *Spartina* [13], *Atriplex* and *Salicornia brachiata* [14]. There are still some salt-tolerant grasses surviving at extremely high saline environments that have not been investigated for gene network analysis. Differential expression of genes for downstream signaling, redox homeostasis, transport across membrane etc have been validated [15]. A strong correlation between antioxidant defense system and tolerance towards salinity has been reported through differential gene expression of ROS system in several plant species [16]. The quantitative role of numerous molecular pathways, their qualitative relationships and the overall functional network underpinning plant tolerance to salt stress, on the other hand, remain unknown. As a result, the response mechanisms of stress-tolerant taxa would be more effective in comparison to sensitive species. As a result, closely related, salt tolerant and sensitive plant species of natural habitat are being explored for comparative stress tolerance strategies. After *Arabidopsis*, its related species, *Thellungiella*

has been investigated as an extremophile model since it is tolerant to drought and salt stress [15]. Various strategies/mechanisms are contributing towards salinity tolerance and survival in all the plant species including halophytes, hence, we analyzed the differential expression of antioxidant enzymes and antioxidants in two contrasting halophytic non-model plant species, *Urochondra setulosa* and *Dichanthium annulatum* with different salinity tolerance levels.

Materials and methods

Plant material and experimental conditions

These halophytes have been originally taken from extreme saline sodic Kutch plains of Rann of Kutch, Bhuj, Gujarat, their native habitat in India. Their root cuttings were planted in the macro pots having saline soil of $\text{ECe} \sim 16 \text{ dS m}^{-1}$ in a net house with natural conditions. After establishment of plants with stable growth, different treatments of saline water were imposed in these pots as $\text{ECe} \sim 30, 40, 50 \text{ dSm}^{-1}$ ($\sim 300, 400, 500 \text{ mM NaCl}$ respectively) with three replications in a randomized block design (RBD). One set without saline irrigation served as control. The plants have been maintained in these conditions for one year with continuous saline irrigations and leaves were harvested within 7–10 days of immediate saline irrigation. Soil salinity, ECe of 4 dSm^{-1} represents approximately 40 mM NaCl [17].

Estimation of hydrogen peroxide (HO)

Leaf samples homogenized in 5 ml of chilled 0.01 M phosphate buffer ($\text{pH } 7.0$) was centrifuged at 8000 g for 10 minutes and supernatant was collected. $50 \mu\text{l}$ of supernatant was mixed with 0.01 M potassium phosphate buffer ($\text{pH } 7.0$) with potassium dichromate (5%) and glacial acetic acid (1:3; v/v). This reaction mixture was incubated for 10 min at 100°C and absorbance was read at 570 nm . Reaction mixture without sample was used as blank. H_2O_2 content was calculated as per the method given by Sinha [18] through standard curve of $10\text{--}160 \mu\text{mol H}_2\text{O}_2$.

Estimation of Ascorbate

Ascorbic acid content was estimated by the method of Desai and Desai [19]. Leaf tissue was homogenized with 5% metaphosphoric acid with centrifugation at $10,000 \text{ rpm}$ for 10 min. Few drops of bromine solution and thiourea were added to supernatant with 1 ml of 2,4-dinitrophenylhydrazine solution. Reaction was initiated by keeping samples at 37°C and stopped after 2 hours with 5 ml of conc. H_2SO_4 . Absorbance of colored solutions was measured at 491 nm with standard curve of ascorbic acid.

Estimation of Glutathione

Total glutathione was estimated using 2-nitrobenzoic acid as per method of Salbitani et al [20]. Reaction mixture was prepared with 0.1 M Na-phosphate buffer $\text{pH } 7.0$ containing 1 mM EDTA, $40 \mu\text{l}$ of 0.4% (w/v) 5,5'-dithio-bis (2-nitrobenzoic acid), $100 \mu\text{l}$ of leaf extract and Milli-Q water and incubated for 5 min at room

temperature. Further 50 µl of NADPH (0.4%) and 1 µl of Glutathione reductase (0.5 U) was added in the reaction mix and incubated for another 30 minutes, mixed gently and read the absorbance 412 nm. Total glutathione was calculated by the calibration curve with 0-500 µM standard solution.

Estimation of Lipid peroxidation

Malondialdehyde (MDA) accumulation was measured as an indicator of lipid peroxidation as given by Heath and Packer [21]. One g leaf tissue was macerated in 5 ml TCA (0.1% trichloroacetic acid; w/v). After centrifugation at 8000 g for 15 min, one ml of the supernatant was precipitated by adding 4 ml TCA (20%) having 0.5% 2-thiobarbituric acid (w/v). This was further incubated at 95°C for 30 minutes with intermittent stirring and immediate cooling at 4°C. Supernatant was collected after 10 min with centrifugation at 8000x g and MDA content was measured at 532 nm using molar extinction coefficient of MDA ($155 \text{ mM}^{-1} \text{ cm}^{-1}$).

Biochemical Enzyme activities

Superoxide dismutase (SOD) activity was measured in the irradiated solution at 560 nm using nitro blue tetrazolium following procedure of Beauchamp and Fridovich. Enzyme activity of SOD was quantified on spectrophotometer (SPECORD-210 PLUS, Analytic Zena) and expressed in units/g FW by taking one unit enzyme for inhibition of photo-reduction of one µmol of NBT. Catalase (CAT) activity was determined by measuring H_2O_2 decomposition at 240 nm for 1 minute [23]. A unit of catalase was defined as a decrease in absorption for 3 minutes and $1 \text{ mmol H}_2\text{O}_2 \text{ ml}^{-1} \text{ min}^{-1}$. POX was measured through the rate of guaiacol oxidation in the presence of H_2O_2 at 470 nm [24] and the amount of enzyme required to oxidize one nmol of guaiacol $\text{min}^{-1} \text{ ml}^{-1}$ is defined as one unit of peroxidase activity. Ascorbate peroxidase (APX) activity was calculated using extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ as per method given by Nakano and Asada [25]. Glutathione reductase (GR) was quantified as per unit enzyme for oxidation of one nmol of NADPH min^{-1} [26]. Dehydroascorbate reductase (DHAR) activity was assayed using modified method of Hossain and Asada [27]. The Monodehydroascorbate reductase (MDHAR) activity was based on NADH oxidation with a decrease in A_{340} [28].

de novo RNA sequencing and differential gene expression

RNA isolation, quality check, construction of sequencing libraries and RNA sequencing were completed by Genotypic Technology [P] Ltd. Bangalore, India. Plant samples were sequenced on an Illumina HiSeq sequencer with two biological replicates of each treatment and 150 base pair length paired-end reads were generated. For each treatment sample, averages of 46.08 million raw sequencing reads were generated. FastQC [29] and Cutadapt [30] were used for checking the quality of raw data and further pre-processing, respectively. This raw data has been published [31] and submitted to NCBI with accession numbers PRJNA561259 and PRJNA665324 for *Urochondra setulosa* and *Dichanthium annulatum* respectively. High quality data having Q-score more than 30 (> 99.9% correct) was selected for further assembly and clustering. Assembling of processed reads was done by Trinity program, a graph-based approach. CD-HIT-EST [32] was used to cluster the assembled transcripts with $\geq 95\%$ sequence similarity, thereby, reducing redundancy

without excluding sequence diversity. Functional annotation of the clustered transcripts was done with Uniprot and KEGG pathway databases. Differential expression of genes between these two halophytic plants was quantified using an R package, DESeq.

Validation of genes encoding antioxidative enzymes

Quantitative expression of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), Monodehydroascorbate reductase (MDHAR), Dehydroascorbate reductase (DHAR) and Glutathione reductase (GR) was validated on real-time PCR (Bio-Rad CFX-96™). Trizol reagent (Hi-media) was used for isolation of total RNA of same leaf sample (used for the transcriptomic analysis) as per the manufacturer’s protocols, and treated with DNase I (Thermo Scientific). RNA concentration and purity were analyzed on Nanodrop (Denovix® DS-11+ Spectrophotometer) and first-strand cDNA was amplified from the purified RNA template with iScript™ cDNA synthesis kit as per given protocol. For quantitative Real time PCR, 20µl PCR reaction mixture was prepared using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) in three biological replicates. The genes and their corresponding primers (designed using Primer5 software) are presented in Table 1. Normalization of expressed genes was done against *Actin* gene and $2^{-\Delta\Delta Ct}$ method [33] was utilized for calculating relative expression of validated genes.

Table 1
Primer sequences for qPCR validation

Sr. No.	Halophyte	Gene	Forward primer (5'-3')	Reverse primer (5'-3')
1	<i>Urochondra setulosa</i>	<i>SOD</i>	GGAGGCCATGTCAATCAATC	CACAAGGTCCAGAAGTGCAA
2		<i>CAT</i>	TTGATCCTGCCCGTCATTC	GCCTGCTTGAAATTGTTCTCC
3		<i>POX</i>	GGTAGTAGCCGATTGAGTG TTC	ACTTCTTCCGTGGTCGTTTAC
4		<i>APX</i>	ACCCACCACTTGAAGAACAG	CTACAGTACCCTGCATTGAGAC
5		<i>DHAR</i>	AACGGTGGTGATGGCAAATG	GCATATTCAGGAGGAGTCACAAGAG
6		<i>MDHAR</i>	GGATACCTCTTCCCACAGAATG	GTGCTCAGGATCAGCTCAATG
7		<i>GR</i>	TCTCAATACCTTCACCTGCC	CCACCACGAGCTTATGTTG
8	<i>Dichanthium annulatum</i>	<i>SOD</i>	GCTGGTGATCTTGGAATGTG	CTCTGCCAATGATTGAGTTTGG
9		<i>CAT</i>	CAAGCCGAGCATGTAAGATTG	TGGGTGTTGCCGATAAAGAG
10		<i>POX</i>	AACGACCTGTCCGTGTACTTC	GAAGCAGTCGTGGAAGAAGAG
11		<i>APX</i>	CTGCCTACCAAAGCCTAGTTC	AATCTACTATGCGGGAGTGC
12		<i>DHAR</i>	CCTAGAGGATAAGCTGCATGTG	GATCAGAGATGGAGTTGGGTAC
13		<i>MDHAR</i>	GATGGCTTTACTCGCACTCG	GATATTGAAGAAATCGCTGGGCAC
14		<i>GR</i>	CGTGGGAGACGTTACCAATAG	TTGGGAGAACACAGCAGAAG

Statistical analysis

Variance analysis of the physiological data for spectrophotometric enzyme activities, H_2O_2 , MDA, ascorbate and glutathione contents was done using the SAS (Version 9.3, SAS Institute Inc., Cary, NC, USA) and TUKEY's Honest Significant test at $p < 0.05$ was applied for comparison of mean differences. Significant p-values at $p \leq 0.05$ of differentially expressed transcripts were selected from NGS data for DEGs.

Results

ROS system

Lipid peroxidation, estimated in terms of MDA content, progressively increased with salinity levels and maximum content of 2.98 mmol/g FW was obtained at $\text{ECe} \sim 50 \text{ dS m}^{-1}$ in *U. setulosa*. MDA content increased by $\sim 25\%$ at salinity of $\text{ECe} \sim 30 \text{ dS m}^{-1}$ in both halophytes whereas 96.05% accumulation was observed in *U. setulosa* at highest salinity level of $\text{ECe} \sim 50 \text{ dS m}^{-1}$ (Table 2). In *Urochondra*, H_2O_2 content increased by approximately 1.8 times under saline conditions of $\text{ECe} \sim 50 \text{ dS m}^{-1}$. The rate of increase in H_2O_2 content was 35.17, 37.98 & 77.57% in comparison to control under different levels of salinity (Table 2). In *Dichanthium*, at salinity level of $\text{ECe} \sim 30 \text{ dS m}^{-1}$ H_2O_2 content increased by 46.50% over control. Ascorbate content increased by 31.8, 46.6, 61.4% at three salinity levels, respectively in *U. setulosa* while only 10% increase in AsA was seen in *D. annulatum*. On the other hand, glutathione content was almost double than the control condition in *U. setulosa* at salinity level of $\text{ECe} \sim 50 \text{ dS m}^{-1}$ with 37.8 and 68.7% increase at $\text{ECe} \sim 30 \text{ dS m}^{-1}$ and $\text{ECe} \sim 40 \text{ dS m}^{-1}$ with 54% increase in *D. annulatum* under salinity (Table 2).

Table 2
ROS components in *Urochondra setulosa* and *Dichanthium annulatum* at different salinity levels

Treatments in <i>Urochondra setulosa</i>	mmoles g ⁻¹ FW		µg g ⁻¹ FW	
	H ₂ O ₂ content	MDA content	Ascorbate content	Glutathione content
Control (ECe – 0.69 dS m ⁻¹)	4.37 ± 0.19 ^c	1.52 ± 0.20 ^d	112.36 ± 1.55 ^d	51.14 ± 1.83 ^d
ECe – 30 dS m ⁻¹	5.91 ± 0.40 ^b	1.89 ± 0.31 ^c	148.14 ± 3.61 ^c	70.51 ± 0.95 ^c
ECe – 40 dS m ⁻¹	6.03 ± 0.25 ^b	2.06 ± 0.29 ^b	164.78 ± 4.46 ^b	86.27 ± 3.42 ^b
ECe – 50 dS m ⁻¹	7.76 ± 0.79 ^a	2.98 ± 0.43 ^a	181.44 ± 2.13 ^a	103.11 ± 1.68 ^a
General Mean	6.02	2.11	151.68	77.76
CV	1.66	2.95	1.24	2.91
Treatments in <i>Dichanthium annulatum</i>	H ₂ O ₂ content		Ascorbate content	
	H ₂ O ₂ content	MDA content	Ascorbate content	Glutathione content
Control (ECe- 0.69 dS m ⁻¹)	2.86 ± 0.128 ^b	4.33 ± 0.517 ^b	69.14 ± 2.24 ^b	25.61 ± 0.23 ^b
ECe – 30 dS m ⁻¹	4.19 ± 0.21 ^a	5.41 ± 0.286 ^a	76.41 ± 2.41 ^a	39.47 ± 1.00 ^a
General Mean	3.53	4.87	72.78	32.54
CV	6.75	3.45	4.39	2.60
<i>*values in the column having different superscripts are significantly different at p < 0.05</i>				

As salinity levels increased, the activity of antioxidant enzymes SOD, CAT, POX, APX, DHAR, MDHAR and GR also increased significantly in both the halophytes (Fig. 1). The activity of SOD exhibited significant variations ($p < 0.05$) and increased by 43% at ECe ~ 30 dS m⁻¹, 74% at ECe ~ 40 dS m⁻¹ and 115% at ECe ~ 50 dS m⁻¹ in *U. setulosa* whereas in *D. annulatum* ~ 30% increase in SOD activity was seen at ECe ~ 30 dS m⁻¹. This induced level of SOD activity showed the higher dismutating capability of *U. setulosa* with increase in the salinity level. Catalase activity also increased with increased salinity levels with 49, 82 & 121% increase at ECe ~ 30, 40 & 50 dS m⁻¹ respectively, indicating the protective action of catalase under salt stress in *U. setulosa*. Similarly in *D. annulatum* CAT activity increased to 158% over control plants. A significant increase in peroxidase activity was also observed with increasing salinity but the per cent increase was less in comparison to CAT (Fig. 1). Peroxidase activity increased about 13.7% and 37% at ECe ~ 30 dS m⁻¹ in both the plants respectively with 46% at ECe ~ 40 dS m⁻¹ and 52% increase at ECe ~ 50 dS m⁻¹ in *U. setulosa*. Similar to the increased H₂O₂ content, the activity of APX increased to compensate the increased H₂O₂ in

these halophytes under saline environments and highest ascorbate peroxidase activity (449.89 units/mg protein) was noted at $EC_e \sim 50 \text{ dS m}^{-1}$ which is 117% higher than control in *U. setulosa* with 69 & 75% increase at lower levels of salinity. In *D. annulatum*, APX activity increased by 28% only at $EC_e \sim 30 \text{ dS m}^{-1}$. DHAR activity also increased from 10–43% with increasing salinity levels in *U. setulosa* while only 16% increase was observed in *D. annulatum* at lowest level of salinity (Fig. 1). Similarly, MDHAR activity increased by 9.17 and 13% at lower salinity of $EC 30 \text{ dS m}^{-1}$ in *Urochondra* and *Dichanthium* respectively but at higher levels, it increased by 27 and 44% in *U. setulosa*. On the other hand, slight increase of 18% was observed in GR activity in *D. annulatum* at low salinity levels with 45–60% increase at higher salinity in *U. setulosa*.

Transcriptomic analysis and identification of DEGs for ROS

Illumina paired-end transcriptome sequencing generated about 352.78 million reads through *de novo* analysis as the complete reference genome is not available for these halophytes. In all the treatments, 123,715 clustered transcripts were produced through the high-quality RNA-seq analysis having 1259 bp average length with N50 of 1819 bp in *U. setulosa* while the *de novo* assembly of *D. annulatum* generated 188,353 transcripts having 864.2 bp average contig length with N50 value of 1100 bp. Blasting of these clustered reads was done against *viridi plantae* database (NCBI) and about 64.47% and 65.52%, were found to annotate with database, in *Dichanthium annulatum* and *Urochondra setulosa* respectively. The normalized expression values of DESeq were used to find out the fold change expression for transcripts encoding antioxidative enzymes and ROS system in response to stress. The up-regulated and down-regulated transcripts were selected with log2fold change more than 1 and less than 1 respectively. The gene transcripts encoding for antioxidative enzymes were found as catalase (*Dichanthium*_DN68555_c1_g1_i4; *Urochondra*_DN29893_c0_g1_i1), Super oxide reductase (*Dichanthium*_DN60919_c0_g3_i2; *Urochondra*_DN29046_c0_g1_i3), peroxidase (*Dichanthium*_DN64999_c3_g1_i2; *Urochondra*_DN25708_c0_g2_i1), ascorbate peroxidase (*Dichanthium*_DN61491_c0_g6_i3; *Urochondra*_DN39524_c2_g3_i5), ascorbate glutathione cycle (*Dichanthium*_DN61212_c6_g10_i4; *Urochondra*_DN28738_c0_g2_i4, monodehydroascorbate reductase (*Dichanthium*_DN69181_c1_g1_i1; *Urochondra*_DN39698_c0_g1_i8). The expression of all the above mentioned transcripts was found to be up-regulated with increase in the salinity level (Fig. 2).

Differential expression of genes related to ROS signaling network

Gene ontology analysis revealed that in *U. setulosa*, under biological function ontology of oxidative stress (GO: 0006979), 24, 7, 7 DEGs were up-regulated ($p\text{-value} \leq 0.05$) with down regulation of 4, 10, 15 DEGs at salinity levels of $EC 30, 40$ and 50 dS m^{-1} respectively. Likewise in *D. annulatum*, 13 DEGs were upregulated ($p\text{-value} \leq 0.05$) at $EC 30 \text{ dS m}^{-1}$ coding for peroxidase and ascorbate peroxidase while six DEGs were down regulated. Venn diagram showed that overall 19 unique transcripts were identified including 9 in *D. annulatum* at $EC 30$ and 6, 8, 7 in *U. setulosa* at $EC 30, 40$ and 50 dS m^{-1} respectively coding for catalase, peroxidase, L-ascorbate peroxidase, glutathione peroxidase etc [Supplementary table1]. Similarly under ontology of response to stress (GO:0006950); 16 DEGs at $EC 30$, 16 at $EC 40$ and 21 DEGs were upregulated at 50 dS m^{-1} respectively in *U. setulosa* while 5, 18, 19 were down-regulated. The proteins coding for these transcripts

include heat shock proteins, universal stress proteins, calmodulin binding proteins, Dehydrin/LEA group-2 like proteins, putative trehalose-6-phosphate synthase and Absciscic acid stress ripening6 proteins etc [Supplementary table 2]. In *D. annulatum*, mainly the transcripts coding for calmodulin binding proteins were upregulated with only 4 DEGs and 13 were down regulated. Out of 34 unique transcripts, DEGs for putative USP family protein, Endoplasmic-like protein, Late embryogenesis abundant protein, Absciscic stress ripening protein, Calmodulin binding protein1, ABA-stress protein, Heat shock protein were common at lower salinity level between two halophytic plants (Venn diagram). Transcripts for Protein SET DOMAIN GROUP 41 were common between all treatments in both the plants.

The upregulated DEGs in *U. setulosa* under GO term, Cell redox (GO: 00045454) include 4, 19, 10 while 7, 27, 15 DEGs were downregulated at EC levels of 30, 40 and 50 dS m⁻¹ respectively. These transcripts code for glutaredoxin family proteins, disulfide isomerase, protein disulfide isomerase-like, putative nucleoredoxin, Thioredoxin-like-3-1-chloroplastic with molecular function of cell redox homeostasis [Supplementary table 3]. Similarly in *D. annulatum* 7 DEGs were upregulated coding for Thioredoxin superfamily protein, Glutathione C-13 & C-15, Thioredoxin and disease resistance protein RPM1 with 14 down regulated DEGs. Differential expression of unique transcripts through Venn diagram shows that overall 52 unique DEGs were expressed coding for Glutaredoxin family protein predicted protein, Thioredoxin, Grx_I1-glutaredoxin subgroup III Glutaredoxin-C10, chloroplastic Glutathione reductase chloroplastic Thioredoxin F, chloroplastic Peroxiredoxin Q etc in all the saline treatments where Glutaredoxin family protein Predicted protein was common between two plants.

Under glutathione metabolic process ontology (GO:0033355) five upregulated and four down regulated transcripts were expressed at lower salinity levels of EC 30 dS m⁻¹ in *D. annulatum* with six upregulated and seven down regulated DEGs in *U. setulosa* [Supplementary table 4]. At increasing salt concentrations of EC level 40 50 dS m⁻¹ and 50 dS m⁻¹ in *U. setulosa*, 1 & 7 DEGs were upregulated with 4 and 7 down regulated transcripts. At EC 30 dS m⁻¹ in *D. annulatum* and at EC 50 dS/m in *U. setulosa*, upregulated transcripts code for Ribosomal protein like protein with glutathione transferase activity involved in glutathione metabolic process in cytoplasm as well as ribosome while at salinity treatments of 30 and 40 dS m⁻¹ in *U. setulosa*, the upregulated transcripts code for NAM-like protein including Myb family protein like with cellular location of cytoplasm. It means at highest salt concentration in both grasses, some translational change or synthesis of new protein occurs which might be involved in imparting tolerance at highest concentrations of salt at EC 30 dS m⁻¹ for *D. annulatum* and EC 50 dS m⁻¹ for *U. setulosa*. Total 17 unique DEGs were expressed as we can see from the Venn diagram that Myb family protein-like (Os08g0225900 protein) were common between three saline treatments in *U. setulosa* while transcripts for chloroplastic Ribosomal protein-like Glutathione reductase, Putative glutathione-S-transferase Adenylosuccinate synthetase, chloroplastic AMPSase, Putative tau class GST protein 4 were expressed only in *D. annulatum*.

Quantitative genes expression encoding antioxidative enzymes

The expression profiles of antioxidant genes of these two grasses were also analyzed in leaf tissue on q-PCR. The expression pattern of genes (Fig. 2) encoding antioxidative enzymes followed the same trend as the physiological enzyme activity (Fig. 1) in both halophytic plants. In *U. setulosa* the relative expression of *SOD*

increased from 4.89 REU (relative expression unit) at $ECe \sim 30dS\ m^{-1}$ to 8.32 REU at $ECe \sim 50dS\ m^{-1}$. Similarly, the expression of gene *CAT* increased with increasing salinity *i.e.* from 11.08 REU at $ECe \sim 30dS\ m^{-1}$ to 14.30 REU at $ECe \sim 50dS\ m^{-1}$. Likewise, the relative expression of peroxidase, ascorbate peroxidase (APX), GR, DHAR, and MDHAR also increased 2–3 folds with salinity in both the halophytes. Gene expression levels of these enzymes were parallel to the log2fold change pattern through NGS data. In *D. annulatum* the expression values of APX were higher while the expression levels of catalase, SOD, glutathione reductase, DHAR and MDHAR were much higher in *U. setulosa* (Fig. 2).

Conclusively, through the expression levels of antioxidant enzymes and their physiological activities, it can be summarized that H_2O_2 generated under saline environment is taken care by increased activities of enzymes SOD and through AsA-GSH pathway. In moderately salt tolerant halophyte, *D. annulatum*, which can survive upto salinity of $EC\ 30\ dS\ m^{-1}$, accumulated H_2O_2 is detoxified by higher activities of antioxidant enzymes, SOD, APX and catalase where as in highly salt tolerant (in this experiment surviving at salinity levels of upto $EC\ 50\ dS\ m^{-1}$) halophyte *U. setulosa*, peroxidase takes over catalase to remove H_2O_2 along with DHAR and MDHAR which correlates with the significantly expressed transcripts. This shows that SOD along with AsA-GSH route counteracts the toxic effects of salinity along with other molecular networks or signaling.

Discussion

Environmental stresses influence plant metabolism and physiological activities through osmotic balance, ion homeostasis, signal transduction, redox balance, gene expression and enzyme activity modifications etc [34, 35]. Usually, generation of ROS species and toxicity mechanisms are similar in glycophytes and halophytes; however, the strategies for detoxification and prevention differ in terms of total antioxidant activity and isoenzymic-form expressed in response to salinity. Plants respond to oxidative stress primarily through an endogenous defensive mechanism comprised of various enzymatic and non-enzymatic antioxidants. [36, 37]. A steady-state balance between the antioxidant scavenging system and accumulation of ROS in plant cells, enables the optimal ROS level in the cell for adequate redox biology and normal plant growth regulation processes. In our studies, salt stress induced oxidation stress was scavenged by enhanced activities of these antioxidant enzymes as well as the antioxidants accumulation in both the halophytic plants where SOD activity was more in highly salt tolerant, *U. setulosa* than *D. annulatum*. While comparing both the halophytes, the free radicals generated due to salinity are initially converted to H_2O_2 by SOD as we can see from the accumulated H_2O_2 content and higher expression of SOD. In *D. annulatum*, this H_2O_2 is further reduced by catalase while in *U. setulosa* higher peroxidase activity correlates with H_2O_2 content which further is carried forward by AsA-GSH pathway. Ascorbate and glutathione contents were 2–3 fold higher in *U. setulosa* than *D. annulatum* depicting more detoxification of H_2O_2 at higher salinity levels. Hence, we can briefly summarize that at lower levels of salinity SOD and catalase detoxify the free radicals in *D. annulatum* while at higher salinity levels, it is being dismutated through SOD and AsA-GSH pathway in *U. setulosa* and hence, the later is highly salt tolerant.

During ROS scavenging, The enzyme activities of APX, MDHAR, DHAR, and GR define the AsA-GSH pathway along with the concentration of antioxidant metabolites, ascorbate and glutathione. [38]. Ascorbate

peroxidase enzyme breaks down reactive H_2O_2 into water with release of MDHA, which is quickly dissociated into ascorbate and DHA. Further, glutathione (GSH) catalyzes reduction of Dehydroascorbate (DHA) into ascorbate yielding oxidized glutathione GSSG. This GSSG is finally reduced by glutathione reductase (GR). The reduced of AsA and GSH antioxidants, plays important role in conferring stress tolerance to plants. [38]. Here, we can see that the expression level alongwith enzyme activity of SOD, POX, Asc Peroxidase, DHAR, MDHAR and GR are prominent in *U. setulosa* at higher salt levels of EC 40 dSm^{-1} and 50 dSm^{-1} while the Asc-GR pathway seems to be less active in *D. annulatum* at lower levels of salinity. Superoxide dismutase (SOD) plays an important role to define the specific H_2O_2 'signature' in halophytes and hence, its higher intrinsic activity triggers a cascade of physiological and molecular responses for further stress signaling, thereafter, other antioxidant enzymes detoxify this H_2O_2 [39].

The first effect of ROS accumulation under abiotic stress exacerbate membrane damage through lipid peroxidation which is a non-enzymatic autoxidation processes. Here, we measured lipid peroxidation in form of malondialdehyde (MDA) content which accumulated more in *D. annulatum* (Table 2) than *U. setulosa*, the first being more sensitive to salinity whereas, H_2O_2 content was higher at high salt levels in *U. setulosa* than in *D. annulatum* (Table 2). Superoxide dismutase acts as the first defensive barrier against oxidative stress by dismutating superoxide radicals into less toxic H_2O_2 [40]. In our studies also, we have seen that SOD activity increases with increasing levels of H_2O_2 in both the plants with higher activities in tolerant halophyte *U. setulosa* than the moderately tolerant *D. annulatum*. Catalase defends cells against H_2O_2 by catalyzing it into H_2O and O_2 . Higher catalase activity is correlated with enhanced gene expression reducing the oxidative damage, owing to this enzyme's protective function. In present experiment, the activity of catalase is more than peroxidase in *D. annulatum* than *U. setulosa*, probably, H_2O_2 is being detoxified more through catalase (Fig. 1). In addition to its scavenging role in detoxification of H_2O_2 , peroxidase is also involved in lignification and cell-wall biosynthesis. From the differential expression of DEGs under oxidative stress, we can see peroxidase activity due to oxidative stress involved in hydrogen peroxide catabolic process as the integral component of membrane (Supplementary table 1) and extracellular region including L-ascorbate peroxidase, glutathione peroxidase and catalase activity. In DEG analysis, we found that the transcripts upregulated for peroxidase in *U. setulosa* are mainly involved in biosynthesis of phenylpropanoids which are involved in most of the plant responses against biotic and abiotic stresses. Polymers based on phenylpropanoids, such as lignin, tannins or suberins impart mechanical and environmental resistance in angiosperms and gymnosperms against wounding or drought or other stress. In the present study, the upregulation of these transcripts at high salinity also indicate more membrane stability which again proves the tolerance potential of these halophytes. For cell redox category, protein disulfide oxidoreductase activity along with isomerase activity was upregulated (Supplementary table 3). The families of thioredoxin, protein disulfide-isomerase (PDI), glutaredoxin, DsbA (disulfide-bond forming) and their homologs constitute the protein disulfide oxidoreductases. As per its functional annotation, this protein has isomerase activity too in addition to oxidative and reductive activities. In mungbean also, increased levels of superoxide radicals, H_2O_2 content along with higher activities of enzymes; SOD, CAT, APX, GR have been reported with increased electrolyte leakage and lipid peroxidation due to NaCl [37]. In other plants also like, *Brassica juncea* [41], tomato [42] and *Triticum aestivum* L [43] higher activities of antioxidant enzymes have been reported. The changes in these enzymes are related with plant responses to salt induced iso-osmotic stress via balancing the cellular redox.

H₂O₂ accumulation is extremely toxic to the cell because it is capable of passing through biological membranes and infiltrating other subcellular compartments. As a result, instant detoxification of H₂O₂ through ascorbate peroxidase is critical for a robust ROS system that could also regulate the cellular levels of H₂O₂ in green leaves. In our study also, significantly higher ascorbate peroxidase activity in both the halophytes indicates the scavenging of H₂O₂ through APX pathway. The enhanced SOD activity along with the other major antioxidative enzymes such as GR, MDHAR and DHAR reflects the involvement of these antioxidative enzymes in detoxifying effect of ROS and hence, governing salt tolerance of *Urochondra* and *Dichanthium* at different levels of salinity.

A positive effect of both components, enzymatic and non-enzymatic, in imparting salt tolerance has been explored earlier in various plants including halophytes. For example, significant increases in activities of CAT and APX have been observed in sapodilla rootstock (*Manilkara zapota* (L.) P. Royen) with exposure of rootstocks to diluted seawater of EC 12 dSm⁻² [44]. In salt tolerant and sensitive rice varieties, increasing transcript levels of Mn-SOD and GR genes ($P \leq 0.05$) are positively correlated with NaCl concentrations. At 30 and 90 mM NaCl, APX gene expression levels increased in Mevlutbey rice variety which decreased at higher NaCl levels of 150 and 210 mM [45]. The previous reports on other halophytes such as *Suaeda fruticosa* [46], *Limonium delicatulum* [47], *Sporobolus marginatus* [40], *Haloxylon salicornicum* [48], also revealed similar report for the enhanced enzyme activity under salinity conditions which showed the stronger capability of these halophytes to remove ROS by protecting from oxidative damage and maintained redox homeostasis. ROS activation was linked to varying tolerance in the genus *Juncus* towards salinity and drought in three species viz. *J. maritimus*, *J. acutus* (both halophytes) and *J. articulatus* (salt sensitive) [1]. Oxidative stress was created in all these plants due to water and salt stress but higher malondialdehyde accumulation was reported in *J. articulatus* and whereas the other two halophytes *J. maritimus* and *J. acutus* showed better tolerance in terms of efficient ROS system with increased activities of superoxide dismutase and glutathione reductase. Inter & intra species specific variations were observed for ascorbate peroxidase activity depending on treatment. To overcome the oxidative stress, redox homeostasis was maintained in *Suaeda corniculata* through highly active APX dependent ascorbate-glutathione and peroxiredoxin pathways [49]. The genes of antioxidant system for SOD, APX, GR, DHAR, MDHAR from *Pennisetum glaucoma* (Pg) were transferred in tomato seedlings and a significantly higher activities of these enzymes was observed in the transgenic plants exposed to drought and salinity respectively. Additionally, membrane stability in terms of reduced electrolytic leakage and lower malondialdehyde contents were observed [34].

Generally, ROS in plants regulate signal transduction cascade in response to abiotic stress through downstream signaling of redox-sensitive transcription factors and receptor proteins with direct inhibition of phosphatase [50]. This signaling starts with calcium and phospholipid with further network of transcription factors, serine/threonine protein kinase, NADPH oxidase and MAPK cascades [51] as has been reported in other halophytes including *Sporobolus virginicus* [52], *Mesembryanthemum crystallinum* [53], *Halogeton glomeratus* [54] and *Suaeda fruticosa* [55] through comparative transcriptomics under salt stress. Through such studies, several transcription factors including WRKY, DREB, NAC and basic leucine zipper (bZIP) were evaluated for imparting salt tolerance [56]. In our studies, a significant enrichment of various components of ROS system were revealed [Supplementary tables 1–4]. In total, 66 and 276 differentially expressed genes (DEGs) for ROS scavenging and signaling were discovered at higher salt concentrations in the halophytes, *D.*

annulatum and *U. setulosa* respectively. In response to stress, the proteins coding for these transcripts include heat shock proteins, universal stress proteins, calmodulin binding proteins, Dehydrin/LEA group-2 like proteins, putative trehalose-6-phosphate synthase and Absciscic acid stress ripening proteins etc. in *U. setulosa* and in *D. annulatum* mainly coding for calmodulin binding proteins. We identified few genes for cell redox homeostasis in these two halophytes mainly coding for glutaredoxin family proteins, disulfide isomerase, protein disulfide isomerase-like, putative nucleoredoxin, Thioredoxin-like-3-1-chloroplastic with molecular function of cell redox homeostasis. During stress conditions, genes that regulate redox reactions are mostly involved in protecting the cell behavior and environment. Similar to our findings, the differential expression analysis revealed an increase in glutathione-S-transferase tau1 and glutathione transferase in *Suaeda fruticosa* [55], *Salicornia europaea* [57], *Suaeda maritime*, *Reaumuria trigyna* [58]. GST gene from *Suaeda salsa* was transformed in *Arabidopsis* which enhanced the salt tolerance capacity in transgenics. Increased Glutathione content has also been reported in *Arabidopsis* under salt stress contributing in increasing salt tolerance [59]. In our findings also, most of the genes coding ROS system were expressed upon exposure to salinity involved in various protecting and redox pathways. Cumulatively, we can summarize that at different salt levels, *U. setulosa* tend to maintain membrane integrity and steady levels of ROS with prominent role of SOD and Asc-GSH pathway than moderately salt tolerant halophyte, *D. annulatum*. *U. setulosa* is able to tolerate higher salt concentrations than other halophytes, hence, it can be further explored for gene mining for salt tolerance since the model halophyte, *Thellungiella halophila*, salt cress has been evaluated at lower levels of saline treatments for short durations.

Conclusion

Soil and water salinity is an emerging issue for sustainable agriculture causing monetary loss of billions and other associated social consequences. In this situation, genetic engineering seems one of the fastest methods of speed breeding to restructure the salt tolerant cereal crops. Therefore, grass halophytes, close relative of cereals, could be the one of the options for mining of genes imparting salt tolerance because of conserved domain for salt tolerance from ancient evolution of plant kingdom from the sea. Hence, the finding of our studies for differential expressions of regulatory patterns of antioxidant enzymes in two different halophytes depicts a protective antioxidant system as one of possible strategy of tolerance in saline environments which can be utilized to enhance tolerance against salinity in cereal crop plants through advanced gene transformation technologies. The RNA-Seq data generated would serve as a repository for salt tolerance genes in related plant species and further crop breeding efforts for sustainable production in salt affected soils. Moreover, these halophytes, particularly *U. setulosa* can tolerate sea water salinity and hence, scope of seawater irrigation can be explored in the face of anticipated shortages in freshwater supply, opening new horizon for expanding saline agriculture into inarable regions.

Declarations

Data availability

The raw data is available with the corresponding author which can be shared on request. Transcriptomics data is submitted at <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA561259> and

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Author's Contributions

A.M. formulated the experiments, organized and prepared the main manuscript, supervision of project. N.K. performed QC, gene validation and bioinformatic analysis, wrote the original manuscript draft. C. L. performed total RNA isolation and physiological enzyme analysis. Ar. K. discussed data presentation design. B.L.M. helped in maintaining salinity throughout the experiment and A.K. interpreted results and performed SAS.

Ethical declarations

Conflict of interest

All the authors declare no competing interests.

Ethical approval and consent to participate

This article does not involve any studies on humans/animals conducted by any of the authors.

Consent for publication

All authors have read and approved the final manuscript.

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Figures

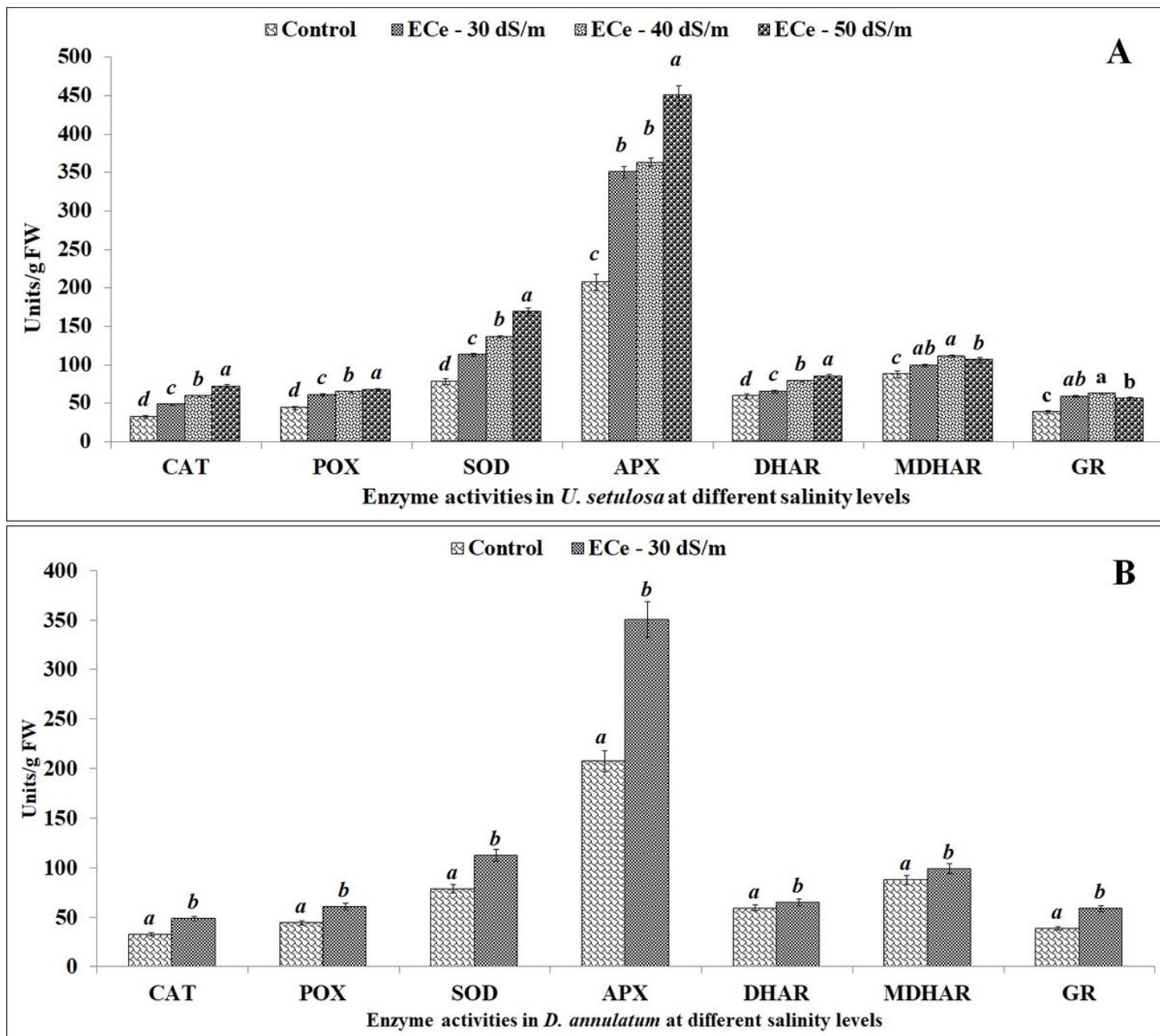


Figure 1

Antioxidative enzyme activities in *Urochondra setulosa* (A) and *Dichanthium annulatum* (B) at different salinity levels. Alphabets in superscript denote significant difference at $p \leq 0.05$

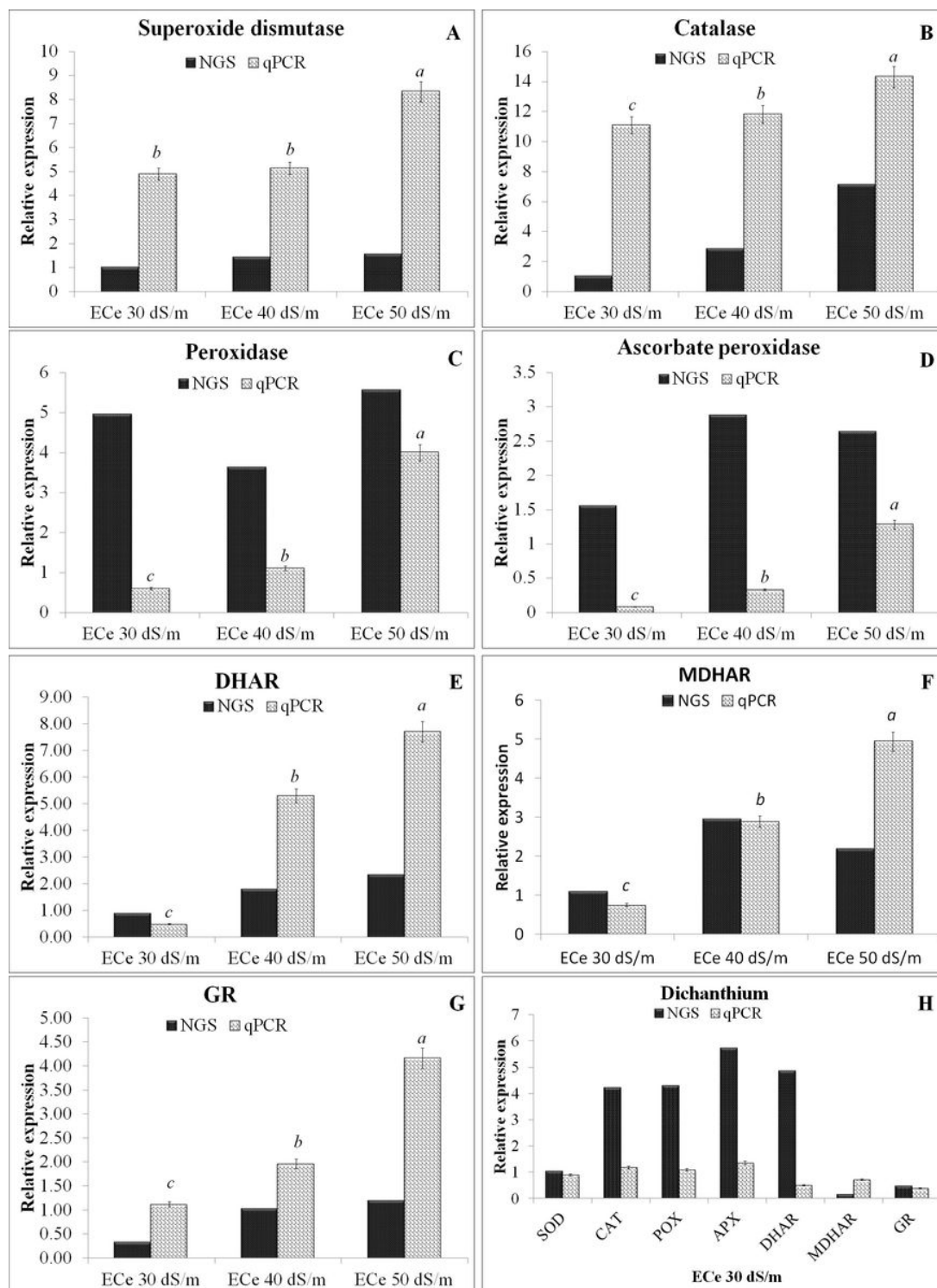


Figure 2

A comparative analysis of gene expression between RNA-seq data and qPCR of SOD (A), Catalase (B), Peroxidase (C) and Ascorbate peroxidase (D), DHAR (E) MDHAR (F) and GR (G) in *U. setulosa* and *D. annulatum* (H). Alphabets in superscript denote significant difference at $p \leq 0.05$

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