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SALINITY STRESS

Multidimensional screening and evaluation of morpho-physiological indices for salinity stress tolerance in wheat

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Abstract

Soil salinity is one of the major constraints to crop production worldwide. The multifaceted nature of salinity tolerance traits complicates plant screening and the identification of salt-tolerant germplasm to be used for the genetic advancement of corps. Many screening criteria have been suggested to distinguish between genotypes. Most of these were applied under controlled environmental conditions and limited to one developmental stage of plants. As a result, most of the reported tolerance could not be validated under field conditions. This study employed a membership function value (MFV) to assess NaCl tolerance of eight wheat (*Triticum aestivum*) genotypes measured at germination, vegetative and reproductive stages, as an integrative tool for the overall plant performance. Salt stresses had an adverse effect on plant physiological (residual transpiration, stomatal density, chlorophyll fluorescence characteristics; leaf N, Na^+ and K^+ content) and agronomical (plant height; biomass; root and tiller number; grain) characteristics. Based on this assessment, plants were divided into three contrasting groups: salt tolerant, moderately salt tolerant and salt sensitive. Although genotypes did not show the same degree of tolerance in germination, glasshouse and field experiments, the variety Yu-07 showed consistently better performance in all trials, whilst Aus19720 was most sensitive in glasshouse and field experiments. These contrasting genotypes could be of a potential value for further studies to uncover the genetic mechanisms governing salt stress response in wheat.

KEYWORDS
agronomy, membership function value, potassium, sodium chloride stress, stomata, yield

1 | INTRODUCTION

Soil salinity is one of the main abiotic stresses limiting crop production worldwide. Approximately 6% of the total land area and 20% of irrigated land are affected by salinity (Ghonaim et al., 2021) comprising about 1125 million ha in the world (Hossain, 2019). The rate of soil salinization is about 3 ha/min (Shabala et al., 2014) due to various natural reasons (rising sea level, low rainfall, high surface evaporation, weathering of native rocks) and human activities (poor irrigation and drainage systems).

Wheat is the second most important cereal crop (FAO, 2021) and responsible for about 20% of the total dietary calorie and protein

uptake worldwide (Shiferaw et al., 2013). It is an important commercial crop for Australia, which accounts for 10% of global wheat exports (Fischer et al., 2014). The demand for wheat is expected to increase by up to 60% by 2050 (Nelson et al., 2010; Shiferaw et al., 2013). However, wheat is a moderately salt tolerance crop and in Australia about 69% of the wheatbelt is affected by soil salinity (Rengasamy, 2002), which results in about a 40% yield reduction (Shabala & Munns, 2012), costing the Australian economy around A$200 million per year (Orton et al., 2018). An effective and feasible way to improve wheat yield is via genetic improvement, by creating salinity tolerant germplasm (Shabala, 2013).

Salinity tolerance is a complex of traits that affects various physiological processes, such as osmotic adjustment, ionic homeostasis (mainly Na^+/K^+), oxidative homeostasis (ROS) and efficient stomata operation (Quamruzzaman et al., 2021a, 2021b; Zhao et al., 2020). Osmotic tolerance is associated with improving water uptake capacity of plants, thus enhancing cell expansion. Ion homeostasis contributes to ionic balance (mainly K^+/Na^+) and assures optimal metabolic activity of key organelles. Oxidative stress tolerance minimizes ROS damage to plant's cellular structures and macro molecules like DNA, enzymes and lipids. It is also causally related to salinity-induced programmed cell death (Shabala, 2009). Stomatal closure is associated with reducing transpiration for better water saving under salinity stress conditions (Hedrich & Shabala, 2018; Niu et al., 2018).

Many attempts have been made to improve the salt-tolerant wheat, but success in breeding programs has been limited due to the lack of superior donor germplasm, making a single gene approach unsuccessful (Flowers, 2004; Liang et al., 2018). The reliance on polygenetic pyramiding often means the relative contribution of key traits that confer salinity tolerance changes with plant ontogeny (Ashraf & Akram, 2009). Finally there is a lack of effective screening methods. Different methods have been developed for screening wheat for salinity tolerance based on plant growth and yield, salinity damage score, germination and physiological mechanisms including K^+/Na^+ discrimination. Methods suitable for use in controlled environments are concomitant with a need for a large number of populations and require a high of salinity stress (Munns & James, 2003). A membership function value (MFV) has been used as an effective screening method for diverse abiotic stress tolerance including salinity (Wu et al., 2019), drought (Chen et al., 2012) and mercury tolerance (Liu et al., 2017). The MFV method is a process to combine multiple screening methods and requires only simple mathematical calculation. This method is applicable for screening a large number of accessions (Chen et al., 2012; Wu et al., 2019; Mohamed et al., 2020) at any growth stages and growth conditions.

Most prior studies focused on plant screening under controlled environments, with less attention given to field trials; this fact is often considered as one of the major hurdles in the breeding process (Dadshani et al., 2019; El-Hendawy et al., 2009). Under field conditions plants must deal with other abiotic (e.g. drought, heat) and biotic (e.g. disease, insect) stresses concomitant with soil heterogeneity, fluctuations of air temperature and rainfall at the same time with salinity stress (Dadshani et al., 2019; Moustafa et al., 2021).

The present study was designed to validate the use of MFV as an integrative tool for overall plant performance to assess NaCl tolerance of eight wheat (*Triticum aestivum*) genotypes measured at germination, vegetative and reproductive stages, under controlled (glasshouse) and field conditions. A secondary objective was to identify genotypes with higher salinity tolerance that could be utilized as donors to breed salt-tolerant cultivars.

2 | MATERIALS AND METHODS

2.1 | Plant materials

A total of eight different wheat accessions were obtained from Australian and Chinese National GeneBanks. These were selected based on the salinity damage scores from our previous GWAS experiment (Quamruzzaman, Manik, Shabala, Cao, et al., 2021a; Quamruzzaman, Manik, Shabala, & Zhou, 2021b). The full list and a short description of these genotypes are given in Table S1.

2.2 | Phenotypic analysis

2.2.1 | Germination experiment

Germination tests were carried out following the procedure described below. The seeds of these genotypes were surface sterilized with 70% ethanol for one minute and washed three times with distilled water. Then seeds were kept at room temperature for 24 h. Thirty uniform seeds of each genotype were selected and germinated in 9-cm Petri dishes. Seeds were placed on a double layer of blotting paper and treated with either 150 mM NaCl solution or distilled water (control). Each Petri dish was considered as an experimental replication. Seeds were considered to have germinated when the radicle length was ≥2 mm.

The number of germinated seeds were recorded after seven days of treatment. The shoot length (SL), root length (RL), shoot fresh weight (SFW), root fresh weight (RFW), seedling dry weight (SDW) and relative water content (RWC) were also measured after 7 days of treatment. Abnormal seedlings with much faster (excessively tall seedling) or slower growth (excessively short seedling) were removed and only uniform seedlings from each replication were used to record the SL (cm), RL (cm), SDW (g) and RWC (%).

2.2.2 | Glasshouse experiment

Two sets of glasshouse pot experiments were conducted between February 2020 and December 2020. Pre-germinated seeds were sown in plastic pots filled with potting mixture described by Fan et al. (2016). A spilt plot design was used for both experiments with salinity treatment as main plots and genotypes as subplots, and

three plants per pot. Salt treatment (150 mM NaCl) was applied at the 2–3 leaf stage and continued through till harvesting.

The first experiment was conducted using three replications for measuring of leaf Na^+ and K^+ contents at early growth stages. After 2.5 months of salt treatment plants were harvested. Leaves of three different physiological ages old, intermediate and newest fully open (usually the flag leaf) - were used for measuring the leaf ionic content. Salinity tolerance was also assessed during the vegetative stage by visual observation of leaf injury (based on leaf yellowing and wilting; 0 = no damage and 10 = all dead) (Hasanuzzaman et al., 2018).

The second experiment was carried out using four replications to assess grain yield and yield components. Plants were harvested at maturity and the following parameters recorded: plant height (cm), number of tillers, biological yield (BY, grain weight +straw weight), grain yield (GY), thousand seed weight (TSW) and harvest index (HI).

2.2.3 | Field experiment

The eight genotypes were further evaluated under field conditions during April 2020 to December 2020. The experiment was arranged as a split plot design with treatment as main plots and genotypes as subplots. The field site was located at the Tasmanian Institute of Agriculture, Mt Pleasant Laboratories at Prospect, Tasmania, Australia (41.44°S 147.14°E). Daily minimum and maximum air temperatures and rainfall throughout the entire period of the experiment were obtained from an automatic meteorological station (weather station no. 091237) located close to the experimental site. Details of weather are presented in Figure S1.

Seeds were sown in round concrete tanks (2100 mm diameter by 450 mm depth). The tanks were filled with sandy loam soil and the bottom of each tank contained 50 mm coarse gravel overlaid with drainage matting. Each tank was divided into four equal parts, with one part containing 85 seeds of each genotype. Salt treatments were started when plants reached the 2–3 leaf stage. Initial salt treatment was applied using raw salt (2.7 kg NaCl/tank, 2 split with one week interval) between the rows and diluted by watering from top of the plants to bring the soil solution up to 150 mM NaCl. Control plots were watered using tap water. Three replicates were applied for both salinity treatment and controls. Salinity levels were monitored by measuring electric conductivity (EC) of the soil leachate. Subsequent treatments of NaCl solution were applied based on EC value of leachate water after rainfall to keep the salinity of the leachate at 150 mM.

2.3 | Sampling and measurements

2.3.1 | Physiological measurements

The third fully open leaves of three plants were collected from the field experiment. The cut end was immediately sealed with a nail polish and taken to the laboratory to measure initial leaf fresh weight (W_i). Samples were then placed in a controlled dark room at 20–21°C and 58% relative humidity. Leaves were weighed again after 24 h (W_{24}) and placed in a dry oven at 40°C for 72 h and reweighed (W_d). Residual transpiration (RT) was measured per dry weight basis by using the following formula (Hasanuzzaman et al., 2017).

$$\text{Residual transpiration} = \frac{(W_i - W_d) - (W_{24} - W_d)}{W_d}$$

where W_i = Initial fresh weight; W_{24} = Fresh weight after 24 h; W_d = Dry weight. The measured residual water loss was expressed in mg H_2O cm^{-2}.

The relative water content of sample was measured using the following formula

$$\%\,\text{RWC} = \frac{\text{FW} - \text{DW}}{\text{FW}} \times 100$$

Where FW = sample fresh weight, DW = sample dry weight.

Stomatal density (SD) was quantified on the abaxial leaf surface of the third fully open leaf. The leaf surface was coated with a thin layer of a clear nail polish. The dried layer of a nail polish was peeled off using fine forceps and placed on a microscopic glass slide and covered with a coverslip. The imprints were examined under an optical microscope with a 40×objective lens, and stomatal density was determined within the field of view and expressed per surface area.

Leaf area (LA) was measured from the thrid fully open leaf at 1.5 months after salt treatment. LA was recorded using the LI-3100C Licor leaf area meter.

Relative chlorophyll content (SPAD values) and maximum photochemical efficiency of photosystem II (PSII, chlorophyll fluorescence *Fv/Fm* value) were measured on the third fully open leaf by using a Minolta Chlorophyll Meter SPAD-502 (https://www.konicaminolta.com.au/) and Chlorophyll Fluorometer (OS-30p, Opti-Science, USA), respectively. Later, SPAD values was transformed to associated specific leaf nitrogen content (SLN, g m^{-2}) values by following the equation of Van Oosterom et al. (2010). Details for calculation of SLN are given in the Table S1.

$$\text{Ln}(\text{SLN}) = 0.041 \times \text{SPAD} - 1.87,\ n = 75,\ R^2 = 0.90,\ p < .001$$

Three types of leaves (flag leaf, intermediate leaf and old leaf) from glasshouse experiments and the third fully open leaf from the field experiments were selected for the measurement of Na^+ and K^+ contents. Dried leaf samples of all germplasm were weighted to approximately 100 mg, ground and placed into 15 ml digestion tubes. After that 10 ml (100 mM) acetic acid was added to each tube and the tube heated at 90°C in a digestion block for 3 h (Chen et al., 2020) with periodical shaking. Samples were then cooled down, and 1ml of digested solution was taken to determine Na^+ and K^+

ion concentrations using LAQUA Twin Na^+ and K^+ meter (HORIBA Advanced Techno Co., Ltd.), respectively.

The normalized difference vegetation index (NDVI) and normalized difference red edge (NDRE) were measured using Crop Circle model ACS-430 from Holland Scientific (Lincoln, NE, USA) at 1.5 months after salt treatment from 0.5m above the canopy. NDVI and NDRE data were recorded by measuring the canopy reflectance between wavebands 670 ± 5.5 nm (red reflectance), 730 nm (red edge reflectance) and 780 ± 10 nm (near-infrared reflectance). Ground cover was estimated by photos of plants taken with Nikon D3500 DSLR camera and processed in ImageJ software (Rasband, 2012). Ground cover photos were taken from 0.50 m above the canopy during periods of cloud cover.

2.3.2 | Yield and yield components

To record yield and yield components, plants were air-dried for about three weeks. Amongst the measured traits were biological yield (BY), grain yield (GY), harvest index (HI) and 1000 seeds weight (TSW). To determine the grain yields, all spikes were threshed using a belt thresher Agriculex Lbt-2 Belt Thresher, Canada. The thousand seeds were counted by SeedCount SC5000 (Next Instruments, Condell Park, NSW, Australia) and weighted by a digital scale.

2.4 | Salt tolerance evaluation

The membership function value (MFV) was used to evaluate the overall plant performance under saline conditions. The MFV was calculated from the relative value of salinity and control treatment (salinity tolerance coefficients) for every studied trait. Salt tolerance coefficients (STC) were calculated using the formula

$$\text{STC} = \frac{\text{Value for the NaCl treated plant}}{\text{Value for the control}} \times 100$$

MVF value was calculated using the following formula for every trait described by Wu et al. (2019)

$$\text{MVF1} = \frac{\text{STC}_i - \text{STC}_{\min}}{\text{STC}_{\max} - \text{STC}_{\min}}$$

$$\text{MVF2} = 1 - \frac{\text{STC}_i - \text{STC}_{\min}}{\text{STC}_{\max} - \text{STC}_{\min}}$$

Where STC_i is the salt tolerance coefficient of a specific genotype. $\text{STC}_{\min}$ and $\text{STC}_{\max}$ are the minimum and maximum salt tolerance coefficient value amongst all genotypes, respectively.

MFV2 was used for the traits that are inversely related for salinity tolerance (lower the value, higher the tolerant to salt, for example leaf Na^+ content and RT).

The MFV produces the value ranged between 0 and 1. MFV of all traits for each germplasm were averaged together and ranked the degree of salinity tolerance amongst wheat germplasms (from 1 to 8).

2.5 | Statistical analysis

Analysis of variance (ANOVA) and Pearson correlation for salinity tolerance traits were performed using IBM SPSS software package (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp). Mean separation was determined at $p \leq 0.05$ by using Tukey's test (Tukey, 1977).

3 | RESULTS

3.1 | Phenotypic characterization at germination experiment

Significant growth and biomass reduction was observed due to salt treatment within the eight genotypes (Figure 1). The lowest reduction of shoot fresh weight (37%), root length (32%), shoot length (31%) and seedling dry weight (24%) were observed in genotype Yu-07. The highest reduction of SL (83%), RL (51%) and SFW (86%) were found in genotype Revenue. In the case of seedling dry weight, the genotype Yu-07 and Revenue produced 24% and 49% less seedling dry weight compared to control treatemnt respectively. The reduction in relative water content ranged between 7 and 9% across the genotypes and was not significantly different.

Salt tolerance coefficient value was used to find out the correlation amongst morpho-physiological traits of wheat. Most of the traits were found to positively correlated with each other (Table 1). As expected, SFW demonstrated highly significant correlation with SL, RL and SDW (at $p < 0.01$). The SDW also showed significant relationship with SL and RL.

3.2 | Phenotypic characterization in glasshouse experiment

Salinity damage scored (SDS) was assessed through visual symptoms of leaf wilting and chlorosis. Control plants did not show any obvious leaf wilting or chlorosis. SDS showed a wide range of variations amongst the eight germplasm at the early growth stage of plants under stress conditions. SDS ranged between 1.67 (tolerant) to 8.67 (sensitive).

Leaf ionic content (Na^+, K^+ and K^+/Na^+ ratio) was measured at the vegetative growth stage of plants (Figure 2a–c). Based on the leaf Na^+ content, genotypes were divided into two groups, group 1 Na > 15 mg g^{-1} and group 2 Na < 15 mg $g^{-1)}$. Genotypes H-243, H-132, Aus19720, Aus19402 were in group 1 and Revenue,

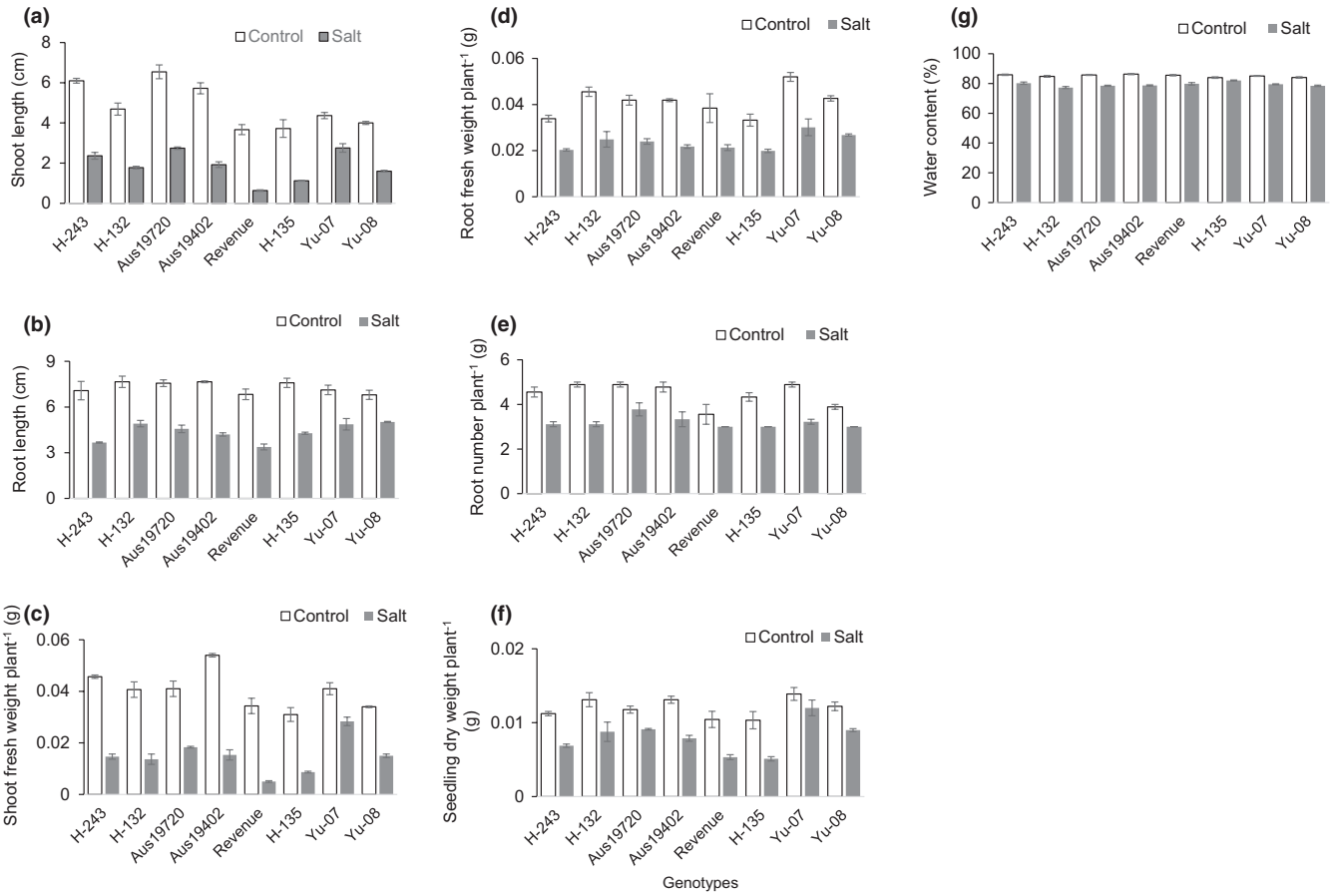

FIGURE 1 Impact of salt stress on germination stage of eight wheat genotypes. (a) shoot length, (b) root length, (c) shoot fresh weight, (d) root fresh weight, (e) root number, (f) seedling dry weight (shoot + root), (g) water content. Means were separated by Tukey's test at $p \leq 0.05$. Vertical bars indicate ± standard error of the mean ($n = 3$)

TABLE 1 Pearson's correlation coefficient (*r*) for phenotypes measured in germination trial

	SL	RL	SFW	RFW	RN	SDW	RWC
SL	1						
RL	0.660	1					
SFW	0.973**	0.742**	1				
RFW	0.225	0.419	0.302	1			
RN	−0.555	−0.222	−0.386	0.114	1		
SDW	0.892**	0.751*	0.927**	0.178	−0.226	1	
RWC	−0.148	−0.109	−0.097	0.575	0.007	−0.417	1

*,**Correlation is significant at the 5% and 1% level.

H-135, Yu-07, Yu-08 were in group 2. Between these two groups, the highest and lowest leaf Na^+ content was observed from H-243 and Yu-07, respectively. In contrast, genotypes from the group 1 and group 2 showed 10% and 41% reduction in leaf K^+ content compared to the control, respectively. The highest and lowest K^+ content was recorded in H-132 and Yu-08, respectively. Consistent with this, Yu-07 and H-243 exhibited higher and lower K^+/Na^+ ratio compared with other genotypes under salinity treatment.

Salinity stress reduced the agronomic traits for all genotypes in glasshouse conditions. The maximum (31%) and minimum (5%) plant height reduction was noticed in Aus19720 and Aus19402, respectively, compared with control (Figure 2d). On the other hand, genotypes from the group 1 showed 89% and group 2 74% relative changes in tiller number. The exception was noticed in genotype Aus19402, which produced equal number of tillers both in salt and control conditions. The most significant decline in tiller numbers were observed in Revenue (31%) (Figure 2f).

The biological yield decline ranged from 5% to 40% across the eight genotypes. As expected, a reduction in agronomic and physiological characteristics lead to a decrease in grain yield. The latter varied from 1.6 ± 0.23 g to 6.4 ± 1.16 g, and the maximum and minimum

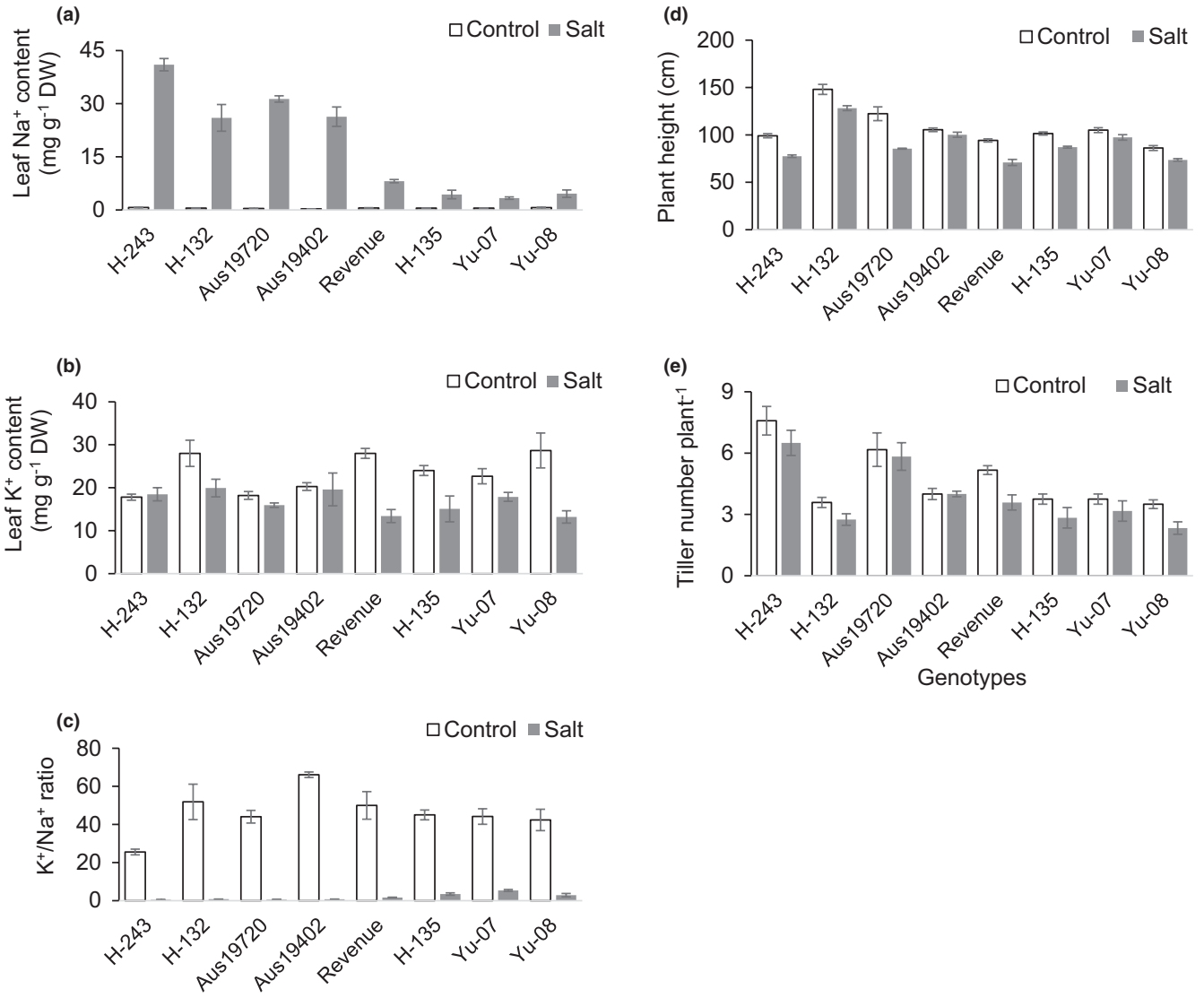

FIGURE 2 Effect of salinity stress on leaf ionic content and morphological traits in glasshouse experiment. (a) leaf Na^+ content, (b) leaf K^+ content, (c) K^+/Na^+ ratio, (d) plant height, (e) tiller number. Means were separated by Tukey's test at $p \leq 0.05$. Vertical bars indicate ± standard error of the mean ($n = 3$ for a, b, c and $n = 4$ for d, e)

grain yield reduction was observed in Aus19720 (45%) and Yu-07 (12%), respectively. These two genotypes also exhibited a similar trend in declining of TSW. In contrast, no significant difference was noticed either in genotypes or between the treatments in the harvest index (Figure 3).

3.2.1 | Correlations

Leaf Na^+ and K^+ content showed strong and positive correlation with the number of tillers (Table 2). Na^+ content showed strong but negative correlation with K^+/Na^+ ratio, whilst the K^+/Na^+ ratio produced significant correlation with TSW. Plant height showed a strong positive correlation to GY, BY, TSW and HI. Similarly, BY and GW exhibited strong correlation with each other ($r > 0.9$).

3.3 | Phenotypic characterization in field experiment

3.3.1 | Morphological and physiological phenotypic evaluation

After 1.5 months of salt treatment, genotypes were divided into three group, that is highly injured, moderately injured and less injured. Out of eight genotypes, H-243, Aus19720, Aus19402 displayed strong leaf injury. Moderate injury was noticed in H-132 and Revenue. The less injury was recorded in H-135, Yu-07 and Yu-08.

Salinity stress led to a reduction in measured agronomic traits during the vegetative and maturity stage of plants (Figure 4). The genotype H-243 showed the less reduction (~29%) whilst Yu-07 showed higher reduction (~55%) in LDW than the

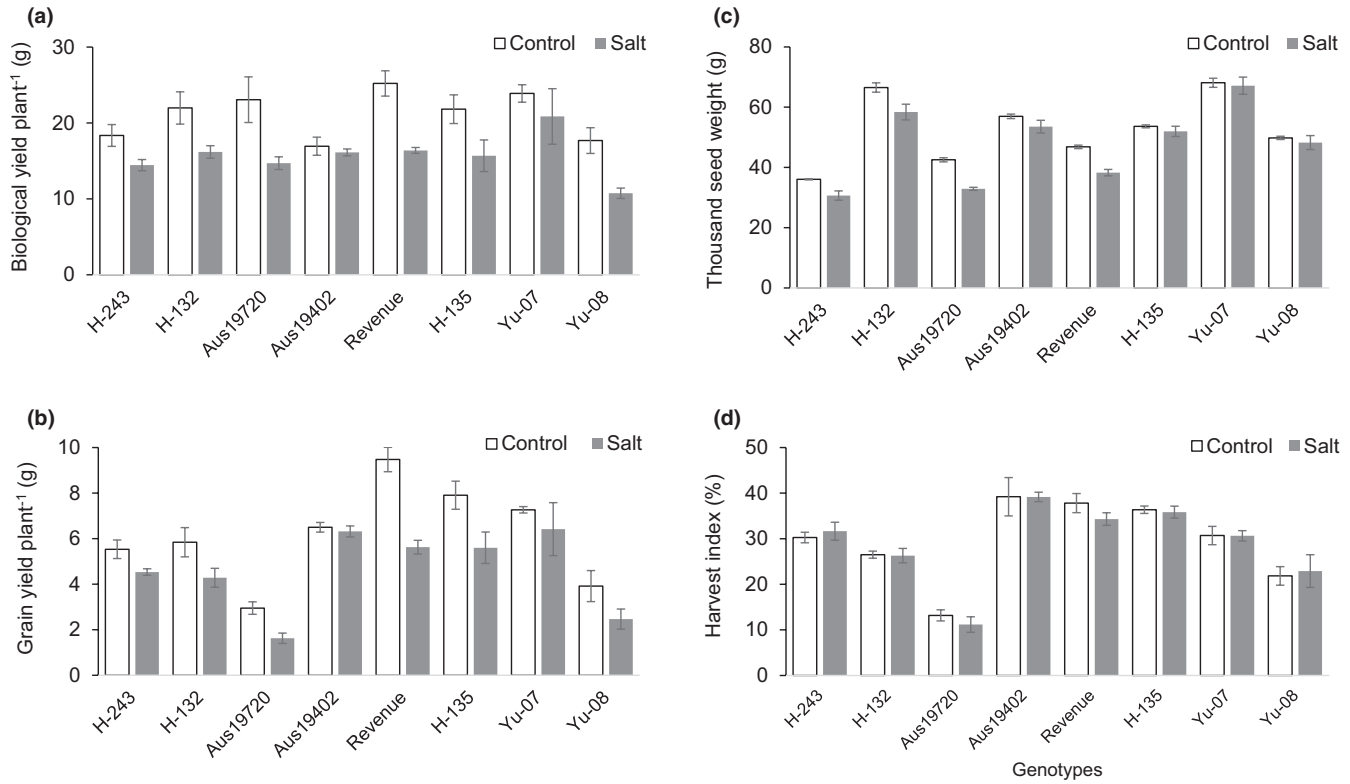

FIGURE 3 Influence of salinity stress on yield and yield-related traits in glasshouse conditions. (a) biological yield, (b) grain yield, (c) thousand seed weight, (d) harvest index. Means were separated by Tukey's test at $p \leq 0.05$. Vertical bars indicate ± standard error of the mean ($n = 4$)

TABLE 2 Pearson's correlation coefficient (r) for phenotypes measured in glasshouse trial

	Na^+	K^+	K/Na	PH	Tiller	BY	GY	TSW	HI
Na^+	1								
K^+	0.779*	1							
K/Na	−0.795*	−0.315	1						
PH	−0.135	0.070	0.431	1					
Tiller	0.828*	0.881*	−0.343	0.116	1				
BY	0.354	0.652	0.087	0.724*	0.643	1			
GY	0.260	0.593	0.144	0.798*	0.514	0.971**	1		
TSW	−0.495	−0.179	0.725*	0.865**	−0.183	0.411	0.537	1	
HI	−0.239	0.055	0.292	0.639	−0.228	0.356	0.569	0.700	1

*,**Correlation is significant at the 5% and 1% level.

other genotypes. The LA ranges between 8.3 ± 1.38 cm^2 and 17.8 ± 2.16 cm^2 in control, and 4.5 ± 0.09 cm^2 and 10.9 ± 2.12 cm^2 in salt-treated plants. The relative changes (% of control) in LA of salt affected plants ranged between ~54 and ~90%. Similar to leaf dry weight, genotype H-243 and H-132 showed ~30% reduction in LA. The relative changes in the tiller number ranged between 61 and 90%, and the greatest reduction was observed in Aus19402 (Figure 4d). The genotypes H-243, H-132, Aus19720 and Aus17402 developed a 10% higher reduction in tiller number than the group Revenue, H-135, Yu-07 and Yu-08. However, the genotype Aus19720 produced the maximum number of effective tillers both in control and salt-treated plants.

Salinity caused a reduction in the relative water content with an exception in Revenue and Yu-07 (Figure 5a). Based on the significant impact of salinity stress, genotypes were grouped into two categories. The first category had four genotypes, that is H-243, H-132, Aus19720, Aus17402 that showed a significant reduction in RWC. The genotypes from the second category did not show any significance difference between control and stress.

The stomatal density increased significantly across the genotypes in stressed plants (Figure 5b). Increased in SD due to salinity stress was ranged from 30 ± 2.01 to 48 ± 2.3 stomata/mm^2. In control plants, SD ranged between 26 ± 1.45 and 34 ± 2.03 stomata per mm^2. The genotypes H-243, Aus19720 and H-135 had better

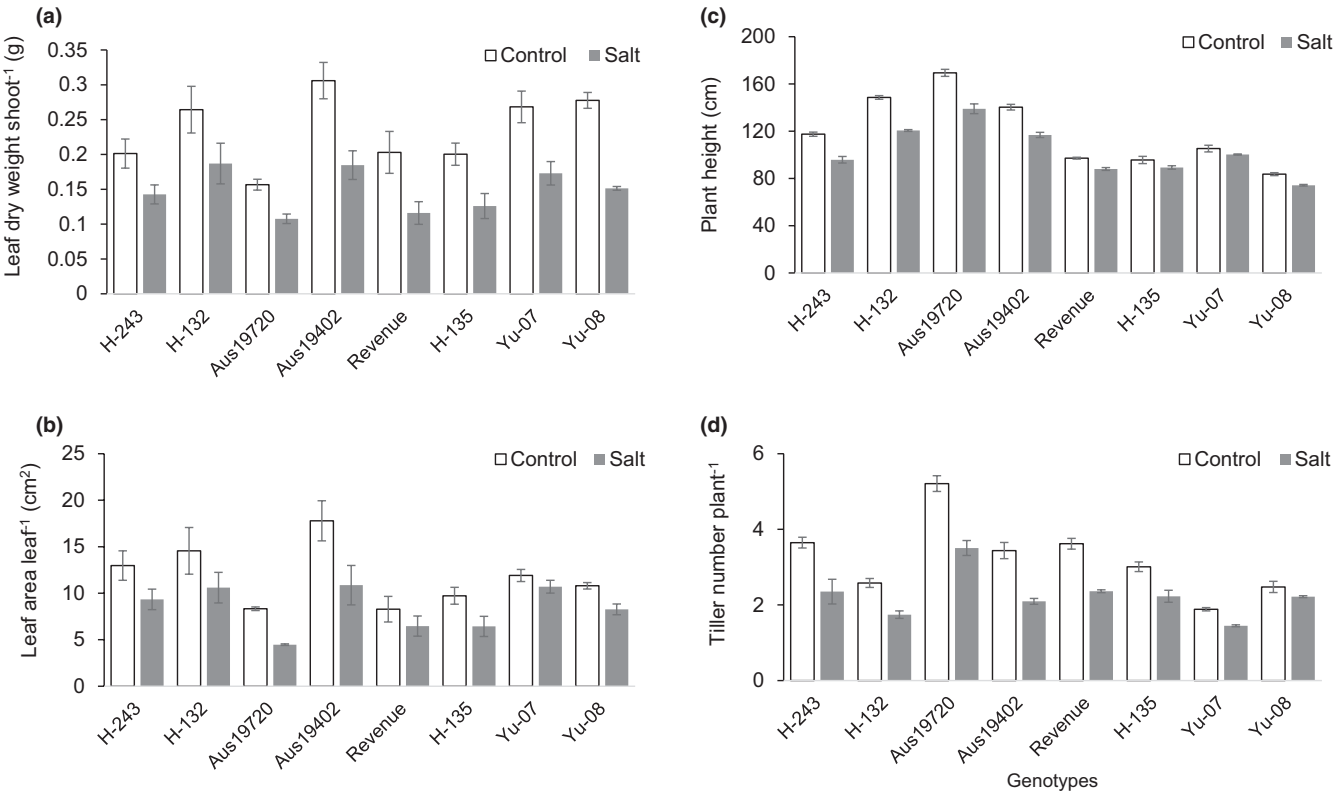

FIGURE 4 Effect of salinity stress on agronomic traits in field experiment. (a) leaf dry weight, (b) leaf area, (c) plant height, (d) number of effective tillers. Plant height and total tiller number were recorded at harvesting time. Any tiller with panicle was considered as an effective tiller. Means were separated by Tukey's test at $p \leq 0.05$. Vertical bars indicate ± standard error of the mean ($n = 3$)

SD than group Revenue, Yu-07 and Yu-08, which were significantly better than Aus19402 and H-132, which were the most sensitive. Amongst the genotypes, the highest and lowest increment in the stomatal density were recorded in H-243 (74%) and Aus19402 (11%), respectively, compared with control.

Salinity stress increased the rate of residual transpiration in the fully open 3rd leaf of all genotypes except Revenue. The genotype Revenue showed 7% reduction in the residual transpiration. Based on the relative changes of RT (% control), the genotypes H-135, H-243 and Yu-07 performed better than Yu-08, Aus19720 and H-132; the worst performing (in term of water saving under salinity conditions) was Aus19402 (Figure 5c). Under stress conditions, the genotype Yu-08 transpired the highest amount of water (1.03 ± 29 mg H_2O cm^{-2}) and Revenue transpired the lowest amount of water (0.39 ± 07 mg H_2O cm^{-2}). Salinity also slightly reduced maximum photosynthetic efficiency of PSII (chlorophyll fluorescence *Fv*/*Fm* ratio) values although the relative changes were modest (90 to 99% of control) (Figure 5d).

Salinity caused a significant increment of SLN content of almost all genotypes except H-243 (Figure 6a). The maximum increment in SLN was observed in genotype Aus19402 (44%) and a minimum increment was quantified in H-243 (7%). On the other hand, about a 3-fold of variation was found in leaf Na^+ accumulation amongst the genotypes under saline conditions. Based on the leaf Na^+ content, all genotypes were divided into two groups, that is higher accumulation group (1st group) and lower accumulation group (2nd group). The genotypes Revenue, H-135, Yu-07 and Yu-08 accumulated average 7.40 ± 0.59 (mg g^{-1} DW) which was significantly better than H-243, H-132, Aus19720 and Aus19402, which accumulated average 16.01 ± 0.82 (mg g^{-1} DW) (Figure 6b). Obviously, the genotype Yu-07 and Aus19720 accumulated the lowest and the highest amount of Na^+, which were 6.67 ± 0.48 (mg g^{-1} DW) and 19.10 ± 0.61 (mg g^{-1} DW) higher than control, respectively. Conversely, H-135 and Yu-08 accumulated similar absolute amount of leaf Na^+.

The effect of field saline conditions on leaf K^+ content was complex (Figure 6c). Compared to control, most of the genotypes significantly decreased leaf K^+ accumulation, whilst three genotypes namely H-135, Yu-07 and Yu-08 increased their K^+ content. The genotype H-135 and Yu-07 significantly increase the leaf K^+ accumulation with a relative change of 109% and 142% of control. The highest reduction in K^+ content (29%) was reported for genotypes H-243, Aus19720 and Aus19402. In relation to leaf Na^+ and K^+ content, the highest and lowest K^+/Na^+ ratio was recorded in Yu-07 (22% of control) and H-243 (6% of control). The similar value of relative changes (10%) in K^+/Na^+ ratio was observed in Aus19704 and Revenue (Figure 6d).

Salinity stress had the adverse effect on spectral traits across the genotypes (Figure 7). The relative value (% control) of NDVI ranged between 67% (H-243) to 101% (H-132). The highest and lowest value of NDRE followed the similar trend of NDVI. Similarly, lowest relative value of ground cover was observed in H-243 (56% of control).

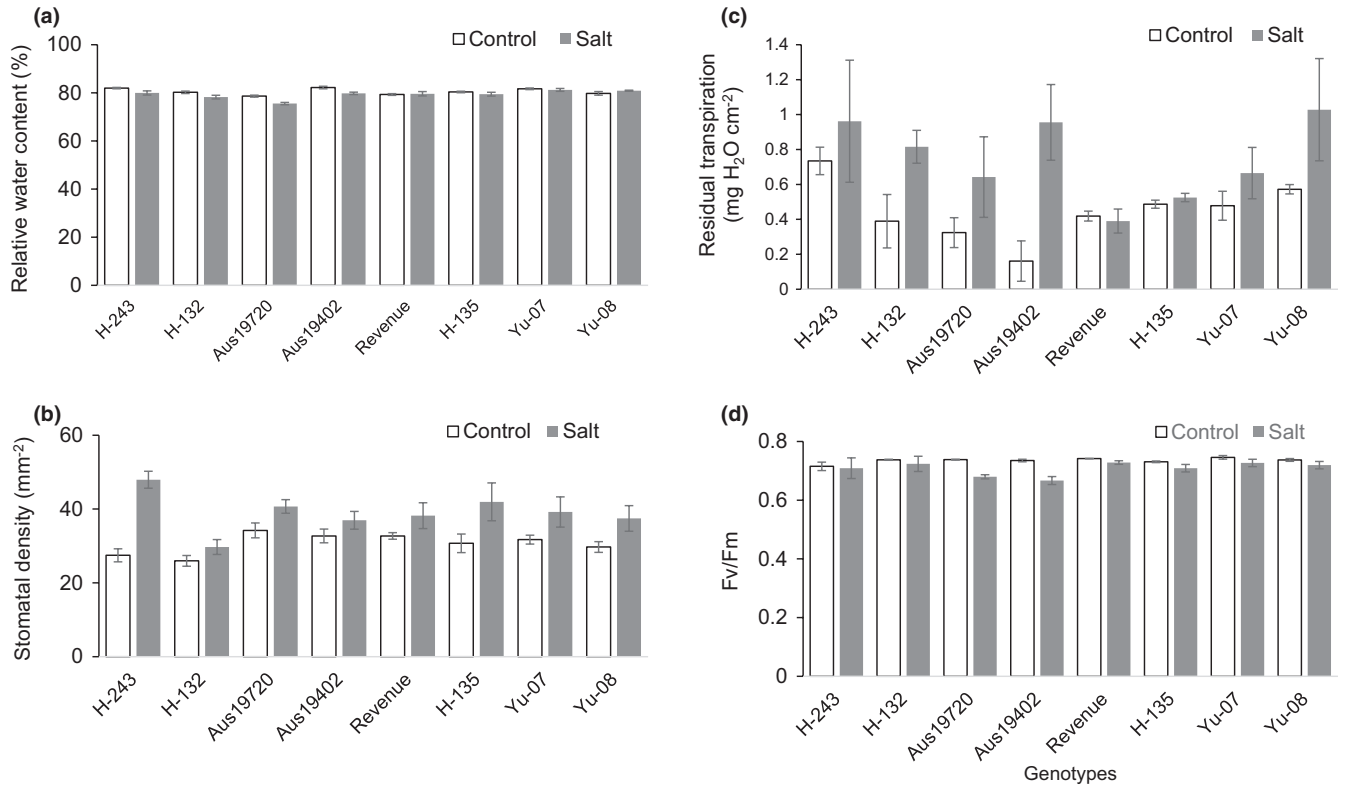

FIGURE 5 Impact of salinity stress on photosystem and gas exchange related traits in field conditions. (a) relative water content, (b) stomatal density, (c) residual transpiration, (d) *Fv/Fm*. Means were separated by Tukey's test at $p \leq 0.05$. Vertical bars indicate ± standard error of the mean ($n = 3$)

3.3.2 | Phenological evaluations

Most of the genotypes showed no significant differences between control and salt stress in tillering and stem elongation phenology (Table S5). In contrast, head emergence showed a significant difference between salt stress and control treatment. A minor reduction was observed in all genotypes for head emergence under salt stress compared with control (Table S5). Amongst genotypes, Aus19720 took the longest for head emergence, both in control and salty plots. Alternatively, earlier head emergence was recorded in genotype Yu-08.

3.3.3 | Yield and yield-related phenotypic evaluation

Salinity caused a significant reduction in grain yield of all genotypes except H-135 and Yu-07 (Figure 8a). The highest and lowest grain yield was reported on Revenue (5.10 ± 0.37 g) and H-243 (2.18 ± 0.08), respectively. In terms of a relative value (% of control), H-135 exhibited the least reductoin (97%) whilst Aus19402 showed the greatest reduction (65%). The 1st group of genotypes exhibited 24% decline in grain yield, whilst plants in the 2nd group showed only 12% reduction in grain yield. Consistent with this, 2nd group of genotypes exhibited 4% higher in biological yield compared to 1st group of genotypes (Figure 8b).

G enotype showed nonsignificant difference between stress and nonstress plants for thousand seed weight. Salinity stress increased harvest index in genotypes H-135 and Yu-07 (Figure 8d).

3.3.4 | Correlations

Most of the STC values calculated from field salt conditions were correlated with each order (Table 3). Only a few traits demonstrated significant correlation with each other. The *Fv/Fm* displayed significant correlation with RT, LA and SLN. RWC showed significant relationship with a leaf K^+ content and tiller number. Obviously, K^+ significantly correlated with K^+/Na^+ ratio along with GC, PH and TSW. Spectral traits significantly correlated with each other. A significant negative correlation was observed between RT and grain yield. LDW also showed a significant relationship with RWC and Na^+.

3.4 | Principal component analysis

The response of the genotypes to salt stress varied at different developmental stages as well as in different growing environments. Yield performance is the most crucial index to select suitable genotypes. Therefore, principal component analysis (PCA) was performed to visualize the relationship amongst major traits in field and glasshouse experiments using salt tolerance coefficient values for

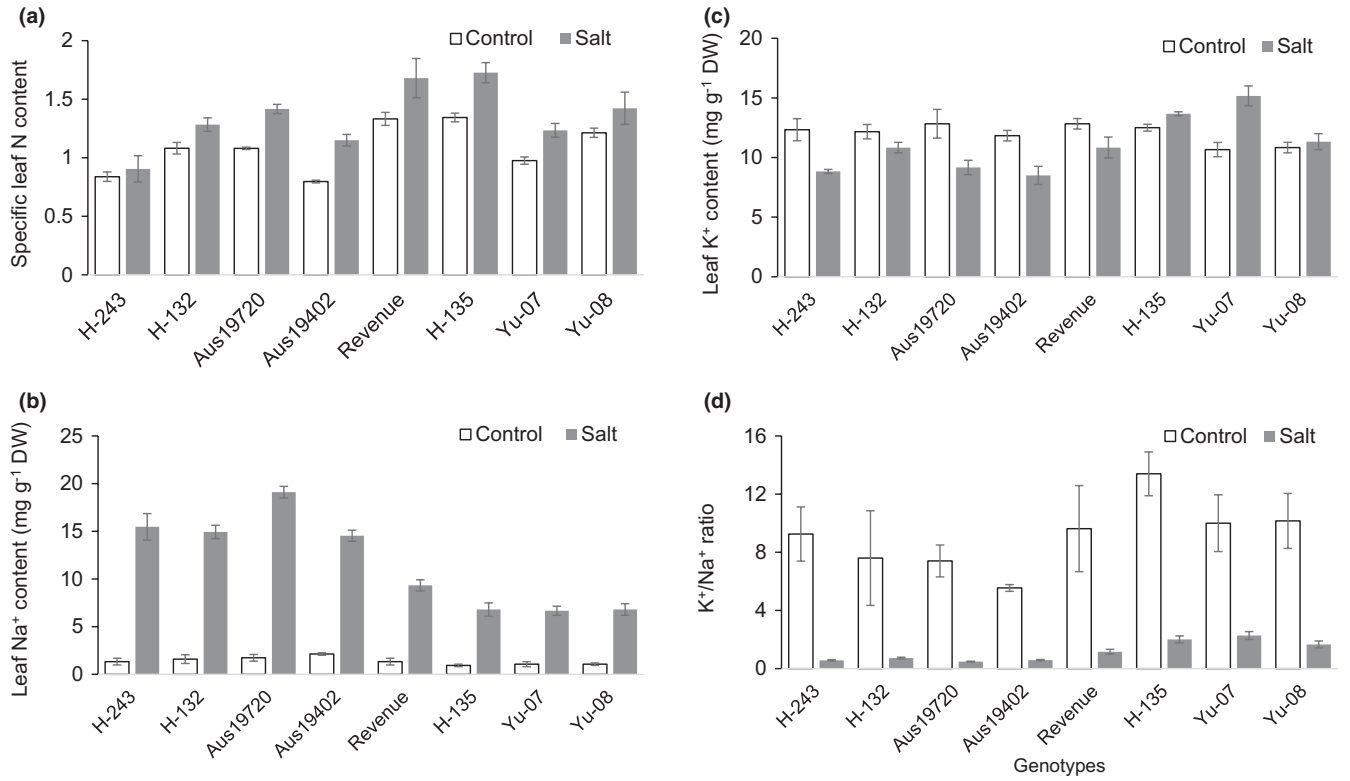

FIGURE 6 Effect of salinity stress on leaf ionic content of eight genotypes in field condition. (a) specific leaf N content, (b) leaf Na^+ content, (c) leaf K^+ content, (d) K^+/Na^+ ratio. Means were separated by Tukey's test at $p \leq 0.05$. Vertical bars indicate ± standard error of the mean ($n = 3$)

grain yield. The PC analysis was executed based on three growth stages: germination, vegetative and maturity. Data from the germination experiment was used as a germination stage for both field and glasshouse experiments. The two components (PC1 and PC2) together explained 55.7% and 64.5% of data variability in filed and glasshouse conditions, respectively (Figure 9). Field PCA plot indicated that LA, SDS, TSW, RFW, HI, *Fv/Fm* and RWC-G (germination) are positively associated with grain yield. The PCA plot also indicted GY had a strong relationship with *Fv/Fm*, RFW and RWC (germination). Alternatively, SDW, PH, SL, SFW, HI and BY positively correlated with GY in glasshouse environment.

3.5 | Ranking and grouping of wheat genotypes

All morpho-physiological parameters studied in three experiments were combined through membership function value (MFV) to evaluate variation in salinity tolerance amongst the wheat germplasms. The recorded traits were standardized by salt tolerance coefficient (STC) and the STC were used to calculate MFV. Furthermore, MFV was used to rank the wheat genotypes on the basis of a degree of salinity tolerance. The higher mean of MFV, the higher the salinity tolerance (Wu et al., 2019).

Table 4 and supplementary materials provide details of MFV evaluation. In germination experiments, Yu-07 and Yu-08 showed higher mean of MFV whilst Aus19720 and Revenue showed lower mean of MFV. In a glasshouse experiment, highest MFV was observed in Yu-07 and AUS19402 and lowest by Aus19720 and Revenue. For the field experiment, Yu-07 and H-135 had highest MFV. In contrast, Aus19720 and Aus19402 displayed the lowest mean of MFV. Based on the results, all 8 genotypes were grouped into three categories, that is highly tolerant (MFV > 0.5), moderately tolerant (MFV between 0.3–0.5) and sensitive (MFV < 0.3) to salinity. Interestingly, genotype Yu-07 showed consistently superior tolerance throughout all three experiments, suggesting similar mechanism controlling salt tolerance in Yu-07 in different environments and growth stages. The genotype H-135 showed a similar trend with Yu-07. Aus19720 was sensitive in both glasshouse and field experimental trials.

4 | DISCUSSION

Under saline conditions, genotypes showed significant differences in their growth and physiological parameters. There are, however, a variety of methods to measure plant stress due to salinity. The MFV is a method to integrate a range of screening observations in both large and small numbers of genotypes at any growth stage and growth environment. There are no trait limitation for this method (Chen et al., 2012; Mohamed et al., 2020; Wu et al., 2019). Therefore, the MFV method was used to screen salinity tolerant genotypes based on different morpho-physiological traits in

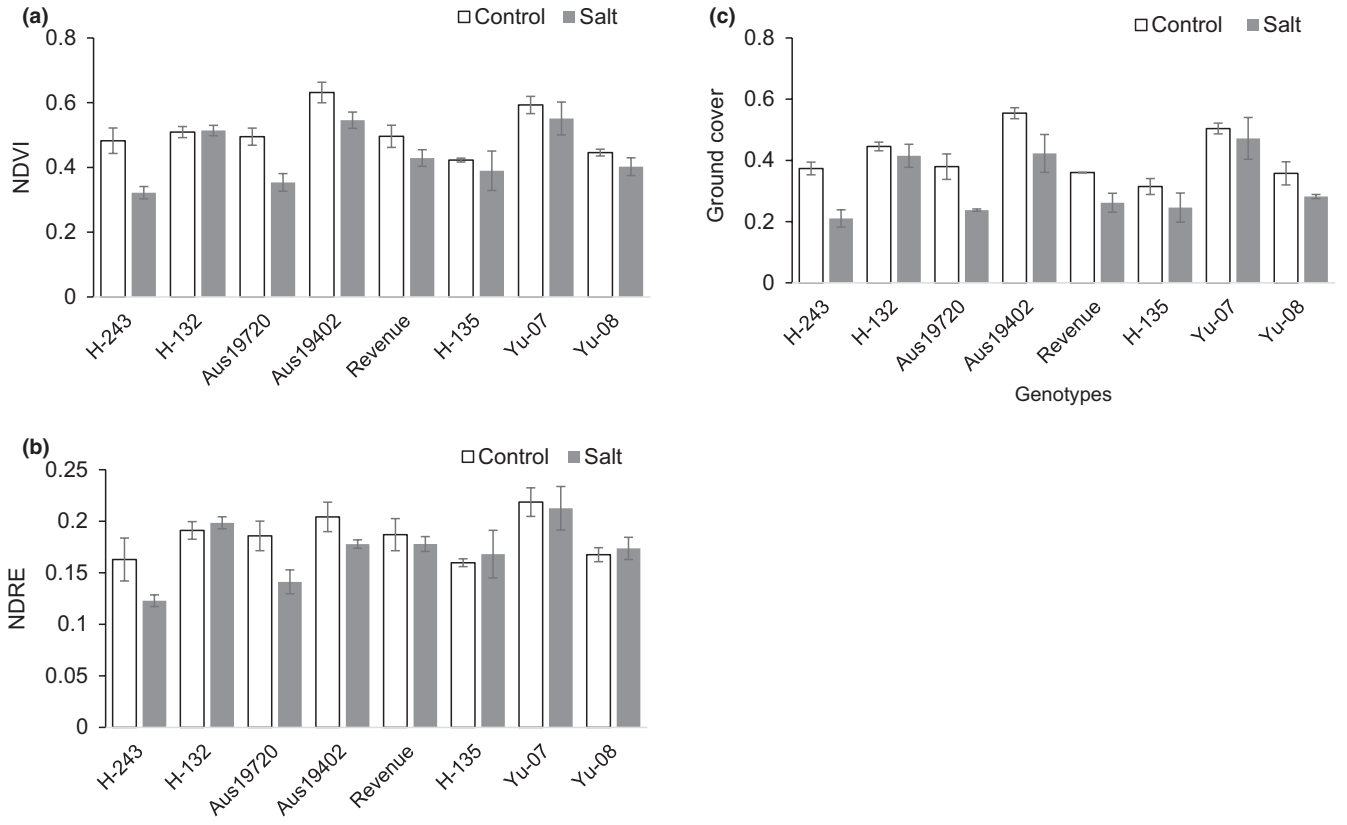

FIGURE 7 Influence of salinity stress on spectral traits under field conditions. (a) NDVI, (b) NDRE, (c) ground cover. Means were separated by Tukey's test at $p \leq 0.05$. Vertical bars indicate ± standard error of the mean ($n = 3$)

different environmental conditions. The higher the mean of MFV from all traits the higher salinity tolerance.

4.1 | Examination of agronomic and physiological traits at vegetative growth stage

Nondestructive measurements are becoming more commonly used to assess abiotic and biotic stress tolerance in plants (Chen et al., 2020; Choudhury et al., 2019), as destructive measurements are generally associated with increased labour requirement and the loss of plants (Flavel et al., 2017; Gandhi et al., 2015; Li, Adhikari, et al., 2020; Li, Zhang, et al., 2020). In the present study, nondestructive measurements of SDS, NDVI, NDRE, ground cover, *Fv/Fm* and SLN were utilized to assess plant performance.

Visual symptom scoring is also widely used as a screening method (Darko et al., 2017; Hura et al., 2017). Leaf chlorosis and wilting has been commonly used to assess biotic and abiotic stress tolerance in many cereals (Chen et al., 2020; Choudhury et al., 2019; Fan et al., 2016; Kang et al., 2019; Xu et al., 2012). In the present study, visual scoring was used to assess salinity tolerance in wheat. A regression analysis demonstrated that ranking of genotypes based of MFV displayed strong relation with SDS both in field and glasshouse conditions, indicating SDS is a reliable trait in field and controlled environment conditions (Figure S2).

. The application of spectral reflectance and remote sensing technologies has been promoted as a reliable technique for effective crop growth and developmental assessment (Sultana et al., 2014; Tan et al., 2020). Abiotic stress causes a severe decline of NDVI, NDRE and ground cover in many crops such as wheat (Thapa et al., 2019). In the present study, almost all genotypes from the tolerant group showed decreased reduction in spectral traits, indicating better light interception by plants and potentially increased photosynthesis. Salinity stress reduced chlorophyll fluorescence *Fv/Fm* ratio value in the sensitive genotypes. The *Fv/Fm* ratio measures maximal photochemical efficiency of PSII (Rapacz & Hura, 2004), where damage to PSII (also referred to as photoinhibition) leads to lowering the *Fv/Fm* value (Adhikari et al., 2019). This parameter seems to be more appropriate as a proxy to measure detrimental effects of salinity on leaf photochemistry, as leaf chlorophyll content (SPAD) data may both decrease (Santos, 2004) and increase (Hasanuzzaman et al., 2018) in chlorophyll content in salt-treated plants. This comes from the fact that with possible increase of chlorophyll degradation under saline conditions, salinity may also reduce the cell size and thus condense chlorophyll in mesophyll tissues.

Leaf production and expansion (leaf area) are essential for overall photosynthetic performance and growth. Leaf dry weight is widely used as an indicator of salinity tolerance of plants. (Nia et al., 2012; Saddiq et al., 2021). Salinity stress reduced the leaf area and leaf dry weight in all tested genotypes. However, most of the genotypes from the tolerant group had better leaf area and moderate leaf dry

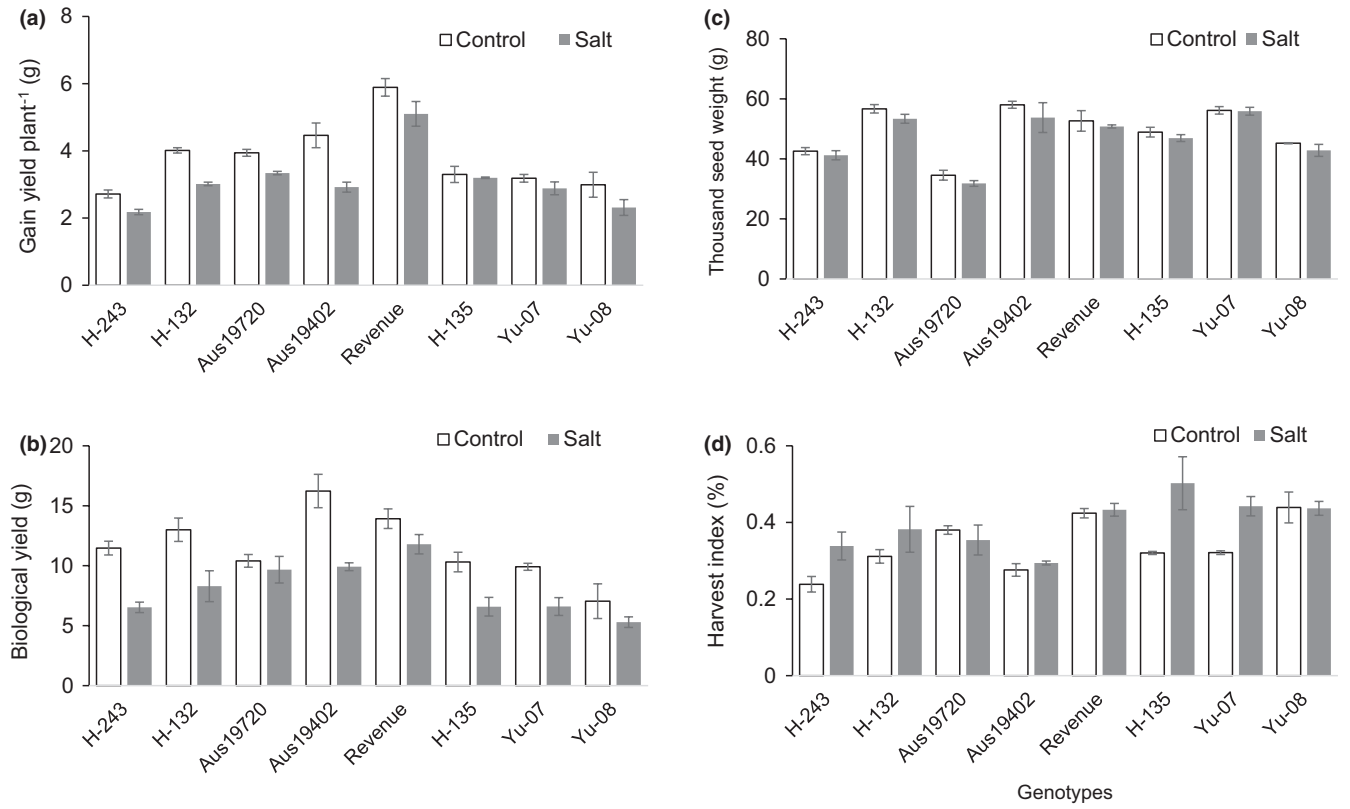

FIGURE 8 Influence of salinity stress on yield and yield-related traits under field conditions. (a) grain yield, (b) biological yield, (c) thousand seed weight, (d) harvest index. Means were separated by Tukey's test at $p \leq 0.05$. Vertical bars indicate ± standard error of the mean ($n = 3$)

weight. The general pattern of plant response to salt stress is a reduction of leaf surface due to the decrease in cell wall extension (Munns et al., 2000; Parida & Das, 2005). This was likely due to the reduction in cell turgor pressure, associated with K^+ loss and aquaporin conductance (Zhao et al., 2020). The decrease of leaf area development led to reduction in light interception (Perez et al., 2019) and photosynthesis (Kumarathunge et al., 2020). This result is supported by the spectral measurement in the present study.

Stomata play an important role in the trade-off between transpiration and uptake of CO_2 for photosynthesis. Stomata function is believed to be an important adaptive tool in plant salt stress tolerance. Stomatal number and behaviour may change during the stress conditions (Hasanuzzaman et al., 2018). In the present study, most of the tolerant genotypes increased their stomatal density (SD). The latter process may be driven by both developmental and physiological mechanisms. Salinity causes a decrease in epidermal cell size thus making stomata denser. Stomata density is also controlled at developmental level by peptide signalling. EPF1 and EPF2 (EPEDERMAL PATTERNING FACTORS) inhibit the stomatal development whilst EPFL9/STOMAGEN trigger the stomatal formation (Hasanuzzaman et al., 2018; Hunt et al., 2010; Lawson & Blatt, 2014). The knockout of the inhibitor and overexpression of promoter mutant greatly increased stomatal densities in model plants (Doheny-Adams et al., 2012). Both mechanisms could contribute to reported changes in SD in our study. In other species like barley such increase in SD is usually associated with greater stomatal conductance as well as CO_2 assimilation efficiency – hence, increased biomass (Hasanuzzaman et al., 2018; Zhu et al., 2014).

Reduced nonstomatal (residual) transpiration is another important mechanism of a plant's salinity tolerance. Almost all genotypes showed increase in residual transpiration (RT) relative to control. However, in the majority of tolerant genotypes the increase in RT was marginal compared with the sensitive lines. This suggested that in the tolerant genotypes transpiration through the leaf cuticle was reduced. As a result, the lower RT led to a higher RWC in tolerant genotypes. The RT had a significant negative correlation with grain yield, indicating the lower the RT the higher the GY. A previous study has shown that reducing RT could be a potentially useful mechanism for enhancing performance of salt grown plants (Hasanuzzaman et al., 2017).

Maintaining intracellular K^+ homeostasis is crucial for different types of physiological activities such as enzyme activation, stabilization of protein synthesis, neutralization of negatively charged proteins, formation of membrane potential and maintenance of cytosolic pH homeostasis (Dreyer & Uozumi, 2011; Shabala, 2003). Potassium is also an important determinant of the cell fate, with salinity-induced cytosolic K^+ loss being causally linked to programmed cell death under wide ranges of abiotic stress conditions (Demidchik et al., 2010; Shabala et al., 2007) including salinity. Conversely, at cellular level, overaccumulation of Na^+ is associated with toxicity

TABLE 3 Pearson's correlation coefficient (*r*) for phenotypes measured in field trial

	LDW	RT	RWC	SD	LA	SLN	*Fv/Fm*	Na^+	K^+	K/Na	NDVI	NDRE	GC	PH	Tiller	BY	GY	HI	TSW
LDW	1.000																		
RT	−0.121	1.000																	
RWC	−0.766*	−0.421	1.000																
SD	0.370	−0.371	−0.060	1.000															
LA	−0.236	−0.437	0.707	0.071	1.000														
SLN	−0.297	0.681	−0.275	−0.669	−0.416	1.000													
Fv/Fm	0.047	−0.776*	0.576	0.466	0.720*	−0.841**	1.000												
Na^+	0.811*	−0.168	−0.701	0.513	−0.485	−0.420	−0.002	1.000											
K^+	−0.229	−0.381	0.870*	−0.122	0.733	−0.064	0.390	−0.621	1.000										
K/Na	−0.528	−0.151	0.671	−0.220	0.654	0.149	0.195	−0.826*	0.928**	1.000									
NDVI	−0.302	0.042	0.421	−0.596	0.434	0.178	0.207	−0.687	0.579	0.557	1.000								
NDRE	−0.445	−0.204	0.648	−0.394	0.478	−0.019	0.406	−0.705	0.637	0.626	0.923**	1.000							
GC	−0.144	0.045	0.325	−0.571	0.548	0.160	0.179	−0.629	0.707*	0.637	0.936**	0.793*	1.000						
PH	−0.531	−0.412	0.702	−0.120	0.601	0.121	0.315	−0.740*	0.836**	0.881**	0.425	0.574	0.429	1.000					
Tiller	−0.473	−0.376	0.714*	−0.032	0.432	−0.310	0.351	−0.462	0.657	0.685	0.352	0.572	0.354	0.521	1.000				
BY	−0.246	−0.255	0.077	−0.454	−0.244	0.189	−0.253	0.058	−0.156	−0.124	−0.211	−0.168	−0.234	0.043	0.104	1.000			
GY	0.035	−0.790*	0.284	0.204	0.262	−0.167	0.407	−0.074	0.550	0.397	0.026	0.228	0.034	0.702	0.278	0.227	1.000		
HI	0.374	−0.336	−0.003	0.576	0.299	−0.294	0.474	0.032	0.440	0.270	0.139	0.227	0.170	0.356	0.012	−0.711*	0.507	1.000	
TSW	−0.043	−0.581	0.533	0.377	0.686**	−0.1411	0.712*	−0.298	0.717*	0.600	0.163	0.266	0.287	0.693	0.271	−0.313	0.574	0.613	1.000

*,**Correlation is significant at the 5% and 1% level.

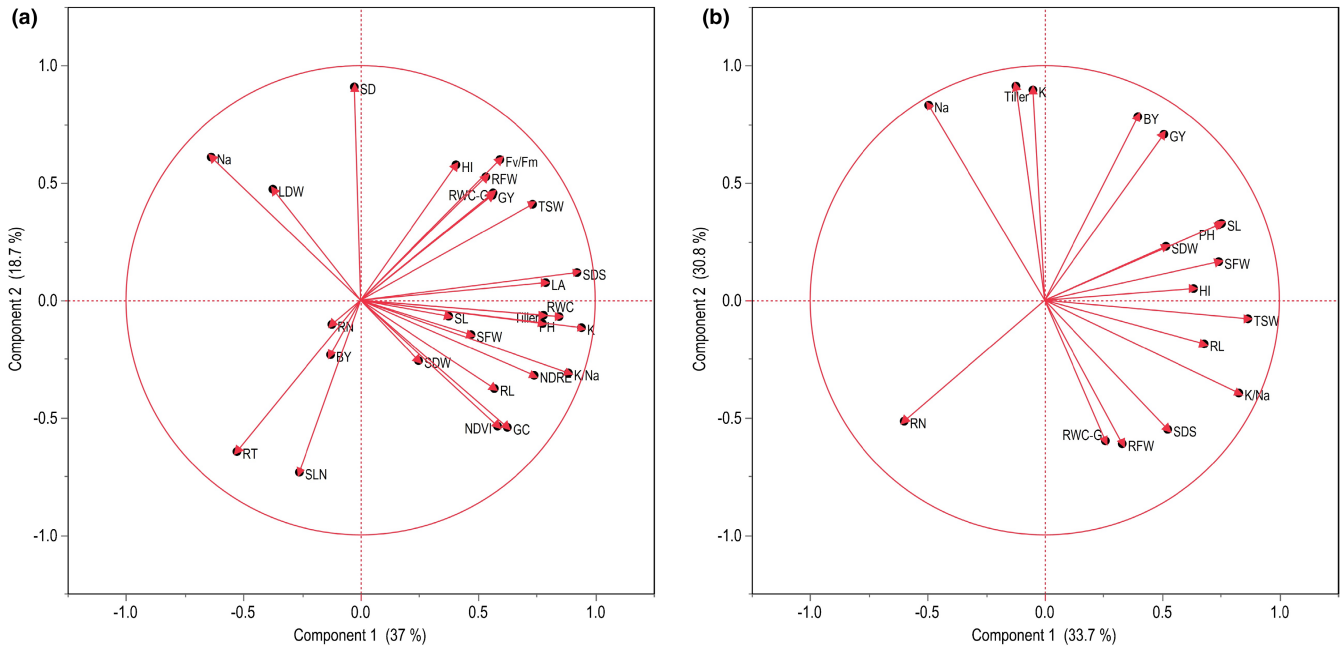

FIGURE 9 Principal component analysis (PCA) of major traits measured at three developmental stages under salt stress conditions. (a) PCA field environment, (b) PCA glasshouse environment. RWC-G: relative water content at germination stage

TABLE 4 Ranking of genotypes based on MFV in three different experiments

Genotype	Germination experiment		Glasshouse experiment		Field experiment	
	Mean MFV	Rank	Mean MFV	Rank	Mean MFV	Rank
H-243	0.360	MT	0.534	T	0.354	MT
H-132	0.303	MT	0.436	MT	0.473	MT
Aus19720	0.501	T	0.197	S	0.259	S
Aus19402	0.203	S	0.713	T	0.264	S
Revenue	0.243	S	0.238	S	0.533	T
H-135	0.402	MT	0.568	T	0.616	T
Yu-07	0.684	T	0.813	T	0.761	T
Yu-08	0.669	T	0.480	MT	0.578	T

Note: Genotypes were ranked based on the member function value. MFV over 0.5 = tolerant (T), MVF between 0.3 to 0.5 = moderately tolerant (MT), MFV below 0.3 = sensitive (S).

Abbreviation: MFV, Membership function value.

in the plants (Munns, 2002; Roshandel & Flowers, 2009; Roy et al., 2014). Adaptation to salinity occurs at various levels of a plant structural organization (from molecular to cellular and whole-plant level) and includes a broad array of anatomical, physiological and biochemical alterations. Amongst these, exclusion of Na^+ from the shoot has long been considered as an important tolerance mechanism in glycophytes (Gupta & Huang, 2014; Munns & Tester, 2008). To minimize or prevent the toxic effect of salt ions (Na^+), ion transporters and channels play critical roles in maintaining cytosolic ion homeostasis under saline stress conditions. This involves an orchestrated operation of Na^+/H^+ exchangers at the tonoplast (NHX) and plasma (SOS1) membranes, removal of excessive Na^+ from the shoot by HKT transporters, and control of passive Na^+ uptake mediated by nonselective cation (NSCC) (Almeida et al., 2013; Dubcovsky et al., 1996; Julkowska & Testerink, 2015; Keisham et al., 2018). Consistent with this, the presence of the Na^+-selective transporter *TmHKT1;5-A* in durum wheat significantly decreased leaf Na^+ concentrations and increased grain yield by ~25% (Munns et al., 2012). In bread wheat a Na^+ transporter candidate gene *TaHKT1;5-D* restricts transport of Na^+ from the roots to the leaves and is associated with shoot Na^+ exclusion and the maintenance of the K^+:Na^+ ratio in leaves (Byrt et al., 2014).

Na^+ content in leaves under glasshouse conditions was much higher than field Na^+ content, most likely due to including old leaves into analysis. Plants protect younger leaves from harmful ionic effect via partitioning Na^+ in older leaves (Munns, 2005;

Wang et al., 2012). However, field K^+ and Na^+ content exhibited significantly positive and negative correlation with spectral traits, respectively. This is indicative of K^+ conferring higher leaf chlorophyll content. Field K^+ and Na^+ content also had the same positive and negative relationship with PH, respectively. In glasshouse experiment, K^+/Na^+ ratio also positively correlated with GY and TSW, indicating higher K^+/Na^+ ratio is an important trait of wheat salinity tolerance. With this context, K^+ is a critically important nutrient element to contribute in photosynthesis and grain yield (Tränkner et al., 2018).

4.2 | Examination of yield and yield-related components

Grain yield is the crucial trait for varietal evaluation in plant breeding. As expected, salinity stress leads to reduction of grain yield and other yield associated traits. Tolerant genotypes showed higher MFV almost in all yield associated parameters in both environmental conditions. Under field conditions, grain yield was negatively correlated with residual transpiration, suggesting that reducing the RT facilitated better water saving and higher photosynthesis. This statement is supported by other physiological attributes where most of the physiological traits showed positive correlation with GY. Previous studies reported that improving plant physiology could lead to higher grain yield in crops (Munns, 2005; Shabala & Munns, 2012). In addition, GY in glasshouse conditions was highly and significantly correlated with PH and BY, indicating less reduction in plant biomass associated with higher GY under salinity conditions. The increase in yield has been found to be largely attributable to improved partitioning of biomass to the grain in wheat (White & Wilson, 2006). This higher grain yield in wheat plants were also correlated with higher number of tillers and TSW.

4.3 | Salinity tolerance varies with growth conditions

Plants employ various types of salt stress tolerance mechanisms (Deinlein et al., 2014; Gupta & Huang, 2014), but their ability to resist salt stress varies widely amongst different species and cultivars (Quamruzzaman, Manik, Shabala, Cao, et al., 2021a; Quamruzzaman, Manik, Shabala, & Zhou, 2021b). Wheat is a moderately salt-tolerant crop and has a certain level of salt tolerance criteria (Maas & Hoffman, 1977). Based on the MFV method, eight genotypes have been divided into three categories: tolerant, moderately tolerant and sensitive groups. Not all the tolerant genotypes displayed consistent rankings throughout three experiments. The discrepancy amongst the field and controlled environment responses could be due the environmental factors. In controlled environments plants are continuously surrounded by adequate growth conditions (Dadshani et al., 2019; El-Hendawy et al., 2009) whilst growth profile under field conditions is subject to considerable environmental variation. Soil heterogeneity, other biotic and abiotic stresses, rainfall and fluctuations of air temperature at the same time with salinity in field conditions could contribute to inconsistency of results (Dadshani et al., 2019; Moustafa et al., 2021). However, the evaluation of wheat genotypes under field conditions is a crucial assessment approach since plants must be able to tolerate these variations as well as salinity per se.

Last but not least, previous studies demonstrated that little or no correlation has been found for salinity tolerance at germination and later growth stages (Ashraf & McNeilly, 1988; Munns & James, 2003). This is true for the three genotypes namely Aus19720, Aus19402 and Revenue in the present study, indicating it could be dependent on cultivars as well as crop species. Conversely, other studies suggested that seed germination is the first stage of crops growth and importance for plants withstanding in stress conditions. During germination the plant's physiology as well as growth and development are significantly affected by salinity stress. Thus, salt tolerance at the germination stage is critical for the successful establishment of plants in saline conditions (Bakhshandeh et al., 2020; Li, Adhikari, et al., 2020; Li, Zhang, et al., 2020; Wu et al., 2019). Therefore, germination testing is frequently implemented for evaluations of salinity tolerant in wide ranges of crop species (Dadshani et al., 2019; Rajabi Dehnavi et al., 2020). It was demonstrated that the majority of the wheat genotypes show consistency in terms of tolerance and sensitivity throughout different growth stages of plants (El-Hendawy et al., 2005). This was also the case in our work, with five genotypes showing consistent tolerance to salinity stress throughout three experiments in the present study.

5 | CONCLUSION

The membership function value (MFV) is a reliable tool for screening of wheat genotypes under distinctly different environmental conditions. Almost all types of morpho-physiological traits from different growth stages can be used in the MFV model. SDS and MFV analysis demonstrated that Yu-07 and H-135 were consistently tolerant genotypes and Aus19720 was the most sensitive genotype to salinity stress. These genotypes could potentially be used in wheat salt tolerance breeding program, and for better understanding of salt stress tolerance mechanisms in plants. Further work is required to understand the cellular and molecular mechanisms of contrasting genotypes to successfully use in breeding program.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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