Morpho-physiological and gene expression responses of wheat by *Aegilops cylindrica* amphidiploids to salt stress

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Running title: Salt tolerance in wheat by Aegilops amphidiploids

Abstract

Aegilops cylindrica Host is one of the most salt-tolerant species in the Triticeae tribe. Amphidiploid plants derived from hybridization of 'Roshan' × *Aegilops cylindrica* and 'Chinese Spring' × *Ae. cylindrica* genotypes contrasting in salt tolerance were assessed for their morpho-physiological responses and the expression patterns of two genes related to ion homeostasis under 250 mM NaCl. Results showed that salt stress caused significant declines in both their morphological and phenological traits. Moreover, salt stress reduced not only their chlorophyll content but also their root and shoot K contents and K/Na ratios, while it led to significant enhancements in the remaining traits. Similar to *Ae. cylindrica*, the amphidiploids subjected to salt stress exhibited significantly higher H_2O_2 levels, root and shoot K contents, and root and shoot K/Na ratios accompanied by lower root and shoot Na contents and MDA concentrations when compared with the same traits in the wheat parents. Quantitative Real-Time PCR showed significant differential expression patterns of the *NHX1* and *HKT1;5* genes between the amphidiploids and their parents. The transcript level of *HKT1;5* was found to be higher in the roots than in the shoots of both the amphidiploids and *Ae. cylindrica* while *NHX1* exhibited a higher expression in the shoot tissues. The consistency of these data provides compelling support for the hypothesis that active exclusion of Na from the roots and elevated vacuolar sequestration of Na in the leaves might explain the declining Na levels in the shoots and roots of both the amphidiploids and *Ae. cylindrica* relative to those measured in wheat parents. It is concluded that the hybridized amphiploids are potentially valuable resources for salt improvement in bread wheat through the backcrossing approach.

Key words: *NHX1, HKT1;5, Ae. cylindrica*, amphidiploid, salt tolerance

Introduction

Soil salinity typically inhibits growth and impairs reproduction in plants through an initial osmotic-stress phase followed by ionic toxicity due to sodium (Na^+) and chloride (Cl^-) accumulation in the cell cytosol to lead ultimately to nutritional deprivation and oxidative stress (Arzani and Ashraf 2016; Munns and Tester 2008). Oxidative stress is caused by

excessive amounts of such reactive oxygen species (ROS) as hydroxyl radical (OH.), superoxide radical (O_2^-), singlet oxygen (O_2), and hydrogen peroxide (H_2O_2). The latter possibly plays a role in salt-stress by inducing K^+ efflux from plant cells (Rubio et al. 2020). Briefly, activation of nonselective cation channels (NSCC) activates NADPH oxidase to increase the production of apoplastic hydrogen peroxide, whereby, NSCC-mediated K^+ efflux is promptly promoted (Demidchik et al. 2007). This is further entrenched by the fact that halophytes employ such salt-tolerance mechanisms as Na^+ influx into the root, reduction of Na^+ translocation, compartmentation of toxic ions into vacuoles, and sodium exclusion (Flowers and Colmer 2015; Maathuis et al. 2014; Arzani 2008).

The ability of photosynthetically active plant tissues to maintain intracellular Na and K homeostasis is one of their major salt stress tolerance mechanisms (Pan et al. 2020; Shabala et al. 2017; Shabala and Pottosin 2014; Wu et al. 2018). Impairment of the intracellular Na and K homeostasis, however, results in inhibited cell division, photosynthesis, growth, and development (Yan et al. 2013; Duan et al. 2015). K^+/ Na^+ ratio has been recognized as a critical physiological mechanism that contributes to the overall salt tolerance of plants (Rubio et al. 2020); in this mechanism Na^+ does not accumulate to toxic levels in the leaves by discriminately excluding Na^+ but accumulating K^+ in soil solution (Arabbeigi et al. 2014; Kiani et al. 2015; Shabala et al. 2013; Zhu et al. 2016). Recently, Arino-estrada et al. (2019) used the positron emission tomography (PET) to demonstrate the dynamic uptake and transport of Na in seedlings and to reveal the significant genetic diversity in terms of salt transport.

The high-affinity potassium transporters (HKTs) play a role in the long-distance transport of Na^+ via phloem and xylem. In addition, the intracellular Na^+/H^+ antiporters (NHXs) excel at Na^+ homeostasis (Yarra 2019). Munns et al. (2012) demonstrated the significant contribution of *Nax2* locus (*TmHKT1;5-A*) to reducing leaf Na^+, that led to an improvement of 25% in durum wheat grain yield under saline soil conditions when compared with their near-isogenic lines lacking the *TmHKT1;5-A*.

A typical salt-excluding halophyte is the grass *Ae. cylindrica* with excellent salt tolerance. The tonoplast Na^+/H^+ antiporter, *NHX1* and the HKT-type protein are critical Na^+ transporters contributing to salt tolerance in plants. Motivated by our previous works of screening the hyper-salt tolerant genotypes of *Ae. cylindrica*, (Kiani et al. 2015), hybridizing wheat and a hyper salt-tolerant *Ae. cylindrica* accompanied by cytological and molecular assessments of the synthesized amphidiploid plants (Kiani et al. unpublished data), and that of Arabbeigi et al. (2018) who tested and identified the expression patterns of some of the

most significant genes (namely, *AecSOS1*, *AecNHX1*, *AecHKT1;5*, and *AecVP1*) involved in Na^+ homeostasis in *Ae. cylindrica*, the current study was implemented to investigate the physiological responses and expression patterns of the genes *HKT1;5* and *NHX1* in the amphidiploids derived from the hybridization of 'Roshan' × *Ae. cylindrica* and 'Chinese Spring' × *Ae. cylindrica* when exposed to 250 mM NaCl.

Materials and methods

Plant materials

Wheat × *Ae. cylindrica* amphidiploid plants were developed through hybridizing wheat (*T. aestivum* L.) cultivars ('Chinese Spring' and 'Roshan') and *Ae. cylindrica*. For this purpose, a hyper-salt-tolerant genotype of *Ae. cylindrica* (USL26) originated from the shores of the Urumia Salt Lake in Northwest Iran (Arabbeigi et al. 2014; Kiani et al. 2015) was used as the male parent. Before and after pollination (24 hr each), the uppermost internodes were injected with a mixed solution (1:1) of 25 mg L^{-1} gibberellic acid (GA_3) 10 mg L^{-1} 2.4- and dichlorophenoxyacetic acid (2,4-D). Fourteen days after pollination, the immature embryos were cultured in the Murashige and Skoog (MS) basal medium (Sigma-Aldrich M9274) containing 0.8% agar and 2% sucrose. Subsequently, the colchicine treatment (Nemeth et al. 2015; Arzani and Darvey 2001) was carried out at the 4-5 tiller stage. The roots were then placed in a 0.1% (w/v) solution of colchicine supplemented with 2% DMSO (dimethyl sulfoxide) and 2 drops of Tween-20 per 100 mL of the solution at the 2-4 tiller stage for 5 h at 20° C. The roots were ultimately washed with tap water for 1 h before being grown under greenhouse conditions (Arzani and Darvey 2001).

Field experiment

A field experiment was carried out during 2017–2018 at the research farm of Isfahan University of Technology, located at Lavark (32□300N, 51□200E; 1,630 m asl), Iran, with a mean annual temperature and precipitation of 15º C and 140 mm. Prior to sowing, surface soil samples from the experimental site were taken from a depth of 0□30 cm, air-dried, and sieved through a 2-mm screen in order to determine their physico-chemical characteristics through analysis in the laboratory according to standard methods. The top 60-cm layer was found to be a clay loam soil (pH 7.5) with a bulk density of 1.48 g/cm3 and an average EC of 4 dS m^{-1} at the sowing date. At the harvesting date, however, the average soil EC of the salt-treated experimental plots was measured at 20.4 dS m^{-1}. A split-plot design with three

replications incorporating a randomized complete block arrangement was used. Salt treatments (normal/control and 250 MM NaCl) were assigned to the main plots and the genotypes to the sub-plots. Each plot contained two rows 2-m long spaced 30 cm from each other with plants spaced 10 cm from each other within the rows. Irrigation was applied via a drip system throughout the period from sowing to the late grain-filling stage. Salt treatment was initiated at the 4-tiller stage using initially an irrigation water containing diluted NaCl (125 mM) but continued with raising NaCl concentration to 250 mM.

Morphological traits

Agronomic traits including number of tillers, plant height, number of spikelets per spike (spikelet/spike), spike length, number of spikes per plant (spike/plant), flag leaf width (FLW), flag leaf length (FLL), flag leaf area (FLA), fresh flag leaf weight (FFLW), dry flag leaf weight (DFLW), plant fresh weight (FW), and plant dry weight (DW) were evaluated under the two control and salt stress conditions. The number of productive tillers per plant was determined prior to harvesting. Plant height from the ground level to the spike tip, excluding the awns, was recorded at maturity. FLL, FLW, and FLA were recorded using a leaf area instrument (Fanavaran Alborz Andisheh Co. Iran). FFLW and FW were determined using ten randomly selected plants. Finally, DFLW and DW were recorded using fresh samples dried in an oven at 70° C for 48 h.

Phenological traits

Days to heading (DH) defined as days from sowing to the emergence of 75% of the heads (spikes) in a plot, days to pollination (DP) representing the number of days from sowing to the emergence of anthers in 75% of the plants in a plot, and days to maturity (DM) denoting the number of days from planting to physiological maturity when the peduncles turned yellow were recorded under both control and salt-stress conditions.

Physiological traits

Enzymatic antioxidants

For enzyme extraction, 0.1 g of leaf sample was ground into a fine powder using liquid nitrogen and transferred into the extraction buffer containing 50 mM sodium phosphate (pH 7.8), 1% polyvinylpolypyrrolidone (PVP), 0.2% Triton X-100, 2 mM EDTA, 2 mM dithiothreitol (DTT), and 50 mM Tris–HCl. The homogenate was subsequently centrifuged at 12,000 g for 30 min before the supernatant was decanted and assayed for enzyme activity.

Ascorbate peroxidase (APX) activity was determined as the decrease in absorbance at 290 nm for 2 min (Nakano and Asada 1981). Catalase (CAT) activity, expressed as mmol H_2O_2 min^{-1} mg^{-1} of protein, was evaluated by measuring the rate of decrease in H_2O_2 absorbance at 240 nm as previously described (Aebi, 1984). The activity of guaiacol peroxidase (GPX) was assessed as the increase of absorbance at 470 nm for 2 min (Herzog and Fahimi 1973). Finally, protein content was determined by using bovine serum albumin as the standard (Bradford 1976).

Lipid peroxidation (MDA)

The thiobarbituric acid (TCA) test was used to measure malondialdehyde (MDA) as the lipid peroxidation product in leaf homogenates (Taulavuori et al. 2001). Briefly, 0.30 g of fresh leaf sample was homogenized in 5 ml of 0.1% TCA. The homogenate was then centrifuged at 12,000 g for 10 min at 4° C. Four ml of 20% TCA containing 0.5% thiobarbituric acid (TBA) was mixed in 1 ml of the supernatant, heated for 30 min at 95º C in a water bath, and immediately cooled on ice. After centrifugation at 10,000 g for 10 min, the absorbance of the extract was read at 532 nm and the values were corrected for non-specific turbidity by subtracting from absorbance at 600 nm. An extinction coefficient of 155 mM^{-1} cm^{-1} was used to calculate the MDA content using the following Equation:

$$MDA\ (nM) = \Delta A_{(532-600)} / 1.56 \times 10^5$$

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) was determined following the procedure proposed in Velikova et al. (2000). For this purpose, fresh leaf tissue (0.30 g) was homogenized in 5 ml TCA. The homogenate was subsequently centrifuged at 12,000 g for 10 min at 4° C and the supernatant was neutralized with 5 M K_2CO_3 (pH =5.6) in the presence of 100 μL of 0.3 M phosphate buffer (pH=5.6). The reaction was allowed to continue for 1 h in the dark; absorbance was ultimately measured at 390 nm. H_2O_2 concentration was calculated using the standard curve based on known concentrations of H_2O_2.

Carotenoid and chlorophyll content

The chlorophyll (a+b) and carotenoid (x+ c) contents of leaf tissues (0.33 g) in 80% acetone extract were determined spectrophotometrically using the redetermined extinction coefficients and equations introduced by Lichtenthaler and Buschman (2001). Briefly, about 0.5 g of fresh flag leaf was cut from mid-section, ground and mixed in 5 mL of 80% acetone,

and stored in the dark for 5 min. The solution was then filtered and the absorbance of the filtrate was read at 645, 663, and 470 nm using 80% acetone as the blank. The quantities of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids were calculated and expressed in mg g^{-1} FW using the following equations:

chlorophyll $a = \frac{[(12.7 \times abs\ 663) - (2.69 \times abs\ 645)]}{1000 \times w}$

chlorophyll $b = \frac{[(22.9 \times abs\ 645) - (4.69 \times abs\ 663)]}{1000 \times w}$

total chlorophyll= chlorophyll a + chlorophyll b

carotenoids $= \frac{[(1000 \times abs\ 470) - (1.82 \times chl\ a) - (85.02 \times chl\ b]}{198}$

Leaf and root Na and K contents

Root and shoot samples were dried for 48 h at 80º C to constant weights. To determine leaf and root Na and K contents, relevant samples (0.2 g dry weight) were incinerated in a muffle furnace at 550º C for 4 h. The ash thus obtained was then dissolved in 10 ml of 2 N HCl and its volume was made to 100 ml. Using a standard curve, the mineral contents were determined by flame photometry (Jenway PFP7, UK) (Houshmand et al. 2005) and the K/Na ratio was accordingly calculated.

Gene expression analysis

The sown seeds were allowed to germinate for 7 days at 4º C before they were transferred into pots 30 cm in height and 20 cm in diameter, which had been filled with a mixture of soil and sand (coarse crushed) at a ratio of 3:1 and placed in a greenhouse with average day/night temperatures of 25 ± 3/17 ± 3 ºC, respectively; a relative humidity of 60–65%; and a photoperiod of 12 h with a photosynthetic photon flux density (PPFD) of about 480 µmol m^{-2} s^{-1}. The seedlings were initially watered with tap water, which was continued throughout the experiment only for the control pots (EC = 1.5 dS m^{-1}). For the salt treatment pots, however, salt stress was initiated by gradually adding 250 mM NaCl upon the emergence of the second leaf. A three-factor factorial experiment was conducted to assess the effects of NaCl treatments (0 and 250 mM NaCl at 24 h and 7 days for the gene expression assay) on two tissue types (root and shoot) of five genotypes with four replicates (two for each of the biological and technical) of 10 plants. Roots and shoots from each experimental unit were sampled, snap-frozen in liquid nitrogen, and immediately stored at –80º C until use.

RNA extraction and cDNA synthesis

The plant tissue sample (100 mg) was ground into a fine powder using liquid nitrogen. Total RNA was isolated using the CTAB method (Murray and Thompson 1980) with minor modifications. Briefly, the powder was homogenized with the extraction buffer (2% CTAB, 20 mM EDTA pH 8.0, 2% PVP-40, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, and 10% of β-mercaptoethanol added immediately prior to use) and the tube was incubated at 65° C for 10 min. Eight milliliters of chloroform was then added to the tube before its vigorous inversion and centrifugation at 10,000 g for 10 min at 4° C. The supernatant was transferred to a new tube, mixed with an equal volume of chloroform:phenol (1:1), and centrifuged at 10000×g for 10 min at 4° C. An equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added to the supernatant obtained from centrifugation, which was then mixed and centrifuged at 10,000 g for 10 min at 4° C. Subsequently, the supernatant was mixed with LiCl (8M ¼ vol concentration) in a new tube and incubated at –20° C for 4 hours. The precipitated RNA was pelleted by centrifugation at 12,000 g at 4º C for 20 min, the pellets were subsequently washed twice with ice-cold 70% ethanol, dried, and finally suspended in RNase-free water.

DNase treatment of total RNA was carried out by Sina kit, Iran, according to the manufacturer's instructions. The purity of the total RNA was quantified using an ND-1000 NanoDrop (Germany). Synthesis of cDNA from l µg RNA was carried out using Suprime -Script RT premix (GeNet Bio, Korea) according to the manufacturer's instructions.

Quantitative Real-Time RT-PCR

Using the gene-specific primers of the *HKT1;5* and *NHX1* genes (namely, 5′-TCCTCCTTG\GACTTGATGTAG-3')/5′-CCATAGACCTTGACCTGAT-3' and 5'-GAATGCCACTCAGATCCAGC-3'/5'-GCTGCTGGGTGGCTTAGTGC-3', respectively), the expression profiles of the above genes were analyzed by conducting qRT-PCR in 20 µl of a reaction mixture containing 1 µl of diluted cDNA, 5 µl of 2x Fast SYBR Green PCR Master Mix (Applied Biosystems), 0.2 µl of 1 µM each of the reverse and forward primers, and 13.6 µl of RNAase free water. Amplification was performed on a StepOnePlus™ Real-Time PCR system (Applied Biosystems) according to the following cycling protocol: 94° C for 4 min, 42 cycles of 95º C for 30 s, 60º C for 35 s, 72° C for 1.5 min, and 72° C for 10 min. A housekeeping gene, β-actin (ACTB), was used as the reference to normalize the expressions of the genes studied. The data of real-time RT-PCR were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The relative expression levels of the target mRNA

in both roots and shoots were calculated using the ΔΔCT method and expressed relative to the values in the shame (normal tissues) after normalization.

Statistical analysis

The data obtained were tested for homogeneity and normality of variance before being subjected to analysis of variance (ANOVA) using SAS version 9.3 (SAS Institute 2011) in which genotype and treatment were considered as fixed effects and replication as a random effect. The Fisher's LSD test ($LSD_{0.05}$) was used to detect significant differences among the means of the treatments. Correlation and linear regression analyses were performed to delineate the relationships among the traits using SAS PROC CORR and SAS PROC REG, respectively. Principal component analysis (PCA) was carried out on the data on physiological responses to salt stress (250 mM NaCl) obtained for the amphidiploids and their parents; the Kaiser's rule of Eigenvalue exceeded 1.0 using PROC FACTOR (method = prin) in SAS 9.3. The first two PCs (PC1 and PC2) were plotted to construct the genotype-by-trait biplot using Stat Graphics.

Results

Morphological traits

The results of ANOVA showed that salt stress had significant effects on all the traits except the number of spikelets per spike, spike length, number of spikes per plant, FLL, and FLW (Table 1). The overall means of the number of tillers and plant height were observed to reduce by 26.60% and 17.86%, respectively, while the overall means of FW and DW decreased by 49.54% and 65.61 %, respectively, in 250 mM NaCl treatment (Table 2). Significant interaction effects of salt stress by genotype were only detected for the number of tillers, FW, and DW traits (Table 1).

Salt stress was found to cause a significant reduction in growth and morphological traits (Table 3), whilst the amphidiploids derived from both 'Chinese Spring' × *Ae. cylindrica* and 'Roshan' × *Ae. cylindrica* crosses were less affected compared to the wheat cultivars (i.e., female parents) but more affected than their wild male parent. The F_1 amphidiploid plants derived from the 'Chinese Spring' × *Ae. cylindrica* cross recorded average numbers of tillers equal to 33 and 24.06 tillers per plant under control and salt stress conditions, respectively, whereas the 'Chinese Spring' recorded a mean number of 12.61 tillers per plant in the control and 9.06 in the salt stress treatment. Although the highest reduction in plant height was

observed in ‘Chinese spring’ (16.18), both *Ae. cylindrica* and ‘Chinese Spring’ × *Ae. cylindrica* hybrid plants were affected by salt stress as evidenced by reductions of 13.19% in *Ae. cylindrica* and 14.02% in ‘Chinese spring’ × *Ae. cylindrica* amphidiploids (Table 3). Drastic reductions of 50.91% and 42.71% due to salt stress were observed in the number of spikes per plant in the female parents (wheat cultivars), while the number of spikelets per spike and spike length were comparatively less affected (Table 3). On the other hand, salt stress caused only a minor and insignificant reduction in these traits in either *Ae. cylindrica* or the amphidiploid plants. Likewise, the highest reduction in flag leaf area (27.38%) due to salt stress was observed in ‘Chinese Spring’ (Table 3). Comparisons based on DFLW indicated no significant changes in the case of *Ae. cylindrica* and ‘Chinese Spring’ × *Ae. cylindrica* hybrid plants as a result of salt stress, while considerable reductions (29.41 % and 26.32 %, respectively) were observed in ‘Chinese Spring’ and ‘Roshan’ as the female parents (Table 3). Although all the genotypes exhibited significantly decreased FW and DW values due to salt stress, lower reductions in these traits were observed in ‘Roshan’ × *Ae. cylindrica*, ‘Chinese Spring’ ×*Ae. cylindrica* amphidiploids, and their male parent (*Ae. cylindrica*) under salt stress (Table 3).

Phenological traits

The amphidiploid plants derived from the ‘Roshan’ × *Ae. cylindrica* and ‘Chinese Spring’ × *Ae. cylindrica* crosses as well as their parents exhibited significant changes in phenological traits (DH, DP, and DM) when subjected to 250 mM NaCl stress compared to those grown under the control conditions (Table 1). The amphidiploid plants derived from ‘Roshan’ × *Ae. cylindrica* and ‘Chinese Spring’ × *Ae. cylindrica* crosses showed significant declines in DH due to salt stress, with mean values of 161.33 and 157.80 days under the control and salt stress conditions, respectively (Fig. 1). Moreover, the average values 170.13 and 185.73 days obtained for DP and DM under the control conditions declined to 165.27 and 176.87 days, respectively, under salt stress (Fig. 1).

Compared to the common wheat (*T. aestivum* L.) cultivars (i.e., ‘Roshan’ and ‘Chinese Spring’), *Ae. cylindrica* exhibited lower DH, DP, and DM values in both control and salt stress conditions (Fig. 2). However, the interspecific hybrid plants obtained from the ‘Roshan’ × *Ae. cylindrica* and ‘Chinese Spring’ × *Ae. cylindrica* crosses exhibited intermediate phenology (Fig. 2).

Physiological traits

Results of the analysis of variance showed that all the physiological traits except CAT and APX activities were significantly affected ($p<0.01$) by salt stress (Table 1). The genotypes differed significantly with respect to the studied physiological traits except for APX activity (Table 1). Salt stress led to significantly increased GPX activity, Chl_a, Car, MDA, and H_2O_2 content, while the other two antioxidant enzymes (namely, APX and CAT) showed no significant effects of salt stress (Fig. 3a). Compared to the control conditions, salt stress led to increases of 26.82% and 7.09% in the overall means of Chl_a and Car, respectively (Figs. 3b and c). The highest increases in Chl_a (27.10 %), Tchl (3.35 %), and Car (10.52%) due to salt stress were recorded for *Ae. cylindrica* (Table 4). In addition, *Ae. cylindrica* and 'Chinese spring' exhibited the lowest and highest reductions in Chl_b content (31.94 % and 42.62%, respectively) under salt stress when compared with recorded values in the control treatment.

Significant differences in leaf and root Na and K contents were observed among the genotypes under the control and salt-stress conditions (Table 1). Compared to the control treatment, salt stress was observed to raise Na contents in both leaf (from 0.49 to 1.07 mM g^{-1} DW) and root (from 0.59 to 1.51 mM g^{-1} DW) (Fig. 4). In addition, salt stress caused a significant decrease in K content in both root (48%) and leaf (62%) tissues, with the leaf K content being evidently higher (Figs. 4a and b). Moreover, salt stress caused significantly decreased K/Na ratios in both root and leaf tissues (Fig. 4c). Nonetheless, the amphidiploid plants and *Ae. cylindrica* exhibited lower Na contents, higher K contents, and elevated K/Na ratios in both their root and leaf tissues than those measured for the wheat cultivars tested (Table 4). Compared to the female parents, the amphidiploids and wild parents exhibited higher Na accumulations in their roots than in their leaves (Table 4).

Salt stress led not only to significant increases in leaf MDA from 5.37 to 8.10 nM g^{-1} FW but also to meaningful enhancements of 1.27 –1.63 mM g^{-1} FW in leaf H_2O_2 in the control and stressed plants, respectively (Figs. 5a and b). Moreover, its effect on MDA depended on genotype, with the highest observed in the female parents (namely, 115.9% in 'Roshan' and 149.30% in 'Chinese Spring' cultivars) (Table 4). An overall increase of 27.82% was observed in leaf H_2O_2 content of the genotypes tested (Fig. 5b), while much higher enhancements were observed in *Ae. cylindrica* and amphidiploid plants than in the female parents (Table 4).

Fig. 6 shows the linear relationships between leaf dry matter (% control) and leaf mineral status (Na, K content, and K/Na ratio) under salt stress. Clearly, dry matter is significantly and negatively correlated with Na content (r^2 =0.40, p < 0.01) while it is significantly and positively correlated with K content (r^2 = 0.58, p < 0.01) and K/Na ratios (r^2 =0.33, p < 0.01). The genotypes superior with respect to dry matter such as the interspecific hybrid plants and *Ae. cylindrica* exhibited the lowest Na contents but higher K contents and K/Na ratios in their leaves when subjected to salt stress with 250 mM NaCl (Fig. 6).

Under the control conditions, FW and DW were positively correlated with Car, CAT, APX, and GPX activities as well as leaf K/Na ratio and root K content while they were negatively correlated with H_2O_2 content and leaf Na content. The correlation coefficients calculated for the data obtained under the 250 mM NaCl treatment showed that FW and DW were negatively correlated not only with MDA but with leaf and root Na contents as well but positively correlated with H_2O_2 content and leaf K/Na ratio as well as leaf and root K contents (Table 5). Meanwhile, a positive correlation was established between CAT activity and DW while H_2O_2 was at the same time significantly correlated with root K content as well as leaf and root K/Na ratios but negatively correlated with MDA and leaf Na content. Although leaf Na content showed negative correlations with APX and GPX activities under salt stress, leaf K content was positively associated with them. Finally, leaf and root K/Na ratios were found positively correlated (Table 5).

The results of PCA revealed that approximately 56% and 24% of the variance in data distribution could be explained by PC1 and PC2, respectively, under salt-stress conditions (Fig. 7). Principal Component 1 (PC1) was found more closely correlated with FW DW, leaf K/Na ratio, and root K content as well as CAT and APX activities, but PC2 exhibited higher correlations with Chl_a, Chl_b, Tchl, and Car under the control conditions. Under salt stress, PC1 established negative correlations with H_2O_2, root K content, and K/Na ratio but it was positively associated with leaf Na content. On the other hand, PC2 was positively correlated with PDW but negatively correlated with root Na content. Ranking of the genotypes in the biplot identified amphidiploids and *Ae. cylindrica* as superior genotypes with respect to salt tolerance due to both their low PC1 and high PC2 values (Fig. 7b).

Expression of salt tolerance genes

The ANOVA results revealed the significant effects of salt stress on the expression profiles of both *NHX1* and *HKT1;5* in the roots and shoots (Table 6). More specifically,

HKT1;5 was induced by 2.6 times after 7 days of exposure to 250 mM NaCl as compared with the control (Figs. 8a and b). Comparison of the expression patterns of the genes studied revealed that the expression level of the *NHX1* gene was by around six times higher than that under the control conditions (Figs. 8c and d).

At the tissue level, *HKT1;5* expressed mainly in the roots; indeed, its expression level in the root tissues was 2.4 times higher than that in the shoot tissues (Figs. 8a and b). On the other hand, the transcript level of *NHX1* in the shoots was significantly greater (2.2-fold) than that in the roots (Figs. 8c and d).

The expression pattern analyses also showed significant differences in the responses of amphidiploids and their parents to the 250 mM NaCl stress (Table 6). Compared to 'Chinese Spring' and 'Roshan' cultivars, *Ae. cylindrica* and amphidiploids exhibited much higher *HKT1;5* expressions in the shoot. However, no significant differences were detected between the amphidiploids derived from 'Roshan' × *Ae. cylindrica* and 'Chinese Spring' × *Ae. cylindrica* crosses at the tissue level (Fig. 8a). Neither were any significant differences observed in the *HKT1;5* expression in the root tissues of *Ae. cylindrica* and amphidiploids plants, while these plants were significantly different in this respect from the wheat parents (Fig. 8b). *HKT1;5* transcript accumulations in the roots of *Ae. cylindrica* as well as 'Roshan' × *Ae. cylindrica*, and 'Chinese Spring' × *Ae. cylindrica* amphidiploids, respectively, under salt-stress were 3.7-fold, 4.2-fold, and 4.1-fold relative to those of the corresponding controls (Fig. 8b).

The *NHX1* transcripts of the salt-tolerant *Ae. cylindrica* in both tissues significantly exceeded those of the others. Interestingly, comparable mRNA expression levels were observed for *NHX1* in the roots and shoots of the amphidiploids of the 'Roshan' × *Ae. cylindrica* and 'Chinese Spring' × *Ae. cylindrica* crosses (Figs. 8c and d). Compared to the control, several-fold increases were detected in the *NHX1* transcript levels in the shoots of not only *Ae. cylindrica* but of the amphidiploids of 'Roshan' × *Ae. cylindrica* and 'Chinese Spring' × *Ae. cylindrica* crosses as well (Fig. 8c). Finally, around 4-fold increases were detected in the root tissues of *Ae. cylindrica*, 'Roshan' × *Ae. cylindrica* and 'Chinese Spring' × *Ae. cylindrica* amphidiploids under NaCl stress when compared with the values under the control conditions (Fig. 8d).

Discussion

Salt stress is one of the most limiting factors in the production of such crop plants as wheat. Plant osmoregulation, protection against oxidative damage, and the strategy of maintaining a low level of cytosolic Na content i.e., Na exclusion intracellular compartmentation, and K/Na homeostasis are among the most important mechanisms of salt tolerance (Arzani 2008; Munns and Tester 2008). Wild relatives of wheat are valuable resources of tolerance to abiotic factors, whereby they have paved a long course of evolution via natural selection in the Fertile Crescent as their land of origin (Arzani and Ashraf 2016). *Aegilops* is the closest relative of the *Triticum* genus within the Triticeae tribe. The most salt-tolerant species from the 22 *Aegilops* species is *Ae. cylindrica*. Breeding would have relied primarily upon introgression of wild material, which offers a substantial improvement of salt tolerance in wheat.

In the present study, salt tolerance as well as the gene expression profiles of *HKT1;5* and *NHX1* were studied in the relevant amphidiploid plants derived from the 'Chinese Spring' × *Ae. cylindrica* and 'Roshan' × *Ae. cylindrica* crosses when subjected to salt stress with 250 mM NaCl. It was found that the amphidiploids derived from both interspecific crosses were less affected by salt stress than the wheat cultivars (i.e., the female parents) were and that they were either comparable or inferior to their male parent (*Ae. cylindrica*) with respect to the morphological and physiological traits or the gene expression profiles studied. Since availability of genetic resources of salt tolerance is an indispensable component of salt-tolerance improvement scheme (Arzani and Ashraf 2016), the synthesized amphiploids are potentially valuable resources for salt-tolerance improvement in common wheat through the backcrossing approach.

In the current study, both flag leaf area and plant height were found significantly influenced by genotype and salt stress. These findings are in line with the assumption that genotypes with smaller flag leaf areas and plant heights possess lower yield losses under salt-stress conditions (Chamekh et al. 2016) as a result of low accumulations of toxic ions in their photosynthetically active leaves (Pan et al. 2020). Although salt stress was observed to cause a marked reduction in the growth of both wheat cultivars ('Chinese Spring' and 'Roshan'), the interspecific hybrid plants exhibited significantly lower reductions as did *Ae. Cylindrica* as well. Salt stress is claimed not only to have an important impact on plant biomass but also to stand as a key trait for assessing salt tolerance in crop plants (Munns et al. 2016; Negrao et al. 2017). Studying barley, Han et al. (2018) found tolerant genotypes to exhibit lower

reductions in their dry biomass and less Na^+ accumulated in their roots and shoots than did the other wild or cultivated genotypes investigated. Consistent with our data, Zeeshan et al. (2020) recently reported that the reduction in plant dry weight was much more noticeable in sensitive wheat cultivars subjected to salt stress. Similar results had also been reported by Kiani et al. (2015) and Kumar et al. (2017).

Although plants are potentially capable of escaping salt, there might exist an evolutionary trade-off between salt escape and other salt tolerance strategies. Our findings confirm that while the phonological traits (DH, DP, and DM) declined under salt stress, the amphidiploid plants and *Ae. cylindrica*, with already low growth periods under both the control and salt-stress conditions, exhibited less plasticity. Indeed, shortened life cycle is one of the important strategies that wild species more often use to cope with drought, salt, and temperature stresses. Phenological stages in the current study were observed to occur earlier in response to salt stress, which is consistent with the observations reported by Chamekh et al. (2016). Phenotypically plastic genotypes that adjust phenology through differential gene expressions following salt-stress cues are maintained to offer the valuable germplasm for the salt escape mechanism (Munns et al. 2016).

The superior salt tolerance of the amphidiploids and *Ae. cylindrica* was substantiated by a non-significant decline in their Tchl in contrast to the significant Tchl reduction observed in wheat cultivars. The deleterious effects of salt stress on chlorophyll content in wheat have also been reported elsewhere (Rauf et al. 2010; Kumar et al. 2017; Al-Ashkar et al. 2019).

Significant increases in CAT, APX, and GPX activities were detected in the amphidiploids of both the 'Roshan' × *Ae. cylindrica* and Chinese Spring ×*Ae. cylindrica* crosses while they were more or less declining in their female parents due to the stress (Table 2). Increasing antioxidant enzyme activities such as those of APX, GPX, and CAT provide further support for the above inferences about superior salt tolerance of the amphidiploids over that of the wheat cultivars. In addition, CAT was observed to have a positive correlation with DW, a finding that is in agreement with recent empirical results reported by Al-Ashkar et al. (2019). Scavenging reactive oxygen species or free radicals (namely, $O_2^{\square}$, $OH^{\square}$, and H_2O_2) that are extremely damaging oxidizing agents are modulated by superoxide dismutase (SOD) to remove the toxicity of the superoxide anion. The ROS produced in chloroplasts are reduced to H_2O_2 and O_2 by SOD; the H_2O_2 and its derivatives thus produced are rapidly removed or reduced by APX or GPX (Sharma et al. 2016).

The genotypes investigated in this study showed differential responses to the discriminate K and Na in the soil solution. In addition, a higher Na accumulation was

detected in the roots than in the leaves under salt stress conditions. The discriminative dynamic features to uptake K^+ and Na^+ from the soil solution, the preferential accumulation of K^+, and the exclusion of Na^+ are known as the important mechanisms involved in the salt tolerance of the tribe Triticeae (Shabala et al. 2013; Kumar et al. 2015; Zhu et al. 2016). Generally speaking, it is the goal of wheat breeders in saline-prone regions to develop salt-tolerant cultivars highly capable of Na^+ exclusion, a trait that is found in such wild halophytically close progenitors of wheat as *Aegilops cylindrica* (Colmer et al. 2006; Arabbeigi et al. 2014).

Although salt stress elicited a decline in both root and leaf K contents, K accumulation in the leaves was by several folds higher in the roots under both salt stress and control conditions. However, the amphidiploids and *Ae. cylindrica* contained lower Na but higher K contents and K/Na ratios in both roots and leaves. These findings led the present authors to hypothesize that the amphidiploid plants exploited such superior features as diminishing Na influx into the root, Na retrieval from the xylem, regulating Na^+ efflux from the root, Na xylem loading, Na recirculation in the phloem, intracellular compartmentation of Na into the vacuoles, and Na secretion from the leaf (Arzani and Ashraf 2016; Kronzucker and Britto 2011). In addition, root Na and K content were found in the current study to be correlated positively and negatively, respectively, with DW under salt stress conditions, suggesting the critical role of the root in Na^+ transport and ion homeostasis. Plant root perpetually strives to adapt itself to perfect conformity with the saline environment through multiple strategies, one of which is conceding stress-elicited K^+ efflux for signaling commitments without endangering the cell via programmed cell death (PCD) (Rubio et al. 2020). Moreover, PCD is also reportedly induced under salt stress conditions by SKOR and GORK-mediated K^+ efflux (Arzani 2018).

The highest increase in MDA/lipid peroxidation was observed in the wheat cultivars (namely, 'Chinese Spring' and 'Roshan') while the lowest increase was recorded for the amphidiploids and *Ae. cylindrica* (Table 2). Consistent with our findings, lower levels of lipid peroxidation had been reported in salt-tolerant *Ae. cylindrica* genotypes (Kiani et al. 2015) and salt-tolerant wheat cultivars (Kumar et al. 2017). These findings are also complements to those reported on wheat and barley by Zeeshan et al. (2020) who found that salt-stress induced significant changes in MDA content among their cultivars, with the highest recorded for a sensitive (Sunmate) wheat cultivar. MDA is one of the end products of lipid peroxidation indicating the status of ROS (reactive oxygen species). ROS appear to act as signaling molecules on the one hand, while excessive generation of ROS leads to oxidative

damages to such fundamental biological molecules as proteins, RNA, DNA, and membranes (lipid peroxidation) as well as PCD, on the other (Arzani 2018).

The results of the current study showed that, similar to lipid peroxidation, H_2O_2 increased as a result of NaCl stress; this was accompanied by lower H_2O_2 accumulation in the amphidiploids and the hyper salt-tolerant genotype *Ae. cylindrica*. These results are underpinned by previous reports on the effects of salt stress on H_2O_2 accumulation in *Ae. cylindrica* and wheat genotypes (Sairam et al. 2005; Kiani et al. 2015). H_2O_2 plays an important role in the process of signaling salt-stress induced K^+ efflux from plant cells through non-selective cation (NSCC) channels (Rubio et al. 2020). Furthermore, H_2O_2 reportedly modulates the expressed SKOR, identifying this K^+ channel as a potential target for ROS signaling (Wu et al. 2018). Finally, enhanced levels of H_2O_2 trigger PCD to result in DNA laddering, caspase-3-like protein recruitment, and increased PARP cleavage (Arzani 2018).

Principal component analysis (PCA) serves to simplify a complex dataset of multiple dimensions by coalescing a set of variables into a few principal components (PCs) that capture the highest variance in the data. The relationships among the attributes are then used as efficient screening criteria. According to the biplot analysis of PC1 and PC2 in the current study, the amphidiploids and *Ae. cylindrica* (the male parent) were identified as superior salt tolerant genotypes. Examination of whole data also strengthened the claim that the amphidiploids are superior in terms of their salt tolerance to the wheat cultivars used as female parents.

HKTs and NHXs synergistically regulate Na^+ exclusion in salt-tolerant genotypes. HKTs regulate Na^+ homeostasis by controlling Na^+ uptake into the root (Munns et al. 2006) and by excluding excessive Na^+ from photosynthetic tissues (Arzani and Ashraf 2016). The hyper salt-tolerant USL26 genotype of *Ae. cylindrica* has been similarly reported to possess a high capability for Na^+ exclusion conferred by C and D genomes (Arabbeigi et al. 2014). In the present study, this hyper salt-tolerant genotype was used to develop the amphidiploid plants. The expression analysis of *HKT1;5* and *NHX1* in the tested genotypes revealed that they could serve as potential candidates for Na^+ exclusion from the roots and shoots of 'Roshan' × *Ae. cylindrica* and 'Chinese Spring' × *Ae. cylindrica* amphidiploids and their male parent. The *HKT1;5* and *NHX1* genes exhibited differential expression patterns in the shoot and the root such that *HKT1;5* had a higher expression in the roots than in the leaves but the opposite was true for *NHX1*.

Alteration in plant gene expression is induced by environmental stresses such as salt stress through either epigenetic changes or modulation of the core signaling networks that successively trigger a downstream network of interacting hormones and transcriptional regulators. The whole process leads to the adjustment of plant growth and development, generating molecular, physiological, and phenotypic plasticity in the plant. Previous study has demonstrated that *AtHKT1* facilitates Na^+ homeostasis and modulates K^+ nutrient status in the plant (Zhang et al. 2017; Rus et al. 2004; Oyiga et al. 2018). In *T. aestivum*, *HKT1;5* as the Na^+ selective transporter plays an important role in improving salt tolerance by Na^+ exclusion from the xylem vessels to its parenchyma cells (XPCs), thereby maintaining a higher K^+/Na^+ ratio and reducing leaf Na^+ content (Oyiga et al. 2018; Rus et al. 2004). The results of the present study indicated that *HKT1;5* gained a higher expression in both the roots and shoots under the 250 mM NaCl treatment when compared with that under the control. Interestingly, the gene was to a significant level more strongly expressed in the roots than in the shoots. These findings are consistent with those previously reported, confirming the claimed significance of the genotype- and tissue-dependent patterns in the expression of *HKT1;5* in both wheat (Zamani et al. 2013) and *Ae. cylindrica* (Arabbeigi et al. 2018). The tissue-specific patterns observed can most likely be explained with recourse to the fact that *HKT1;5-A* encodes a plasma membrane Na^+ transporter expressed in the root cells surrounding xylem vessels located at an ideal site for extracting Na^+ from the xylem to reduce Na^+ transfer to the leaves (Munns et al. 2012). *HvHKT1* exhibits a similar function in barley, in which the mRNA of the *HvHKT1* gene is chiefly transcribed in response to salt stress in the roots rather than in the shoots as evidenced by its expression at the plasma membrane of the root cells (stele and epidermis), which is the layer surrounding the xylem (Han et al. 2018).

In contrast to the roots, the shoots exhibited a much higher expression of *NHX1* where it increased more consistently under the 250 mM NaCl treatment. These findings, together with the higher Na accumulation observed in the roots of the amphidiploids and *Ae. cylindrica* genotype as well as the lower root Na content of the wheat cultivars under the NaCl treatment, suggest that certain mechanisms may be involved in promoting Na exclusion from the photosynthetic tissue in *Ae. cylindrica* and its interspecific hybrid progeny. Our results agree well with those of Nawaz et al. (2014) who reported that *NHX1* was more strongly expressed in the shoots of halophytic and glycophytic Brassicaceae species than in their roots. Experimenting with *Suaeda salsa*, however, Ma et al. (2004) reported that the expression of

SsNHX1 was enhanced by NaCl treatment across the entire plant tissues including its shoots and roots.

The results of the current study also indicated that *NHX1* recorded a higher expression in the genotypes studied than did *HKT1;5*. Although salt exclusion from plant tissues is indisputably of utmost importance in handling salt injury, the salt tolerance of tissues is also a critical element of the plant's overall salt tolerance.

Conclusion

The different aspects of the current study lead to the general finding that amphidiploids exhibit more resemblance to their wild parent (a hyper-salt tolerant genotype of *Ae. cylindrica*). More specifically, we might draw the following conclusions from the data obtained. 1) Compared to the female parents, the interspecific hybrid plants and their wild parent were found less affected by salt stress in terms of growth, physiological, and phenological traits; 2) generally speaking, an interplay seems to exist between *HKT1;5* and *NHX1*, which restrict root-to-shoot transport of Na and elevate the vacuolar sequestration of Na in the shoot tissues, respectively, thereby synergistically regulating Na^+ homeostasis in *A. cylindrica* and amphidiploid plants subjected to salt stress; 3) the higher root and leaf K levels and K/Na ratios are associated with the plant's higher salt tolerance; and 4) the synthesized amphiploids might potentially serve as valuable resources for improving salt tolerance in bread wheat by further backcrossing with *T. aestivum* L.

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Table 1: Results of analysis of variance for the traits studied under the control and salt-stress (250 mM NaCl) treatments

Trait	Mean square				
	Salt stress (S)	Genotype (G)	G × S	Residual	CV %
Morphological traits					
Number of tillers	267.94**	395.04**	67.14**	7.71	14.25
Plant height	1378.75**	1371.46**	49.72^{ns}	41.65	9.33
Spikelet/spike	140.83^{ns}	478.11**	164.26^{ns}	53.41	20.55
Spike length	0.08^{ns}	11.97**	0.99^{ns}	0.28	5.49
Spike/plant	25.17^{ns}	169.65**	1.23^{ns}	10.34	27.79
FLL	2.61^{ns}	8.02*	5.90^{ns}	2.42	9.88
FLW	0.002^{ns}	1.41*	0.03^{ns}	0.02	12.62
FLA	46.33*	66.05**	19.63^{ns}	6.63	19.15
FFLW	0.01*	0.05**	0.02^{ns}	0.003	16.04
DFLW	0.006*	0.008**	0.002^{ns}	0.001	22.52
FW	0.01*	0.01*	0.01**	0.002	13.64
DW	0.01*	0.05**	0.003**	0.001	16.60
Phenological traits					
DH	93.63**	498.22**	3.38^{ns}	7.87	1.76
DP	177.63**	195.53**	14.46^{ns}	13.53	2.19
DM	589.63**	207.03**	21.47*	6.23	1.38
Physiological traits					
Chl_a	0.001**	0.003*	0.0002^{ns}	0.0002	15.86
Chl_b	0.003**	0.001**	0.0006^{ns}	0.0003	38.06
Tchl	0.009**	0.002**	0.0005^{ns}	0.0009	21.08
Car	15.07**	4.86**	1.77^{ns}	1.40	11.06
CAT	0.074^{ns}	0.22*	0.30^{ns}	0.098	27.29
APX	0.02^{ns}	0.02^{ns}	0.01^{ns}	0.02	51.62
GPX	1.01*	0.52*	0.62^{ns}	0.29	43.18
H_2O_2	0.45*	0.43*	0.10^{ns}	0.13	26.14
MDA	0.194**	0.021**	0.01^{ns}	0.008	33.65
Leaf Na	0.28**	0.04**	0.002^{ns}	0.001	3.21
Leaf K	12.86**	0.46**	0.42**	0.06	9.678
Leaf K/Na	7.65**	0.34**	0.02^{ns}	0.03	13.24
Root Na	11.08**	0.11*	0.06^{ns}	0.03	8.76
Root K	4.87**	0.09**	0.54**	0.02	5.39
Root K/Na	15.17**	0.16*	0.18*	0.04	13.60

Number of spikelets per spike (spikelet/spike), number of spikes per plant (spike/plant), flag leaf length (FLL), flag leaf width (FLW), flag leaf area (FLA), fresh flag leaf weight (FFLW), dry flag leaf weight (DFLW), plant fresh weight (FW), plant dry weight (DW), days to heading (DH), days to pollination (DP), days to maturity (DM), chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (Tchl), carotenoid (Car), catalase (CAT), ascorbate peroxidase (APX), guaicol peroxidases (GPX), MDA and H_2O_2 content, root and shoot Na and K contents and K/Na ratios.

^{ns} non-significant, * $p<0.05$ and ** $p<0.01$

Table 2: Mean comparisons of morphological traits under the control and salt-stress (250 mM NaCl) conditions

Trait	Control (0 mM NaCl)	Salt stress (250 mM NaCl)	LSD (P<0.05)	Change (%)
Number of tillers	22.48	16.5	2.12	-26.60
Plant height	75.92	62.36	4.92	-17.86
Spikelet/spike	37.73	33.39	5.57	-11.50
spike length	9.62	9.72	0.40	1.04
Spike/plant	12.49	10.66	2.45	-14.65
FLL	16.03	15.06	1.18	-6.05
FLW	1.07	1.02	0.10	4.67
FLA	17.69	15.26	1.96	-13.74
FFLW	0.35	0.31	0.04	-11.43
DFLW	0.15	0.13	0.02	-13.33
FW	40.33	20.35	8.48	-49.54
DW	9.13	3.14	2.72	-65.61

Number of tillers (#), Plant height (cm), number of spikelets per spike (spikelet/spike, #), spike length (cm), number of spikes per plant (spike/plant, #), flag leaf length (FLL, cm), flag leaf width (FLW, cm), flag leaf area (FLA, cm^2), fresh flag leaf weight (FFLW, gr), dry flag leaf weight (DFLW, gr), plant fresh weight (FW, gr) and plant dry weight (DW, gr).

Table 3: Mean comparisons of morphological traits of the parent plants (‘Roshan’, ‘Chinese spring’, *Ae. cylindrica*) and their F_1 hybrid plants (amphidiploids) under control and salt-stress (250 mM NaCl) conditions

Trait	Genotype								
	‘Roshan’			‘Chinese spring’			*Ae. cylindrica*		
	Control	Stress	Change (%)	Control	Stress	Change (%)	Control	Stress	Change (%)
Number of tillers	14.67	13.84	-5.66	12.61	9.06	-28.15	35.00	29.33	-16.20
Plant height	85.09	72.23	-15.11	84.26	70.63	-16.18	48.00	41.67	-13.19
Spikelet/spike	44.38	41.33	-6.87	37.40	35.80	-4.28	22.67	20.33	-10.32
Spike length	10.87	10.63	-2.21	7.97	7.50	-5.90	10.78	10.28	-4.64
Spike/plant	11.59	5.69	-50.91	6.79	3.89	-42.71	21.67	16.33	-24.64
FLL	14.93	14.75	-1.21	16.70	13.02	-22.04	17.70	16.98	-4.07
FLW	1.58	1.37	-13.29	1.27	1.23	-3.15	0.59	0.51	-13.56
FLA	17.95	14.70	-18.11	12.27	8.91	-27.38	10.49	8.66	-17.45
FFLW	0.42	0.35	-16.67	0.36	0.32	-11.11	0.21	0.18	-14.29
DFLW	0.19	0.14	-26.32	0.17	0.13	-29.41	0.10	0.09	-10.00
FW	40.42	22.34	-44.73	40.36	20.32	-49.65	28.20	17.17	-39.11
DW	12.17	4.13	-66.06	10.17	3.14	-69.12	7.08	3.46	-51.13

Trait	‘Roshan’ × *Ae. cylindrica* amphidiploid			‘Chinese spring’×*Ae. cylindrica* amphidiploid			LSD (P<0.05)	
	Control	Stress	Change (%)	Control	Stress	Change (%)	Control	Stress
Number of tillers	18.24	16.21	-11.13	33.00	24.06	-27.09	4.89	5.87
Plant height	78.91	67.67	-14.24	69.33	59.61	-14.02	11.11	12.44
Spikelet/spike	41.33	38.50	-6.85	30.87	21.00	-31.97	21.15	3.37
Spike length	10.13	9.54	-5.82	9.93	9.16	-7.75	1.32	0.60
Spike/plant	11.42	8.22	-28.02	16.27	12.14	-25.38	4.08	6.74
FLL	15.19	14.98	-1.38	16.55	16.48	-0.42	3.24	1.77
FLW	1.48	1.33	-10.14	0.64	0.62	-3.13	0.26	0.26
FLA	15.47	14.49	-6.33	17.27	14.28	-17.31	5.38	4.31
FFLW	0.36	0.31	-13.89	0.35	0.31	-11.43	0.11	0.08
DFLW	0.15	0.13	-13.33	0.19	0.17	-10.53	0.08	0.04
FW	30.36	19.29	-36.42	30.38	16.21	-46.64	3.16	2.19
DW	9.17	4.11	-55.18	8.19	3.31	-59.58	0.07	0.07

Number of tillers (#), Plant height (cm), Number of spikelets per spike (spikelet/spike, #), spike length (cm), number of spikes per plant (spike/plant, #), flag leaf length (FLL, cm), flag leaf width (FLW, cm), flag leaf area (FLA, cm^2), fresh flag leaf weight (FFLW, gr), dry flag leaf weight (DFLW, gr), plant fresh weight (FW, gr) and plant dry weight (DW, gr).

Table 4: Mean comparisons of physiological traits of the parent plants (‘Roshan’, ‘Chinese spring’, *Ae. cylindrica*) and their F_1 hybrid plants (amphidiploids) under the control and salt-stress (250 mM NaCl) treatments

Trait	Genotype								
	‘Roshan’			‘Chinese spring’			*Ae. cylindrica*		
	Control	Stress	Change (%)	Control	Stress	Change (%)	Control	Stress	Change (%)
Chl_a	1.11	1.29	16.22	1.05	1.08	2.86	1.07	1.36	27.10
Chl_b	0.58	0.34	-41.38	0.61	0.35	-42.62	0.72	0.49	-31.94
Tchl	1.69	1.63	-3.55	1.66	1.43	-13.86	1.79	1.85	3.35
Car	9.98	10.12	1.40	9.77	10.04	2.76	10.17	11.24	10.52
CAT	1.33	1.16	-12.78	1.91	0.97	-49.21	0.81	1.16	43.21
APX	0.24	0.30	25.00	0.26	0.25	-3.85	0.17	0.25	47.06
GPX	1.53	1.11	-27.45	1.54	1.36	-11.69	1.28	1.39	8.59
H_2O_2	1.17	1.24	5.92	1.05	1.09	3.81	1.29	2.15	66.22
MDA	5.22	11.57	115.9	3.59	8.95	149.30	3.71	5.27	42.05
Leaf Na	1.25	4.39	199.58	1.37	4.59	173.59	1.09	2.08	120.19
Leaf K	4.07	1.25	-69.35	4.55	1.69	-62.76	4.28	2.40	-43.99
Leaf K/Na	2.78	0.29	-89.66	2.72	0.38	-86.19	4.62	1.16	-74.82
Root Na	1.47	2.12	69.92	1.68	3.72	172.19	0.95	2.41	121.62
Root K	4.39	1.67	-61.84	3.90	1.77	-54.74	3.55	2.15	-39.51
Root K/Na	3.53	0.79	-77.58	2.94	0.48	-83.82	3.29	0.89	-72.84

Trait	‘Roshan’ × *Ae. cylindrica* amphidiploid			‘Chinese spring’×*Ae. cylindrica* amphidiploid			LSD (P<0.05)	
	Control	Stress	Change (%)	Control	Stress	Change (%)	Control	Stress
Chl_a	1.07	1.35	26.17	1.06	1.33	25.47	0.02	0.03
Chl_b	0.65	0.38	-41.54	0.68	0.43	-36.76	0.03	0.05
Tchl	1.72	1.73	0.58	1.74	1.76	1.15	0.05	0.07
Car	10.15	11.09	9.26	10.19	11.21	10.00	1.46	3.30
CAT	0.86	1.48	72.01	1.02	1.17	12.82	0.70	0.40
APX	0.20	0.29	45.00	0.19	0.25	31.57	0.11	0.32
GPX	1.34	1.39	3.73	0.83	1.08	22.72	1.20	0.66
H_2O_2	1.27	1.75	37.87	1.42	1.90	34.07	0.55	0.62
MDA	6.37	7.46	17.02	4.94	7.68	55.45	0.15	0.10
Leaf Na	0.81	2.19	170.96	1.01	1.84	81.92	0.27	0.84
Leaf K	3.44	2.46	-28.49	3.73	2.04	-45.40	1.51	0.67
Leaf K/Na	4.16	1.14	-72.59	3.69	1.12	-69.64	1.79	0.44
Root Na	1.16	2.55	119.64	1.29	2.52	93.75	0.34	0.28
Root K	3.18	1.99	-37.59	3.46	2.03	-41.20	0.89	0.22
Root K/Na	2.79	0.78	-71.93	2.66	0.82	-69.36	1.04	0.16

Chlorophyll a (Chl_a, mg g^{-1} FW), chlorophyll b (Chl_b, mg g^{-1} FW), total chlorophyll (Tchl, mg g^{-1} FW), carotenoid (Car, mg g^{-1} FW), catalase (CAT, mol min^{-1} mg^{-1} protein), ascorbate peroxidase (APX, mol $min^{-1}mg^{-1}$ protein), guaiacol peroxidases (GPX mol $min^{-1}mg^{-1}$ protein), MDA and H_2O_2 content (nM g^{-1} FW), root and shoot Na and K contents (mM g DW^{-1}) and K/Na ratios.

Table 5: Correlation coefficients of the traits studied under the control (above the diagonal) and salt-stress (250 mM NaCl) (below the diagonal) treatments

Trait	FW	DW	Chl_a	Chl_b	Tchl	Car	CAT	APX	GPX
FW	1	0.89^{*}	0.09^{ns}	0.34^{ns}	0.25^{ns}	0.62^{*}	0.70^{**}	0.52^{*}	0.55^{*}
DW	0.52^{*}	1	0.08^{ns}	0.40^{ns}	0.28^{ns}	0.68^{**}	0.56^{*}	0.53^{*}	0.51^{*}
Chl_a	0.33^{ns}	0.19^{ns}	1	0.78^{**}	0.91^{**}	0.48^{ns}	-0.16^{ns}	0.23^{ns}	0.13^{ns}
Chl_b	0.15^{ns}	0.07^{ns}	0.61^{*}	1	0.96^{**}	0.74^{**}	-0.04^{ns}	0.17^{ns}	0.24^{ns}
Tchl	0.26^{ns}	0.14^{ns}	0.88^{**}	0.92^{**}	1	0.67^{**}	-0.07^{ns}	0.21^{ns}	0.21^{ns}
Car	0.12^{ns}	0.09^{ns}	0.93^{**}	0.72^{**}	0.91^{**}	1	0.17^{ns}	0.30^{ns}	0.45^{ns}
CAT	0.45^{ns}	0.51^{*}	0.27^{ns}	0.24^{ns}	0.29^{ns}	0.15^{ns}	1	0.26	0.44^{ns}
APX	0.52^{*}	0.17^{ns}	-0.50^{ns}	-0.39^{ns}	-0.49^{ns}	-0.44^{ns}	-0.34^{ns}	1	0.34^{ns}
GPX	0.38^{ns}	0.25^{ns}	-0.09^{ns}	-0.36^{ns}	-0.26^{ns}	-0.27^{ns}	-0.11^{ns}	0.20^{ns}	1
H_2O_2	0.54^{*}	0.52^{*}	-0.41^{ns}	-0.06^{ns}	-0.24^{ns}	-0.34^{ns}	0.05^{ns}	0.53^{*}	0.02^{ns}
MDA	-0.74^{**}	-0.52^{*}	0.16^{ns}	-0.12^{ns}	0.06^{ns}	-0.05^{ns}	0.32^{ns}	-0.25^{ns}	0.47^{ns}
Leaf Na	-0.83^{**}	-0.52^{*}	0.19^{ns}	0.02^{ns}	0.11^{ns}	-0.02^{ns}	0.18^{ns}	-0.63^{*}	-0.51^{*}
Leaf K	0.62^{*}	0.58^{*}	-0.05^{ns}	-0.01^{ns}	-0.02^{ns}	0.11^{ns}	-0.37^{ns}	0.52^{*}	0.59^{*}
Leaf K/Na	0.78^{**}	0.56^{*}	-0.17^{ns}	-0.10^{ns}	-0.14^{ns}	0.05^{ns}	-0.34^{ns}	0.59^{*}	-0.47^{ns}
Root Na	-0.57^{*}	-0.64^{**}	-0.02^{ns}	0.23^{ns}	0.13^{ns}	0.06^{ns}	-0.34^{ns}	-0.60^{*}	-0.27^{ns}
Root K	0.81^{**}	0.53^{*}	-0.18^{ns}	-0.08^{ns}	-0.13^{ns}	-0.04^{ns}	-0.33^{ns}	0.50^{ns}	-0.16^{ns}
Root K/Na	0.38^{ns}	0.38^{ns}	-0.02^{ns}	-0.23^{ns}	-0.15^{ns}	-0.06^{ns}	0.09^{ns}	0.70^{**}	0.19^{ns}

Trait	H_2O_2	MDA	Leaf Na	Leaf K	Leaf K/Na	Root Na	Root K	Root K/Na
FW	-0.60^{*}	-0.04^{ns}	-0.89^{**}	0.26^{ns}	0.68^{**}	0.40^{ns}	0.63^{*}	0.24^{ns}
DW	-0.68^{**}	0.11^{ns}	-0.66^{**}	0.06^{ns}	0.61^{**}	0.29^{ns}	0.58^{*}	0.26^{ns}
Chl_a	-0.37^{ns}	0.10^{ns}	0.10^{ns}	0.13^{ns}	-0.07^{ns}	-0.39^{ns}	0.05^{ns}	0.25^{ns}
Chl_b	-0.52^{*}	0.41^{ns}	0.33^{ns}	0.23^{ns}	-0.17^{ns}	-0.20^{ns}	0.29^{ns}	0.35^{ns}
Tchl	-0.49^{ns}	0.30^{ns}	0.25^{ns}	0.20^{ns}	-0.11^{ns}	-0.29^{ns}	0.18^{ns}	0.32^{ns}
Car	-0.78^{**}	0.30^{ns}	-0.49^{ns}	0.22^{ns}	-0.27^{ns}	-0.06^{ns}	0.47^{ns}	0.40^{ns}
CAT	-0.27^{ns}	0.27^{ns}	0.67^{**}	0.27^{ns}	-0.64^{**}	0.44^{ns}	0.18^{ns}	-0.15^{ns}
APX	-0.16^{ns}	-0.31^{ns}	0.51^{*}	0.48^{ns}	-0.12^{ns}	0.31^{ns}	0.47^{ns}	0.18^{ns}
GPX	-0.46^{ns}	-0.03^{ns}	0.52^{*}	0.44^{ns}	0.19^{ns}	0.17^{ns}	0.40^{ns}	0.21^{ns}
H_2O_2	1	-0.12^{ns}	-0.40^{ns}	0.03^{ns}	0.38^{ns}	-0.18^{ns}	-0.26^{ns}	-0.06^{ns}
MDA	-0.59^{*}	1	-0.07^{ns}	-0.46^{ns}	-0.28^{ns}	0.02^{ns}	-0.33^{ns}	-0.29^{ns}
Leaf Na	-0.60^{*}	0.73^{**}	1	0.49^{ns}	-0.65^{**}	0.40^{ns}	0.57^{*}	0.22^{ns}
Leaf K	0.40^{ns}	-0.79^{**}	-0.75^{**}	1	0.32^{ns}	0.04^{ns}	0.66^{**}	0.60^{*}
Leaf K/Na	0.53^{*}	-0.76^{**}	-0.92^{**}	0.90^{**}	1	-0.3^{ns}	0.05^{ns}	0.28^{ns}
Root Na	-0.48^{ns}	-0.07^{ns}	0.41^{ns}	-0.08^{ns}	-0.33^{ns}	1	0.42^{ns}	-0.59^{*}
Root K	0.63^{*}	-0.77^{**}	-0.74^{**}	0.68^{**}	0.81^{**}	-0.27ns	1	0.71^{**}
Root K/Na	0.59^{*}	-0.23^{ns}	-0.60^{*}	0.35^{ns}	0.60^{*}	-0.90^{**}	0.64^{*}	1

Plant fresh weight (FW), plant dry weight (DW), chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (Tchl), carotenoid (Car), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidases (GPX), MDA and H_2O_2 content, root and shoot Na and K contents and K/Na^+ ratios

^{ns} non-significant, * $p<0.05$ and ** $p<0.01$

Table 6: Analysis of variance of the effects of NaCl treatments (0 and 250 mM at 7 days) on relative gene expression in plant tissues (shoot and root) in F_1 interspecific hybrids and their parents

Source of variation	Degree of freedom	Mean squares	
		NHX1	*HKT1;5*
Salt stress (S)	1	58.70^{**}	14.79^{**}
Generation (G)	4	1.32^{**}	3.37^{**}
Tissue (T)	1	11.23^{**}	5.99^{**}
S × G	4	0.57^{**}	0.56^{**}
S × T	1	8.54^{**}	9.12^{**}
G × T	4	0.15^{ns}	0.21^{**}
S × G × T	4	0.162^{ns}	1.05^{**}
Residual	40	0.97	0.06^{**}
CV (%)	-	10.68	13.87^{*}

^{ns} non-significant, * $p<0.05$ and ** $p<0.01$

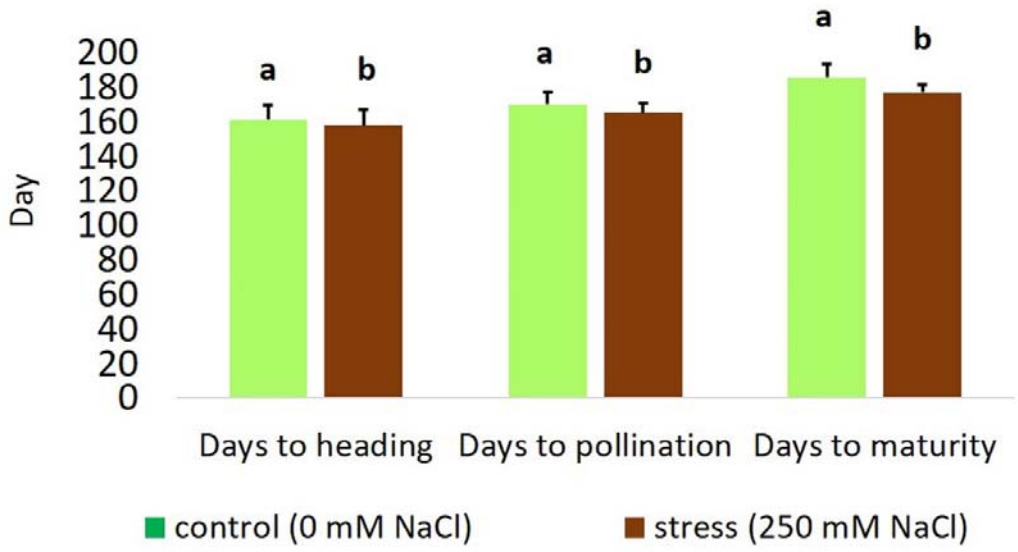

Fig. 1: Effects of salt stress on days to heading, days to pollination and days to maturity. Bars represent means ± SE and bars with the same letter do not significantly differ at $P<0.05$.

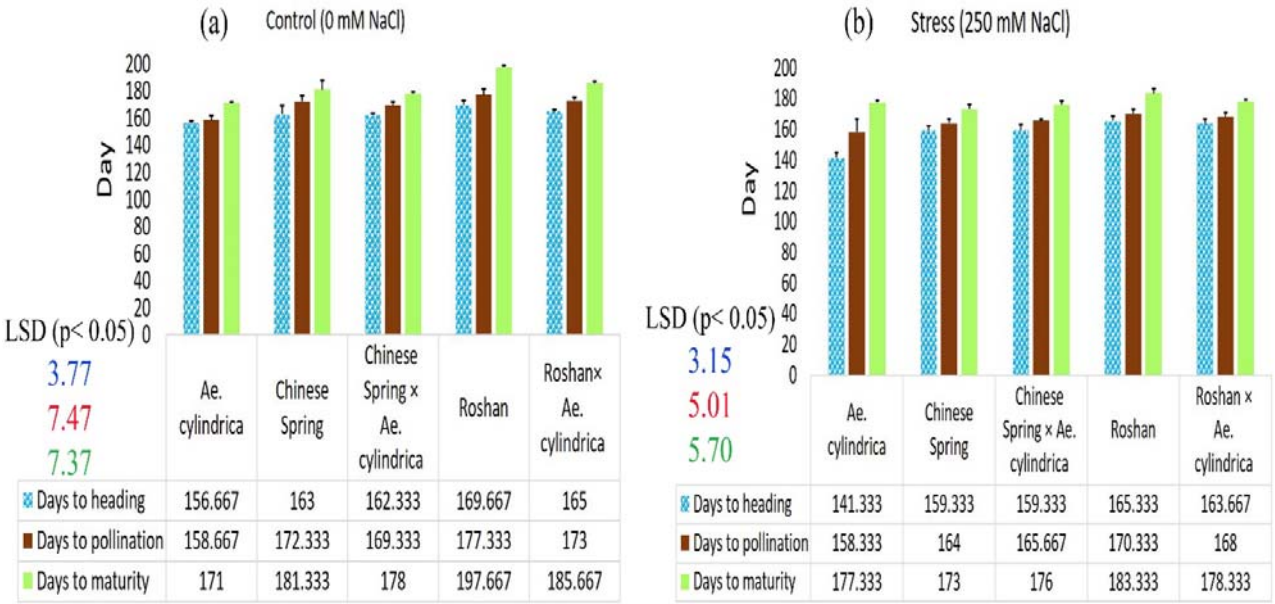

Fig. 2: Mean comparison of phenological traits of parents ('Roshan', 'Chinese spring', *Ae. cylindrica*) and their F_1 hybrid plants (amphidiploids) under (A) control and (B) salt stress (250 mM NaCl) conditions.

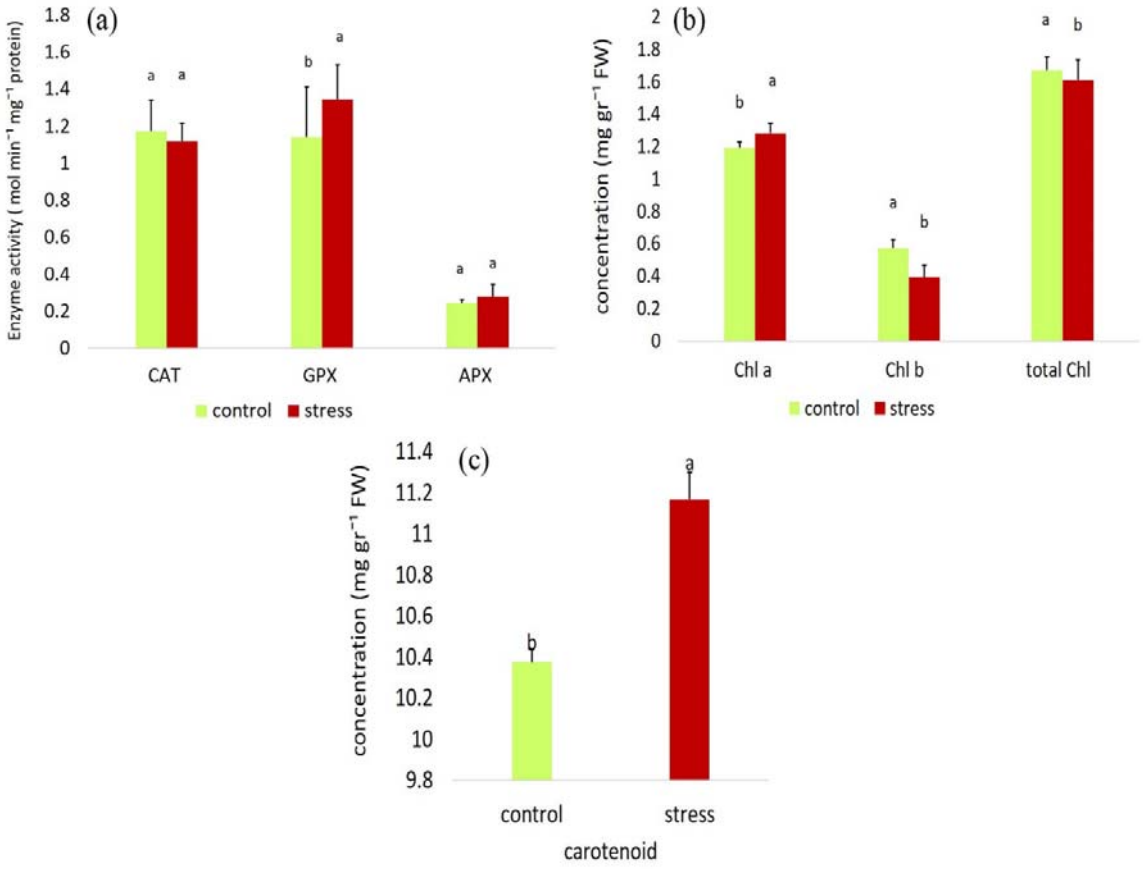

Fig. 3: Effects of salt stress on (a) catalase (CAT, mol $min^{-1}mg^{-1}$ protein), guaiacol peroxidases (GPX, mol $min^{-1}mg^{-1}$ protein), ascorbate peroxidase (APX, mol $min^{-1}mg^{-1}$ protein), (b) chlorophyll a (Chl_a, mg g^{-1} FW), chlorophyll b (Chl_b, mg g^{-1} FW) and total chlorophyll (Tchl, mg g^{-1} FW) and (c) carotenoid (Car mg g^{-1} FW) . Bars represent means ± SE and bars with the same letter do not significantly differ at $P<0.05$.

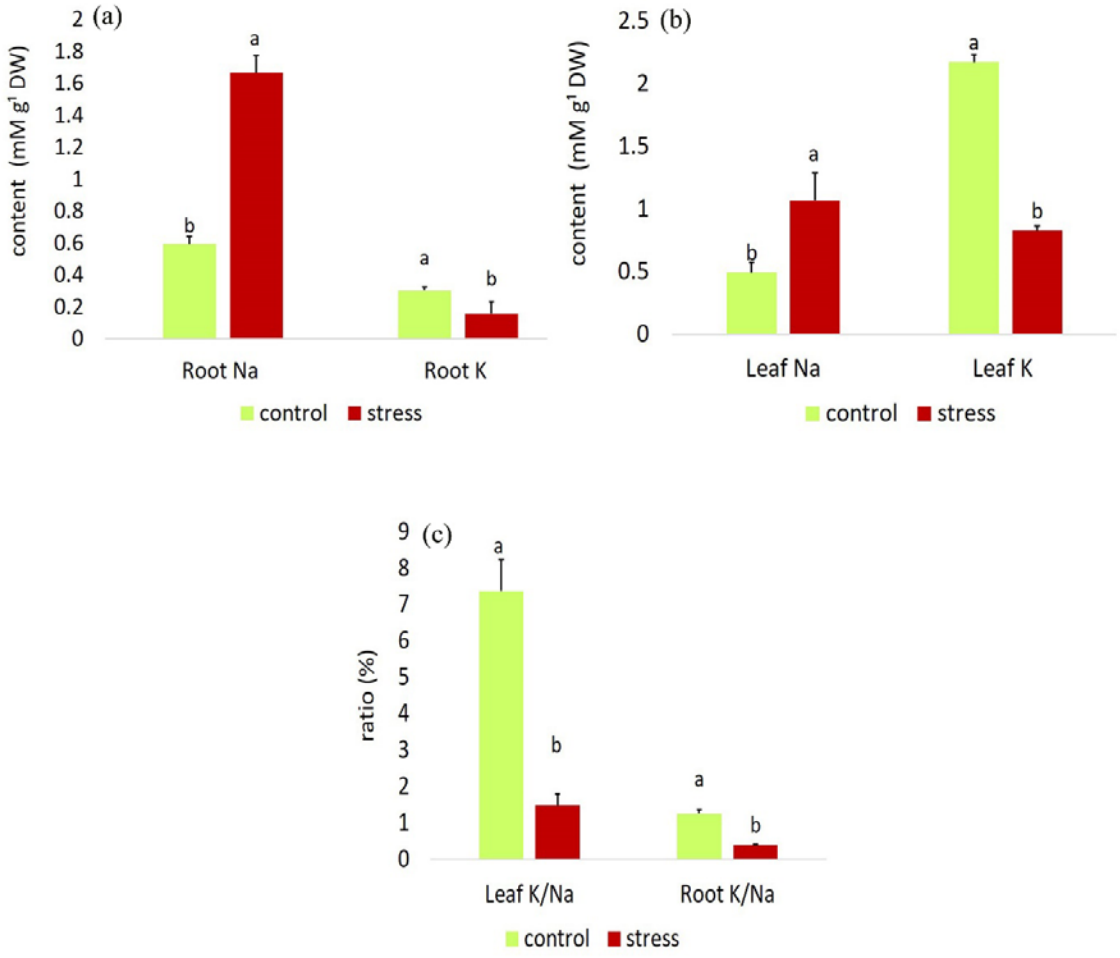

Fig. 4: Effects of salt stress on (a) root Na and K contents (mM g DW^{-1}), (b) leaf Na and K contents (mM g DW^{-1}) and (c) root and leaf Na/K ratios. Bars represent means ± SE and bars with the same letter do not significantly differ at $P<0.05$.

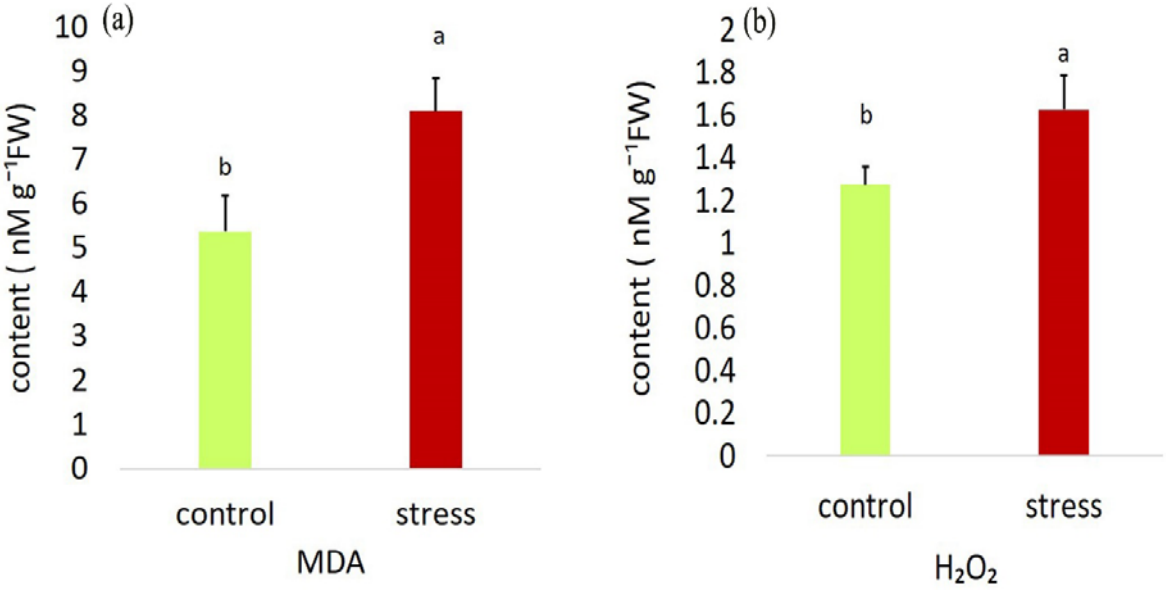

Fig. 5: Effects of salt stress on (a) MDA content (nM g^{-1} FW) and (b) H_2O_2 content (nM g^{-1} FW). Bars represent means ± SE and bars with the same letter do not significantly differ at $P<0.05$.

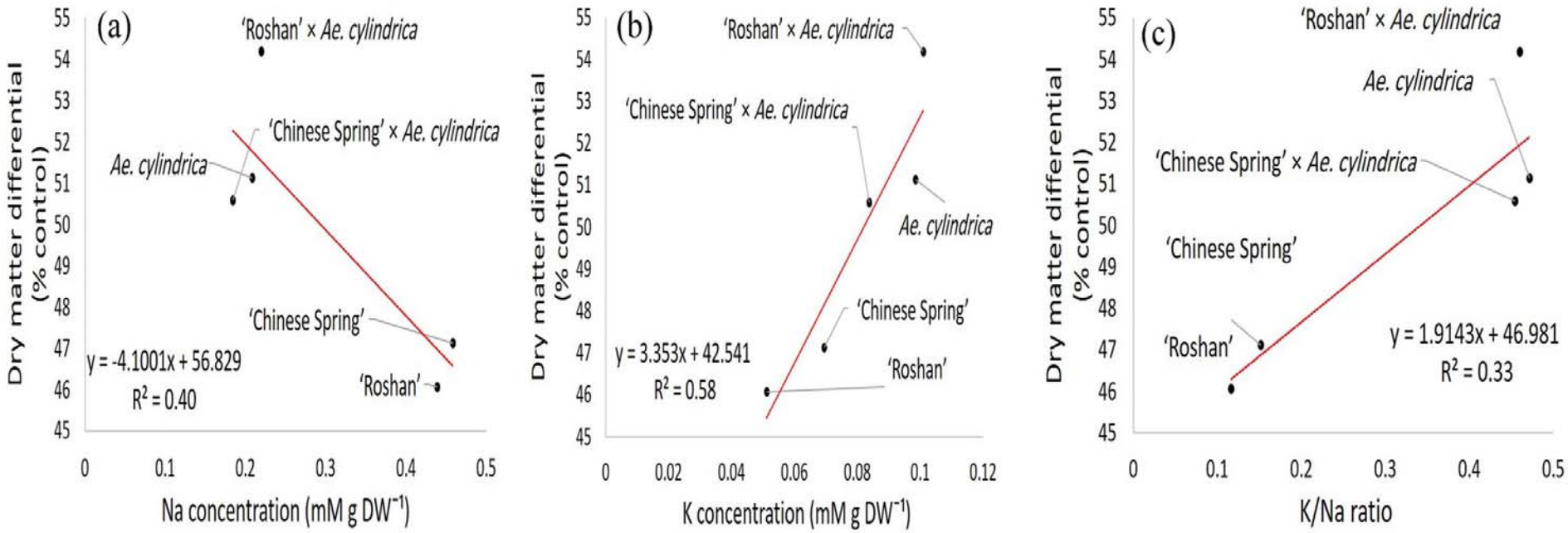

Fig. 6: Relationship between dry matter (% control) and leaf Na (a) leaf K (b) and content and leaf K/Na ratio (c) of F_1 interspecific hybrids and their parents in salt stress condition (250 mM NaCl).

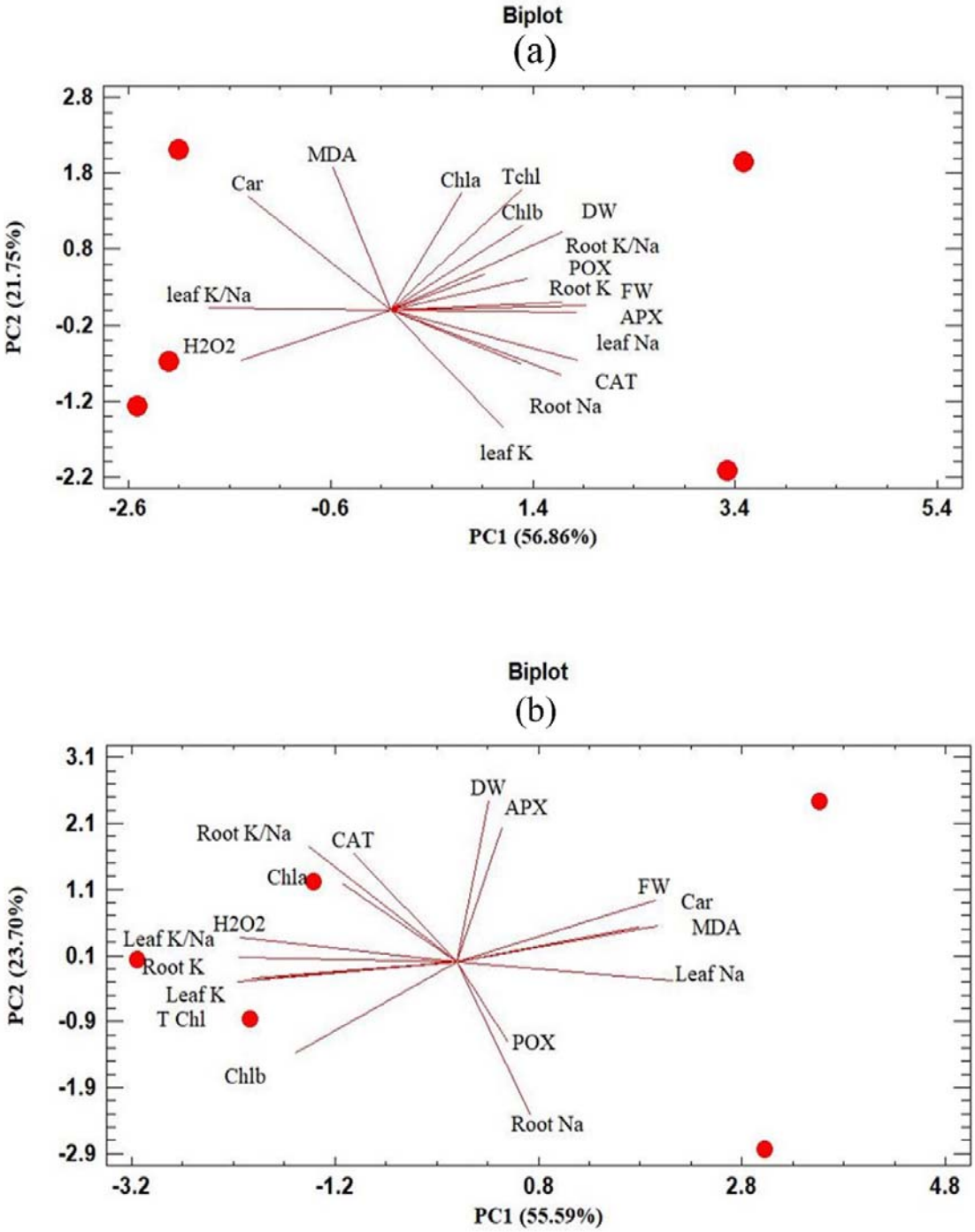

Fig. 7: Bi-plot display of plant fresh weight (FW), plant dry weight (DW), chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (Tchl), carotenoid (Car), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidases (GPX), MDA and H_2O_2 content (nM g^{-1} FW), root and shoot Na and K contents DW^{-1}, and K/Na^+ ratios (mM g) under control (a) and 250 mM NaCl (b) conditions.

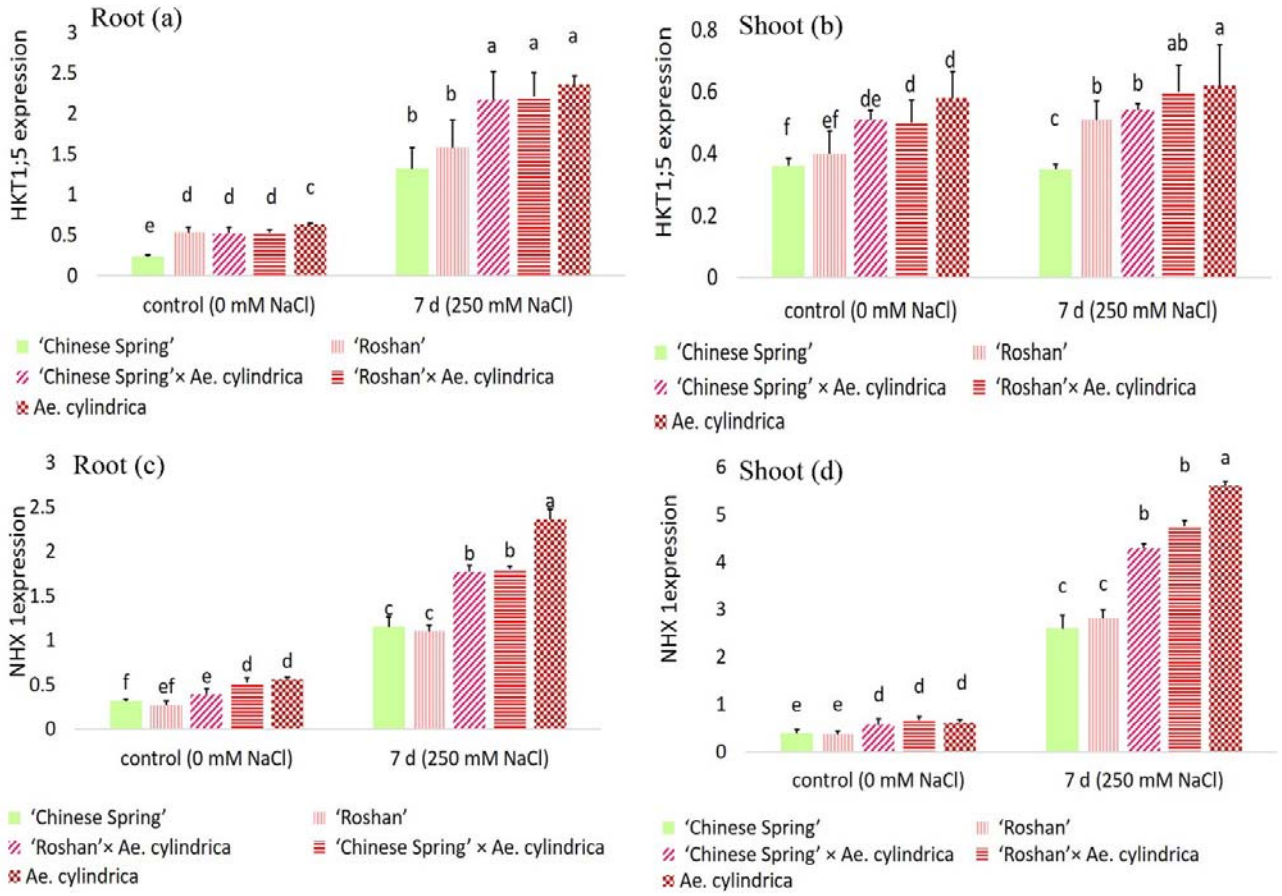

Fig. 8: Expression analysis of the *HKT1;5* gene in shoot (a) and root (b) tissues and *NHX1* gene in shoot (c) and root (d) tissues of F1 interspecific hybrids and their parents. Bars represent means ± SE and bars headed by same letter are not significantly different at P<0.05.