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Detecting Salt Tolerance in Doubled Haploid Wheat Lines

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Abstract: Improving salt tolerance of genotypes requires a source of genetic variation and multiple accurate selection criteria for discriminating their salt tolerance. A combination of morpho-physiological and biochemical parameters and multivariate analysis was used to detect salt tolerance variation in 15 wheat lines developed by doubled haploid (DHL) technique. They were then compared with the salt-tolerant check cultivar Sakha 93. Salinity stress was investigated at three salinity levels (0, 100, and 200 mM NaCl) for 25 days. Considerable genetic variation was observed for all traits, as was high heritability (>60%) and genetic gain (>20%). Principal component analysis indicated the ability of nine traits (root number, root length, root dry weight, shoot length, shoot dry weight, specific root length, relative water content, membrane stability index, and catalase) to identify differences in salinity tolerance among lines. Three traits (shoot length, shoot dry weight, and catalase) were indicative of salt-tolerance, indicating their importance in improving and evaluating salt tolerant genotypes for breeding programs. The salinity tolerance membership index based on these three traits classified one new line (DHL21) and the check cultivar (Sakha 93) as highly salt-tolerant, DHL25, DHL26, DHL2, DHL11, and DHL5 as tolerant, and DHL23 and DHL12 as intermediate. Discriminant function analysis and MANOVA suggested differences among the five groups of tolerance. Among the donor genotypes, Sakha 93 remained the donor of choice for improving salinity tolerance during the seedling stage. The tolerated lines (DHL21, DHL25, DHL26, DHL2, DHL11, and DHL5) could be also recommended as useful and novel genetic resources for improving salinity tolerance of wheat in breeding programs.

Keywords: salt tolerance; genetic variation; wheat breeding; doubled haploid lines; multivariate analyses

1. Introduction

Salinity is considered one of the most important abiotic stressors, threatening food security and affecting human life in arid and semi-arid regions, where almost all irrigated land today is within the ambit of salt effects [1]. Importantly, water shortages in these regions also lead to an increase in the use of brackish water for the production of staple food crops. It is well-known that improving the salt-tolerance of crop genotypes is a much more effective strategy for alleviating the negative effects

of salinity stress on crop production than other agronomic practices (e.g., the application of large quantities of gypsum and the use of effective leaching and drainage systems to remove the salt from the soil). This is because this method can be applied on a large scale, can provide a long-term solution, and is inexpensive for poor farmers. However, success in improving salt tolerance of genotypes has been limited by a number of factors, such as limited sources of genetic diversity in breeding programs, low selection efficiency using simultaneous morpho-physiological and biochemical parameters as screening criteria, and the lack of effective evaluation methods to detect salt tolerance of genotypes using overall salinity levels and multivariable screening criteria [2–6].

Many studies investigated the tolerance of wheat (*Triticum aestivum* L.) into the salinity and found that the tolerated genotypes can survive in the level of 150 mM NaCl [3,5]. High salinity lowers agricultural productivity. Thus, it is necessary to improve wheat germplasm, by introducing new genes or alleles from the rich allelic repertoire found in landraces and some cultivated wheat varieties, to enable higher tolerance for salt-stress [7,8]. Salinity tolerance is a complicated trait, as plants use different mechanisms for handling salt-stress, which can be highly influenced by environmental factors, so it is difficult to select it for breeding programs [9]. Advances in biotechnology have nevertheless allowed progress through the production of doubled haploid lines (DHLs), developed from an anther culture technique, which offers great promise for plant breeding. Anther culture is a method used to obtain haploid embryos using immature pollen microspores in anthers cultivated on nutrition media. This procedure usually needs only a short time to be conducted (only one generation) and could accelerate the production of new varieties with improved traits [10].

The genotype effect is the main limiting factor of in vitro androgenesis. Many wheat genotypes are unable to achieve morphogenesis in anther culture [11]. Anther culture response is a heritable trait and can be transferred into agriculturally desirable material by crossing [12,13]. Genetic research and breeding programs depend on the proper diagnosis of the conditions of quantitative trait inheritance, for traits, such as anther culture response. During the selection process, information about the combining ability of parental components used for crossbreeding is very important. This knowledge is essential for the proper selection of suitable parents, to identify promising hybrids [14]. Four Egyptian bread wheat cultivars, that is, Gemeiza 7, Gemeiza 9, Giza-164, and Giza-168, as well as Line-115, were selected on the basis of anther culture response [10,15], while the check cultivar Sakha 93 was unresponsive of anther culture [16,17].

Generally, salt stress causes a cellular ion balance disturbance, which results in ion toxicity, osmotic stress, and production of reactive oxygen species (ROS) [18], and is reflected in plant growth, induces leaf damage, and eventually leads to death [19]. Salt stress causes an excessive increase in ROS in reaction to salt exposure. Plants tolerant to salt stress evolved a complex set of defense mechanisms, such as osmolyte biosynthesis, intracellular compartmentalization of toxic ions, alterations in ion homeostasis, and ROS scavenging systems [20]. Antioxidant enzymes help to alleviate cellular damage due to oxidative stress [21,22]. Studies have shown that genotypes of salt-tolerant plants generally have enhanced or higher constitutive activity of antioxidant enzymes under salt stress, showing increased activity of antioxidant enzymes and antioxidant contents in response to salt stress, compared to sensitive-cultivars [23,24].

In order to obtain a deeper understanding of the selection of salt-tolerant genotypes, the mechanisms behind the effects of salt on the growth, morphology, physiology, and antioxidative responses of plants must first be identified [25,26]. Salt may affect plant growth indirectly by decreasing the rate of photosynthesis, chlorophyll content, and transpiration, and thereby cause declines in growth [27]. Indeed, under saline conditions, a substantial reduction in photosynthesis has been associated with a decrease in total chlorophyll content and distortion in chlorophyll ultrastructure [28]. Although the factors that limit photosynthesis in salt-stressed plants have been investigated for a number of species, the mechanistic pattern of inhibition remains unclear [29].

In general, the ultimate goal of breeding programs is to improve the size and stability of yield and the quality of traits under stress. To this end, methods of screening for salt tolerance within a large number of genotypes must be quick, cheap, and easy to measure [30–32]. Field evaluation of salt tolerant genotypes requires more cropping seasons for screening and evaluation [33]. Spatial

differentiation in soils impacts field evaluation which results in a high coefficient of variation, which adversely affects the reliability of the results [33]. It is also difficult to measure root traits accurately in the field [34]. Testing the salt tolerance of genotypes in a laboratory setting, where plants are under controlled conditions in small-scale pots, can be a useful indicator, as there is a significant correlation between stress resistance observed in the field and stress resistance observed in the laboratory [32,35,36]. Important laboratory protocols for the screening of salt tolerance in crop plants include seed germination in saline media, exposure of the plant to water stress, determining control of membrane stability, and measuring leaf water content [37]. Although salinity tolerance is determined by polygenic inheritance, most studies still treat salinity tolerance as a single-gene trait and traditionally use visual scoring [9]. Hence, a pyramiding of favorable morphological, physiological, and biochemical traits has been effectively applied for evaluating the salt tolerance of crops in breeding programs [38].

Multi-trait selection in breeding programs and efficient screening methods are important to improving yield. Large datasets from screening tests require correct statistical analysis to formulate conclusions concerning tolerant and sensitive genotypes. Multivariate analysis is a useful tool for identifying sources of genetic variation and discriminating their salt tolerance using accurate and multiple selection criteria. Therefore, a multivariate analysis combining morphological, physiological, and biochemical traits would be most appropriated [2,6,24,38].

Therefore, the aims of our study were to characterize the genetic variance, heritability, and expected genetic advances of different traits as screening criteria for evaluating the salt tolerance of DHL genotypes under different salinity conditions. In addition, particular attention was paid to investigate the efficiency of using multivariable morpho-physiological and biochemical parameters as well as to identify traits that can be employed as credible screening criteria for the selection and improvement of salt tolerance in wheat. We used MANOVA and discriminant function analyses to achieve these goals.

2. Materials and Methods

2.1. Plant Material

Sixteen wheat genotypes were tested in this study. Fifteen DHLs were produced using anther culture technique and selected based on their good grain yield performance. The DHLs were obtained from the Agronomy Department, Faculty of Agriculture, Al-Azhar University, Nasr City, Cairo, Egypt, and published by El-Hennawy et al. [10]. These lines were distributed as follows: 4 DHLs derived from the cross (Line-115 × Gemmeiza-7), 4 DHLs derived from the cross (Line-115 × Giza-164), 5 DHLs derived from the cross (Gemmeiza-7 × Giza-164), and 2 DHLs derived from the cross (Giza-164 × Giza-168). The salt-tolerant check cultivar Sakha 93 was provided by the Agricultural Research Center, Egypt (Table S1).

2.2. Hydroponic Experiment

The salt tolerance of tested genotypes was evaluated in a hydroponic trial. The seeds of each genotype were germinated in trays of washed sand on quarter-strength Hoagland's nutrient solution [39] at an optimal growing temperature (25 °C during the day and 20 °C during the night), photoperiod cycle (16 h light and 8 h dark), and light intensity 4000 Lux. The pH of the nutrient solution was adjusted to 6.0. The solution was replaced once a week and aerated continuously. Both seeds were assigned to the control treatment, and seeds assigned to salinity treatments grew uniformly after 5 days in hydroponic solution without salt. All genotypes were evaluated under 3 salinity levels: 0 mM NaCl, 100 mM NaCl, and 200 mM NaCl. The experiment was carried out in a completely randomized factorial design and replicated 3 times, with 25 seeds for each genotype and replicate. After 25 days of salinity treatment, the plants were harvested.

2.3. Measurements

2.3.1. Shoot Traits

Shoot traits, namely shoot length (SL) and shoot dry weight (SDW), were estimated at harvest (at 30 days after sowing). SL was measured from the soil surface to the tip of the longest leaf. Thereafter, shoots were cut at the crown level, and SDW was recorded after oven drying at 70 °C for 48 h. The ratio of SL to SDW (SLSDW) was estimated by dividing shoot dry weight by SL.

2.3.2. Root Traits

Root traits, such as a number of roots (RN), root length (RL), and root dry weight (RDW), were estimated at harvest. The number of seminal and crown roots was counted. RL was calculated as the average length of each seminal and crown root divided by their number. RDW was recorded after oven drying of fresh roots at 70 °C for 48 h. The specific root length (SRL) was obtained by dividing the RL by RDW. The ratio of shoot dry weight to root dry weight (SRDW) was calculated by dividing SDW by RDW. The ratio of SL to RL (SLRL) was estimated by dividing SL by RL.

2.3.3. Relative Water Content (RWC)

To calculate RWC, 0.5 g of fresh leaves were weighed, and their initial weight (IW) was recorded. The leaves were then soaked in 100 mL distilled water for 4 h. The turgid weight (TW) of leaf samples was recorded. Then, the same samples were oven dried for 48 h at 65 °C. Dry weight (DW) of the samples was taken after confirming that the samples were completely dried out. The RWC was calculated as described by the following equation [40]:

$$RWC = (IW - DW)/(TW - DW) \quad (1)$$

2.3.4. Total Chlorophyll Content (Chl)

A sample of 0.1 g of fresh leaves was taken, cut into 10 pieces, and then was placed in 3 mL methanol. The chlorophyll content was determined as follows: total chlorophyll = 25.8 × A650 + 4.0 × A665. The absorbance was measured at 650 and 665 nm using a spectrophotometer (Ultrospec 2100 Pro, MA, USA). Total chlorophyll was then converted to micrograms of chlorophyll per gram of leaves tissues using the following formula: (μg chlorophyll/mL methanol) × 3 mL methanol/(g tissue), according to Hipkins and Baker [41].

2.3.5. Membrane Stability Index (MSI)

To calculate the MSI, 0.1 g of fresh leaves were sampled and soaked in 10 mL of distilled water. Samples were kept at 40 °C for 30 min, then conductivity (EC1) was recorded using a conductivity meter. Then, the same samples were kept in a boiling water bath (100 °C) for 15 min, and the conductivity was recorded a second time (EC2). The formula MSI = (1 – EC1/EC2) × 100 [42] was used to calculate the MSI.

2.3.6. Antioxidant Assay

To isolate antioxidant enzymes, 0.5 g of fresh leaves were sampled, crushed in liquid nitrogen, and placed in an ice-bath in 4 mL of a homogenizing solution containing 50 mM potassium phosphate buffer (pH 7.8) and 1% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 14,000 rpm at 4 °C for 10 min, and the resulting supernatant was used as the crude extract for 3 enzyme assays (peroxidase (POD), polyphenol oxidase (PPO), and catalase (CAT)).

The CAT activity was measured as described by Aebi [43]. The reaction mixture (3 mL) contained 1.5 mL of 0.1 M potassium phosphate buffer (pH = 7.2), 0.5 mL of 0.075 M H_2O_2, 0.03 mL enzyme extraction, and 0.97 mL distilled water. A decrease in absorbance at 240 nm was recorded after 1 min on the basis of the rate of disappearance of H_2O_2.

POD activity was measured by estimating the enzyme's ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), using the method outlined in Chance and Maehly [44]. The reaction mixture (2 mL) contained 0.22 mL of 100 mM potassium phosphate buffer (pH 6.0 at 20 °C), 0.22 mL of 5% (w/v) pyrogallol solution, 0.10 mL of 0.50% (w/w) hydrogen peroxide solution (H_2O_2), 0.03 mL of enzyme solution, and 1.43 mL distilled water. The mixture was incubated for 5 min at 25 °C, and the absorbance was then measured at 420 nm.

PPO activity was measured as described by Duckworth and Coleman [45]. The reaction mixture contained 0.03 mL of enzyme solution and 1.74 mL of 20 mM catechol solution (which was prepared in 50 mM potassium phosphate buffer, pH 6.8 at 25 °C). The absorbance of the reaction mixture was measured at 420 nm.

All measured traits were grouped into 4 categories: shoot traits (SL, SDW, and SLSDW), root traits (RN, RL, RDW, SRDW, SLRL), physiological traits (RWC, Chl, MSI), and biochemical traits (activities of antioxidant enzymes, such as CAT, POD, and PPO).

2.4. Statistical Analysis

Analysis of variance was conducted, and variance components, including genotypic variance (σ^2_g), genotype × environment ($\sigma^2_{g\times l}$), and residual variance (σ^2_e), were calculated following the methods of Fehr (1987) as follows (Table 1). All variables were calculated using SAS software (Version 9.2, SAS Institute, Inc., Cary, NC, USA), [46] using the following equations:

$$\sigma^2_g = (M_1 - M_2)/rl \quad (2)$$

$$\sigma^2_{g\times l} = (M_2 - M_3)/r \quad (3)$$

$$\sigma^2_e = M_3 \quad (4)$$

where r is the number of repetitions, g is the number of genotypes, and l is the number of salinity levels. Estimates of the variance components σ^2_g, $\sigma^2_{g\times l}$, and σ^2_e allowed the calculation of broad sense heritability (h^2) [47] for all traits.

$$h^2 = \frac{\sigma^2_g}{\sigma^2_p} \quad (5)$$

$$\text{Genetic advance (GA)} = \frac{\sigma^2_g}{\sigma^2_p} \times \sqrt{\sigma^2 g} \times k \quad (6)$$

$$\text{Genetic gain (\%)} = \frac{GA}{\overline{X}} \times 100 \quad (7)$$

where σ^2_p is the phenotypic variance (M1). k = selection differential at 5% selection intensity. The value of k = 2.06.

The genotypic coefficient of variability (GCV) and phenotypic coefficient of variability (PCV) were calculated using the following formulae proposed by Singh and Chaudhary [48]:

$$\text{GCV} = \frac{\sqrt{\sigma^2_g}}{\overline{X}} \times 100 \quad (8)$$

$$\text{PCV} = \frac{\sqrt{\sigma^2_p}}{\overline{X}} \times 100 \quad (9)$$

where $\overline{X}$ is the phenotypic mean for each trait.

Table 1. Analysis of variance and expected mean of squares.

Source of Variation	Degree of Freedom	Mean of Squares	Expected Mean of Squares
Salinity levels (L)	(l − 1)		
Genotype (G)	(g − 1)	M1	$\sigma^2_e + r\sigma^2_{gl} + re\sigma^2_g$
G × L	(g − 1) (l − 1)	M2	$\sigma^2_e + r\sigma^2_{gl}$
Error	gl (r - 1)	M3	σ^2_e

Principal component analysis (PCA) and principal coordinate analysis (PCoA) were performed based on a correlation matrix to reduce the dimensions of data space, and a biplot was drawn using the XLSTAT statistical package (Version 2018, Excel Add-ins soft SARL, New York, NY, USA). Cluster analysis was calculated using PAST software (Version 3.22) [49]. Simple correlation among traits was computed using SAS software (Version 9.2, SAS Institute, Inc., Cary, NC, USA). Path analyses and both direct and indirect path coefficients were calculated using correlation coefficient methods [50], with SDW(y) considered as a response variable and RDW (x_1), SL (x_2), MSI (x_3), Chl (x_4), PPO (x_5), and CAT (x_6) as explanatory variables.

To obtain the residual value, the following equations were used:

$$\begin{gathered}1 = \text{Residual value} + \text{direct effect } (x_1^2 + x_2^2 + x_3^2 + x_4^2 + x_5^2 + x_6^2) \text{ on (y)} + \text{indirect} \\ \text{effect } ((2\ x_1\ r_{12}\ x_2) + (2\ x_1\ r_{13}\ x_3) + (2\ x_1\ r_{14}\ x_4) + (2\ x_1\ r_{15}\ x_5) + (2\ x_1\ r_{16}\ x_6) + (2\ x_2\ r_{23}\ x_3) + \\ (2\ x_2\ r_{24}\ x_4) + (2\ x_2\ r_{25}\ x_5) + (2\ x_2\ r_{26}\ x_6) + (2\ x_3\ r_{34}\ x_4) + (2\ x_3\ r_{35}\ x_5) + (2\ x_3\ r_{36}\ x_6) + \\ (2\ x_4\ r_{45}\ x_5) + (2\ x_4\ r_{46}\ x_6) + (2\ x_5\ r_{56}\ x_6)) \text{ on } (y)\end{gathered} \quad (10)$$

The relative importance (RI%) of each variable to the total variation was estimated according to the following formula:

$$\text{RI\%} = \frac{|\text{CDi}\ |}{\sum \text{i}\ |\text{CDi}\ |} \times 100 \quad (11)$$

where |CDi| is the coefficient of determination

A membership index was applied to characterize the salinity tolerance index (STI) values of tested genotypes. The STI values of tested genotypes were calculated based on 3 effective traits (SL, SDW, and CAT), which were therefore used to calculate the membership index [51]. The membership index value (F_{ij}) was calculated for each trait per genotype, from the ratio of values obtained in salinity stress to the values obtained in the control treatment. Then, the mean membership index averaged over all the traits (F_i) was used as an indicator of tolerance to a given stress.

$$F_{ij} = \frac{x_{ij} - x_{min}}{x_{max} - x_{min}} \qquad \text{and } Fi = \text{average of } F_{ij} \quad (12)$$

where x_{ij} is the ratio of the *i*th genotype, *j*th trait; x_{min} and x_{max} are the minimum and maximum ratio of the trait; F_{ij} is the membership index value of the *i*th genotype, *j*th trait; F*i* is the mean membership index averaged over n traits of the *i*th genotype. Each genotype's salinity tolerance was placed into 5 ranks, classified according to the following criteria: Rank 1: F*i* > 0.8 (highly tolerant, HT), Rank 2: 0.6 ≤ F*i* <0.8 (tolerant, T), Rank 3: 0.4 ≤ F*i* < 0.6 (intermediate, I), Rank 4: 0.2 ≤ F*i* < 0.4 (sensitive, S), Rank 5: F*i* < 0.2, (highly sensitive, HS).

Discriminant Analyses

To confirm the classification of genotypes, the same data used to rank salt tolerance of genotypes were used in discriminant analyses with the group assignment for each genotype. The 3 traits were considered as quantitative variables, and the salinity classes (HT, T, I, S, and HS) were considered as qualitative variables. All genotypes were then given an equal prior probability to be grouped into the 5 levels of salinity tolerance. Discriminant analyses were computed using the XLSTAT statistical package (Version 2018, Excel Add-ins soft SARL, New York, NY, USA).

3. Results

3.1. Analysis of Variance for Studied Traits

Analysis of variance showed significant interaction differences between genotypes and salinity levels for all measured traits, with the exceptions of SLRL, SLSDW, and SRL (Table 2). Genotype and salinity levels were found highly significant as sources of variance for all measured traits, with the exception of PPO (Table 2). The mean performance of all traits showed highly significant differences between tested DHLs and the salt-tolerant check cultivar Sakha93. The linear model explained most of the phenotypic variability (R^2 = 0.56 − 0.87; Table 2).

Table 2. Mean performance of wheat genotypes averaged across all salinity levels and the probability of main effects and their interaction.

Genotypes	RN	RL	RDW	SL	SDW	SLRL	SRDW	SLSDW	SRL	RWC	MSI	CHL	POD	PPO	CAT
DHL2	3.78	9.35	0.032	17.67	0.026	1.91	0.79	0.0015	292.36	85.05	72.37	747.15	0.179	0.026	0.074
DHL3	6.11	6.57	0.030	18.67	0.028	2.86	0.93	0.0015	216.71	91.11	65.99	730.10	0.102	0.020	0.064
DHL5	5.11	7.95	0.034	23.78	0.039	3.09	1.14	0.0016	235.60	90.19	75.12	712.71	0.173	0.031	0.087
DHL7	5.66	6.12	0.033	18.44	0.022	3.05	0.67	0.0012	186.60	92.06	61.34	595.52	0.152	0.020	0.063
DHL8	5.00	6.84	0.025	15.67	0.021	2.36	0.85	0.0014	270.53	89.20	66.00	599.25	0.105	0.027	0.053
DHL11	4.23	7.04	0.031	21.33	0.035	3.15	1.12	0.0016	229.04	85.77	72.98	707.41	0.113	0.033	0.073
DHL12	4.78	6.98	0.027	20.56	0.024	3.02	0.89	0.0012	258.01	91.69	65.15	667.07	0.146	0.022	0.062
DHL14	4.44	7.03	0.024	17.89	0.030	2.56	1.24	0.0017	295.38	90.62	62.54	660.89	0.097	0.026	0.071
DHL15	4.23	8.11	0.024	20.78	0.026	2.98	1.08	0.0013	339.07	92.21	66.10	663.45	0.106	0.034	0.068
DHL21	5.77	7.26	0.032	20.89	0.029	2.89	0.89	0.0014	228.68	83.40	76.99	890.94	0.150	0.028	0.085
DHL22	6.45	7.79	0.024	16.89	0.024	2.23	1.01	0.0015	323.92	90.92	63.28	665.83	0.100	0.024	0.064
DHL23	4.77	6.87	0.028	17.22	0.026	2.64	0.92	0.0015	247.15	94.70	75.58	606.79	0.109	0.033	0.070
DHL25	4.11	8.35	0.032	19.89	0.028	2.43	0.88	0.0014	266.12	87.93	69.12	833.35	0.149	0.028	0.084
DHL26	5.38	6.36	0.027	16.11	0.025	2.56	0.92	0.0016	238.19	84.70	69.52	588.46	0.169	0.030	0.071
DHL29	6.67	6.66	0.030	17.33	0.025	2.65	0.83	0.0014	225.32	89.19	64.58	720.69	0.125	0.024	0.064
Sakha 93	4.78	5.96	0.026	19.44	0.027	3.33	1.04	0.0014	231.59	87.17	74.32	812.75	0.146	0.035	0.071
Mean	5.08	7.20	0.029	18.91	0.027	2.73	0.95	0.0014	255.27	89.12	68.81	700.15	0.133	0.028	0.070
Probability of main effects and their interactions															
Genotype	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Salinity levels	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.031	0.145	0.000
Interaction	0.046	0.008	0.009	0.006	0.000	0.245	0.013	0.196	0.180	0.000	0.013	0.000	0.000	0.000	0.000
R^2	0.87	0.75	0.85	0.88	0.87	0.57	0.82	0.66	0.75	0.80	0.82	0.81	0.82	0.72	0.80

Roots number (RN), root length (RL, cm), root dry weight (RDW, g), shoot length (SL, cm), shoot dry weight (SDW, g), shoot root length ratio (SLRL), shoot root dry weight ratio (SRDW), shoot length shoot dry weight ratio (SLSDW), specific root length (SRL, cm g^{-1}), relative water content (RWC, %), membrane stability index (MSI, %), chlorophyll content (CHL, μg g^{-1} FW), peroxidase (POD, U g^{-1} FW mL^{-1}), polyphenol oxidase (PPO, U g^{-1} FW mL^{-1}), and catalase (CAT, U g^{-1} FW mL^{-1}).

3.2. Phenotypic and Genotypic Coefficients of Variation, Heritability, and Genetic Gain

The results showed that the genetic variance was greater than the interaction variance (genetic variance x environment) for six traits (RN, RL, SL, SLR, SLRL, and SRDW), which suggests that genetic variance has the predominant role in determining these traits (Table 3). Therefore, salinity levels have less impact on determining these traits. The broad sense heritability showed high heritability values (>60.0%) for all measured traits, which varied from 62.71% for SLRL to 93.27% for SL. The ratio of PCV to GCV was approximately equal for most traits. However, the genotypic variance was smaller than the phenotypic variance for all traits. Genetic gain ranged from 22.06% for RWC to 48.00% for POD (Table 3).

Table 3. Estimates of variance components, heritability, genetic gain, and genetic advance as percent of mean, phenotypic, and genotypic coefficients of variability for 16 genotypes tested at three salinity levels.

Variable	Grand Mean	σ^2G	$\sigma^2G\times L$	σ^2P	h^2	GCV	PCV	GA	GG
RN	5.08	1.05	0.09	1.13	92.74	20.19	20.97	2.04	40.06
RL	7.20	2.05	0.81	2.44	83.84	19.85	21.68	2.70	37.45
RDW	0.03	0.00	0.00	0.00	91.54	12.17	12.72	0.01	23.99
SL	18.91	9.52	6.55	10.21	93.27	16.60	17.17	6.24	33.06
SDW	0.03	0.00	0.00	0.00	92.64	18.32	19.04	0.01	36.33
SLRL	2.73	0.15	0.01	0.25	62.71	14.59	18.35	0.65	23.90
SRDW	0.95	0.02	0.00	0.03	89.31	16.12	17.06	0.30	31.38
SLSDW	0.00	0.00	0.00	0.00	75.22	12.39	14.21	0.00	22.25
SRL	255.27	3234.31	2,050,381.86	3,868.25	83.61	22.28	24.36	107.13	41.97
RWC	89.12	24.00	82.36	27.43	87.49	5.50	5.88	9.44	22.06
MSI	68.81	133.99	2204.26	150.44	89.06	16.82	17.82	22.50	32.70
CHL	700.15	10,364.32	137,93,975.86	11,695.23	88.62	14.54	15.45	197.43	28.20
POD	0.13	0.00	0.00	0.00	89.16	24.67	26.13	0.06	48.00
PPO	0.03	0.00	0.00	0.00	81.48	19.77	21.90	0.01	36.76
CAT	0.70	0.00	0.00	0.00	87.86	14.04	14.98	0.02	27.11

Roots number (RN), root length (RL, cm), root dry weight (RDW, g), shoot length (SL, cm), shoot dry weight (SDW, g), shoot root length ratio (SLRL), shoot root dry weight ratio (SRDW), shoot length shoot dry weight ratio (SLSDW), specific root length (SRL, cm g^{-1}), relative water content (RWC, %), membrane stability index (MSI, %), chlorophyll content (CHL, μg g^{-1} FW), peroxidase (POD, U g^{-1} FW mL^{-1}), polyphenol oxidase (PPO, U g−1 FW mL−1), and catalase (CAT, U g−1 FW mL−1), genetic variance (σ^2G), genetic variance × environment ($\sigma^2G\times L$), phenotypic variance (σ^2P), genotypic coefficient of variability (GCV%), phenotypic coefficient of variability (PCV%), broad sense heritability (h^2), Genetic advance (GA), Genetic gain (GG %)

3.3. Principal Component Analysis

Principal component analyses were conducted for all measured traits, salinity levels, and DHLs (Figure 1). PCA resulted in a clear separation between salinity levels and DHLs based on trait combinations to identify the main trait that could be used in selection for salinity tolerance or the trait that explained much of the variation observed in DHLs of wheat. The first four principal components had eigenvalues greater than 1. The first principal component (PC1) explained 31.49% of the phenotypic variation, followed by the second principal component (PC2), which accounted for 23.49% of the variation (Table 4). The third and fourth principal components explained 13.06% and 10.83%, respectively. All measured traits were positively loaded onto PC1 and PC2. The five traits loaded the highest onto PC1 (with scores of >0.30), RN, RL, SL, SDW, and MSI. PC1 had a positive correlation with all studied traits, except for three traits, which showed a negative correlation, RN, SLRL, and RWC (Table 4). The five traits, RDW, SLSDW, SRL, RWC, and CAT, loaded highest onto

PC2 (with scores of >0.30). PC2 had a positive correlation with six traits, RL, SL, SRDW, SRL, RWC, and MSI, and negative correlation with the other nine traits (Figure 1). Plants grown under a high salinity level (200 mM NaCl) exhibited high RN compared with those grown under control level (0 mM NaCl). The overall phenotypic variation in the highest salinity treatment was found to be smaller compared to the two other salinity treatments, which showed high variability along PC2.

3.4. Cluster Analysis and Genetic Relationships of DHLs

A genetic similarity matrix based on Euclidean distance was obtained from the quantitative trait data (Figure 2). Ward's method of agglomeration was used to group all genotypes. The cluster analysis separated the tested DHLs into four major groups, clearly distinguished with a similarity coefficient of 0.481. The first cluster consisted of DHL8, DHL7, DHL23, and DHL26. The second and largest group was made up of six DHLs in two sub-clusters, both consisting of three DHLs. The third cluster included DHL 2, DHL11, and the salt-tolerant check cultivar Sakha93. The fourth cluster consisted of three genotypes, DHL5, DHL21, and DHL25. The distribution of the investigated genotypes in the dendrogram point out that the clustering pattern was related to genetic similarity.

PCoA (two-dimensional) exhibited wide genetic variation among the DHLs compared to the salt-tolerant check cultivar Sakha93 (Figure 3). The first and second principal axes represented 81.15% and 17.68% of the total differences observed, respectively. The genotypes were distributed into all quadrants (four groups). Groups 1 and 2 consisted of three and four DHLs, respectively. Group 3 contained three DHLs. Although group 4 was the largest cluster, consisting of five DHLs, it covered a lower PCoA area compared to the other three groups.

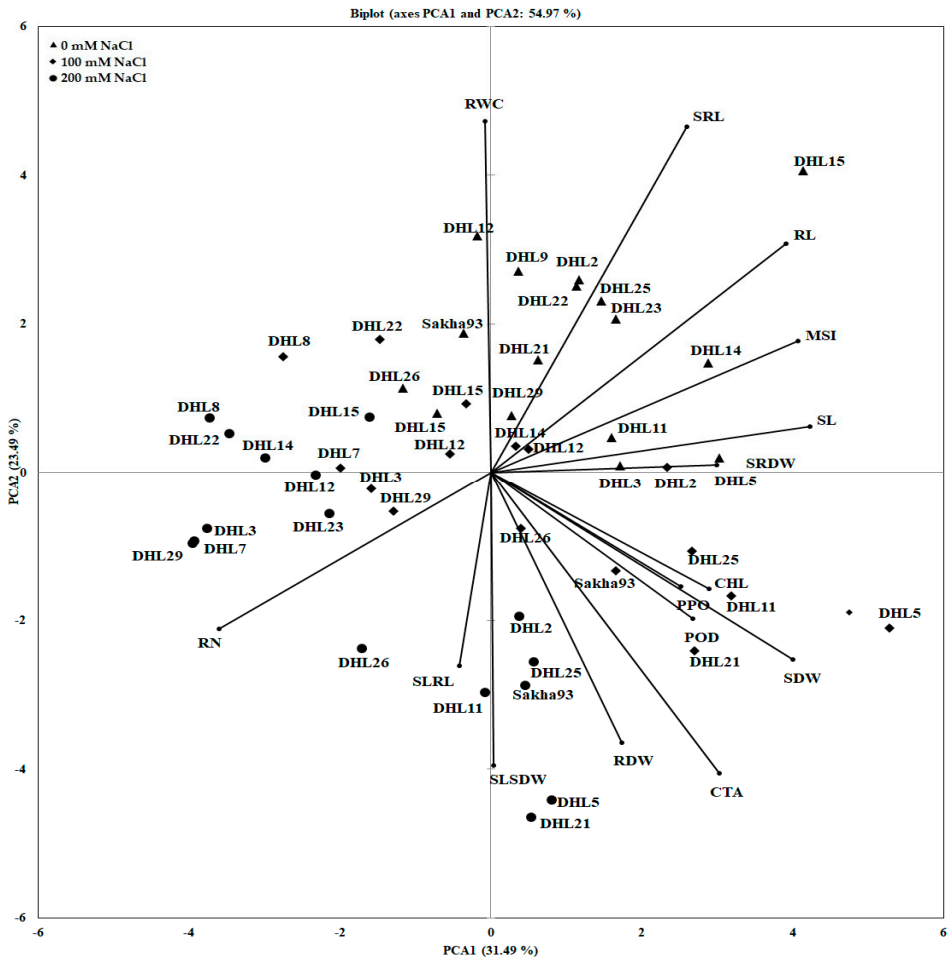

Figure 1. Principal components analysis (based on correlation matrix) of 16 wheat genotypes at three salinity levels. Biplot vectors are trait factor loadings for PC1 and PC2 of 15 measured traits. Doubled haploid line (DHL), roots number (RN), root length (RL), root dry weight (RDW), shoot length (SL), shoot dry weight (SDW), shoot root length ratio (SLRL), shoot root dry weight ratio (SRDW), shoot length shoot dry weight ratio (SLSDW), specific root length (SRL), relative water content (RWC), membrane stability index (MSI), chlorophyll content (CHL), peroxidase (POD), polyphenol oxidase (PPO), and catalase (CAT).

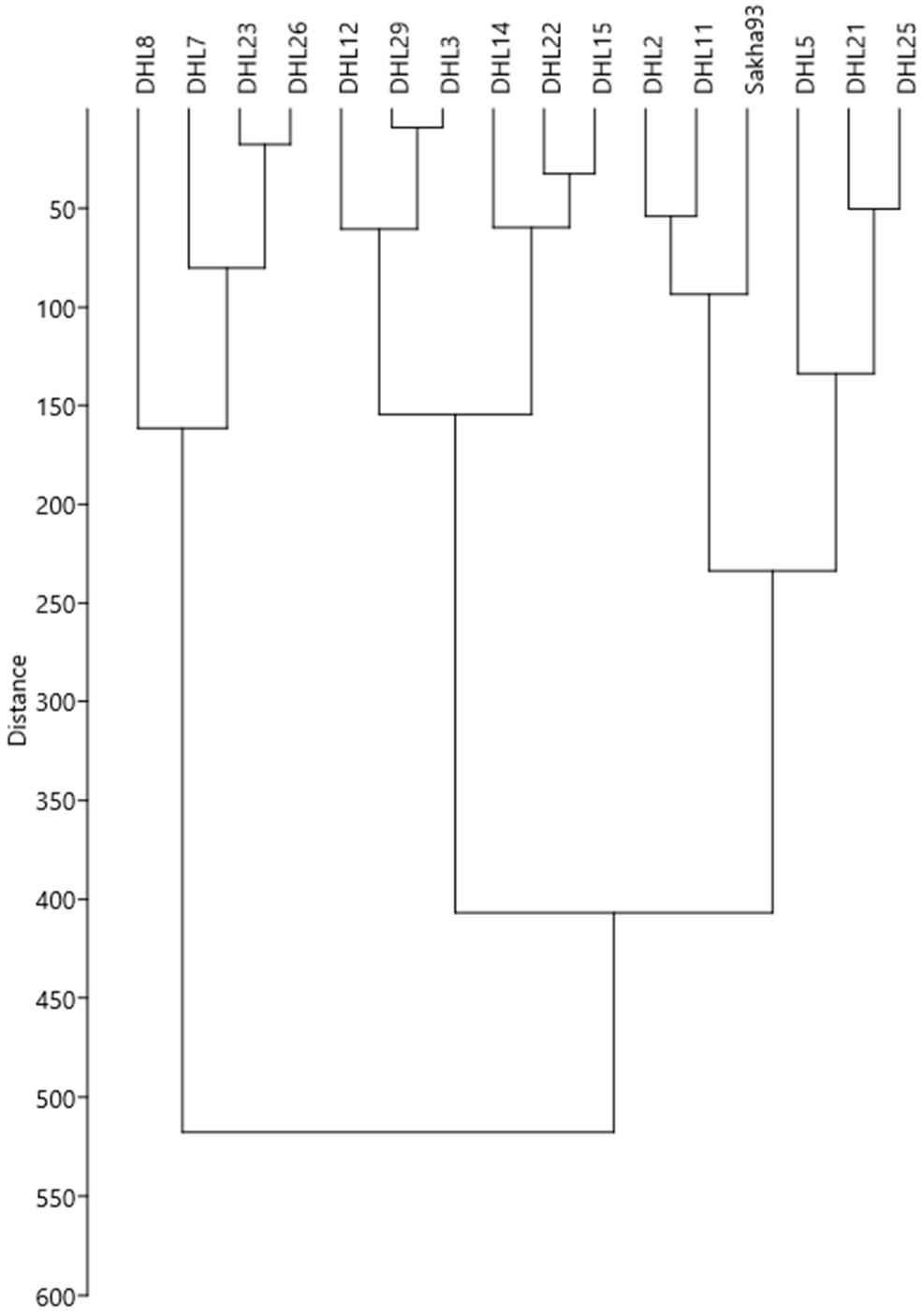

Figure 2. Dendrogram showing clustering of 16 wheat genotypes based on the Euclidean distance for 15 measured traits. DHL: doubled haploid line.

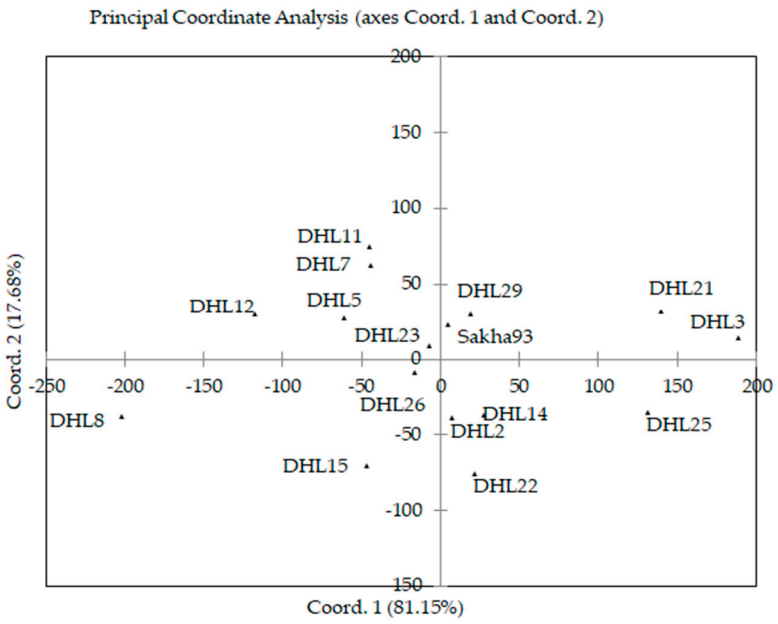

Figure 3. Principal coordinates analysis (PCoA) among the 16 wheat genotypes based on the Euclidean distance for 15 measured traits. DHL: doubled haploid line.

Table 4. Principal component analysis of 16 wheat genotypes, eigenvalues, proportion, and cumulative variance for the first six components for measured traits at three salinity levels (n = 48).

Value	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	4.72	3.52	1.96	1.62	0.88	0.72
Variability (%)	31.49	23.49	13.06	10.83	5.86	4.78
Cumulative %	31.49	54.97	68.04	78.87	84.72	89.51
Component loading *						
RN	**0.48**	0.12	0.00	0.00	0.12	0.06
RL	**0.56**	0.26	0.01	0.10	0.03	0.00
RDW	0.11	**0.36**	0.33	0.00	0.06	0.08
SL	**0.66**	0.01	0.03	0.25	0.01	0.00
SDW	**0.59**	0.17	0.08	0.05	0.08	0.01
SLRL	0.01	0.19	0.00	**0.74**	0.05	0.01
SRDW	**0.33**	0.00	**0.53**	0.07	0.02	0.01
SLSDW	0.00	**0.43**	0.35	0.11	0.05	0.03
SRL	0.25	**0.59**	0.05	0.06	0.00	0.01
RWC	0.00	**0.61**	0.00	0.14	0.04	0.01
MSI	**0.61**	0.09	0.04	0.01	0.00	0.01
CHL	0.31	0.07	0.06	0.02	0.01	**0.49**
POD	0.26	0.11	**0.30**	0.05	0.06	0.00
PPO	0.23	0.07	0.19	0.01	**0.36**	0.00
CAT	0.34	**0.45**	0.00	0.01	0.00	0.00

* values ≥ 0.30 are presented in bold-face and indicates traits important for PC definition; roots number (RN), root length (RL), root dry weight (RDW), shoot length (SL), shoot dry weight (SDW), shoot root length ratio (SLRL), shoot root dry weight ratio (SRDW), shoot length shoot dry weight ratio (SLSDW), specific root length (SRL), relative water content (RWC), membrane stability index (MSI), chlorophyll content (CHL), peroxidase (PPD), polyphenol oxidase (PPO), and catalase (CAT).

3.5. Phenotypic Variation between Genetic Clusters

The attributes of the clusters are shown in Table 5 and Figure 4. The four genetic clusters of the DHLs, which were analyzed for their phenotypic differences, showed significant differences between the four clusters in several measured traits, such as SL, SDW, MSI, CHL, and CAT (Figure 4). Clusters 1 and 4 had significant differences for four traits, that is, SL, SDW, CHL, and CAT. Clusters 3 and 4 were significantly different for CAT only. Clusters 2 and 4 showed significant differences for three traits, MSI, CHL, and CAT. Clusters 2 and 3 were significantly different for MSI only. Clusters 1 and 3 were significantly different for CHL only. Notably, genotypes in cluster 4 had higher values for the four traits, SL, SDW, CHL, and CAT, compared to the genotypes in cluster 1.

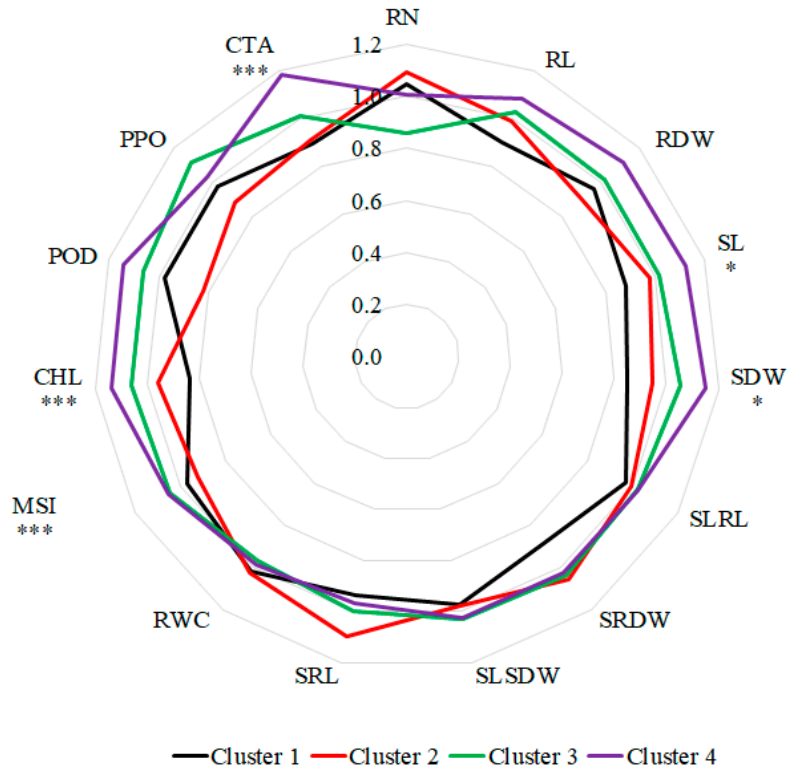

Figure 4. Radar charts comparing 15 traits of the four genetic clusters. Data were analyzed using one-way ANOVA. * and *** indicate significance at $p < 0.05$ and 0.001, respectively. roots number (RN), root length (RL), root dry weight (RDW), shoot length (SL), shoot dry weight (SDW), shoot root length ratio (SLRL), shoot root dry weight ratio (SRDW), shoot length shoot dry weight ratio (SLSDW), specific root length (SRL), relative water content (RWC), membrane stability index (MSI), chlorophyll content (CHL), peroxidase (PPD), polyphenol oxidase (PPO), and catalase (CAT).

3.6. Identification of Traits Related to Salinity Tolerance

In order to understand the best-measured traits and their contribution to salinity tolerance, the relationships between all traits were analyzed (Table 6). The results indicated positive correlations among measured traits in most cases. Results showed a significant positive correlation between SDW and each of RDW ($r = 0.449$), SL ($r = 0.605$), shoot-root dry weight ratio ($r = 0.720$), shoot length-shoot dry weight ratio ($r = 0.524$), MSI ($r = 0.404$), Chl ($r = 0.333$), PPO ($r = 0.390$), and CAT ($r = 0.648$). SL and CAT both showed significant and positive associations with eight measured parameters. The correlation results indicate that RDW, SL, MSI, Chl, PPO, and CAT are important parameters, given their contribution to SDW, as evidence of salinity tolerance (Table 6).

Table 5. Cluster membership, mean, maximum, minimum, and range for 15 measured traits of each cluster.

Cluster	Parameters	RN	RL	RDW	SL	SDW	SLRL	SRDW	SLSDW	SRL	RWC	MSI	CHL	POD	PPO	CTA
	Mean	5.20	6.55	0.028	16.86	0.023	2.65	0.84	0.0014	235.62	90.16	68.11	597.51	0.13	0.028	0.065
Cluster1	Max	5.66	6.87	0.033	18.44	0.026	3.05	0.92	0.0016	270.53	94.70	75.58	606.79	0.17	0.033	0.071
DHL7, DHL8, DHL23, HL26	Min	4.77	6.12	0.025	15.67	0.021	2.36	0.67	0.0012	186.60	84.70	61.34	588.46	0.11	0.020	0.053
	Range	0.89	0.75	0.008	2.78	0.005	0.69	0.26	0.0004	83.92	10.00	14.24	18.33	0.06	0.013	0.019
	Mean	5.45	7.19	0.026	18.69	0.026	2.72	1.00	0.0014	276.40	90.96	64.61	684.67	0.11	0.025	0.066
Cluster 2	Max	6.67	8.11	0.030	20.78	0.030	3.02	1.24	0.0017	339.07	92.21	66.10	730.10	0.15	0.034	0.071
DHL3, DHL12, DHL14, DHL15, DHL22, DHL29	Min	4.23	6.57	0.024	16.89	0.024	2.23	0.83	0.0012	216.71	89.19	62.54	660.89	0.10	0.020	0.062
	Range	2.44	1.54	0.007	3.89	0.005	0.79	0.41	0.0005	122.36	3.02	3.56	69.21	0.05	0.014	0.009
	Mean	4.26	7.45	0.030	19.48	0.029	2.80	0.98	0.0015	251.00	86.00	73.22	755.77	0.15	0.031	0.073
Cluster 3	Max	4.78	9.35	0.032	21.33	0.035	3.33	1.12	0.0016	292.36	87.17	74.32	812.75	0.18	0.035	0.074
DHL2, DHL11, Sakha 93	Min	3.78	5.96	0.026	17.67	0.026	1.91	0.79	0.0014	229.04	85.05	72.37	707.41	0.11	0.026	0.071
	Range	1.00	3.39	0.006	3.67	0.009	1.42	0.33	0.0002	63.32	2.12	1.94	105.34	0.07	0.009	0.003
	Mean	5.00	7.85	0.033	21.52	0.032	2.80	0.97	0.0015	243.47	87.17	73.74	812.33	0.16	0.029	0.085
Cluster 4	Max	5.77	8.35	0.034	23.78	0.039	3.09	1.14	0.0016	266.12	90.19	76.99	890.94	0.17	0.031	0.087
DHL5, DHL21, DHL25	Min	4.11	7.26	0.032	19.89	0.028	2.43	0.88	0.0014	228.68	83.40	69.12	712.71	0.15	0.028	0.084
	Range	1.66	1.10	0.002	3.89	0.011	0.67	0.26	0.0002	37.44	6.80	7.87	178.24	0.02	0.004	0.003

Doubled haploid line (DHL), roots number (RN), root length (RL, cm), root dry weight (RDW, g), shoot length (SL, cm), shoot dry weight (SDW, g), shoot root length ratio (SLRL), shoot root dry weight ratio (SRDW), shoot length shoot dry weight ratio (SLSDW), specific root length (SRL, cm g^{-1}), relative water content (RWC, %), membrane stability index (MSI,, %), chlorophyll content (CHL, μg g^{-1} FW), peroxidase (POD, U g^{-1} FW mL^{-1}), polyphenol oxidase (PPO, U g^{-1} FW mL^{-1}), and catalase (CAT, U g^{-1} FW mL^{-1}).

Table 6. Simple correlation coefficients among 15 measured traits of 16 wheat genotypes at three salinity levels (n = 48).

	RN	RL	RDW	SL	SLRL	SRDW	SLSDW	SRL	RWC	MSI	CHL	POD	PPO	CAT
RL	−0.562 **													
RDW	0.138	0.100												
SL	−0.323 *	0.575 **	0.264											
SLRL	0.273 *	−0.607 **	0.072	0.261										
SRDW	−0.265	0.256	−0.279 *	0.459 **	0.107									
SLSDW	0.201	−0.281 *	0.245	−0.350 *	−0.064	0.357 **								
SRL	−0.564 **	0.845 **	−0.434 **	0.374 **	−0.582 **	0.388 **	−0.370 **							
RWC	−0.292 *	0.294 *	−0.352 *	0.285	−0.060	0.051	−0.557 **	0.450 **						
MSI	−0.388 **	0.675 **	0.171	0.706 **	−0.132	0.307 *	−0.286 *	0.514 **	0.237					
CHL	−0.051	0.327 *	0.313 *	0.386 **	−0.050	0.159	0.013	0.121	−0.240	0.286 *				
POD	−0.062	0.280 *	0.572 **	0.308 *	−0.080	−0.148	−0.025	−0.055	−0.323 *	0.376 **	0.405 **			
PPO	−0.288 *	0.147	0.000	0.163	0.043	0.405 **	0.283 *	0.142	−0.287 *	0.212	0.181	0.206		
CAT	0.030	0.147	0.573 **	0.303 *	0.071	0.279 *	0.462 **	−0.168	−0.519 **	0.188	0.470 **	0.473 **	0.424 **	
SDW	−0.107	0.272	0.449 **	0.605 **	0.166	0.720 **	0.524 **	0.029	−0.212	0.404 **	0.333 *	0.257	0.390 **	0.648 **

* = significant at $p \leq 0.05$, ** = significant at $p \leq 0.01$; roots number (RN), root length (RL), root dry weight (RDW), shoot length (SL), shoot dry weight (SDW), shoot root length ratio (SLRL), shoot root dry weight ratio (SRDW), shoot length shoot dry weight ratio (SLSDW), specific root length (SRL), relative water content (RWC), membrane stability index (MSI), chlorophyll content (CHL), peroxidase (POD), polyphenol oxidase (PPO), and catalase (CAT).

The simple correlation was analyzed further by path coefficient analysis. This analysis involves a method of partitioning the correlation coefficients into direct and indirect effects via alternate characters or pathways (Table S2). The traits suggested to be very important by PC1 and PC2 (SL, CAT, and SDW) were applied in the correlation and path analysis. The components of SDW variation were determined directly and jointly by each factor (Figure 5). Two most important sources of SDW variation were SL (19.10%) and CAT (13.72%). Furthermore, the joint effect of SL with CAT was 9.81%. It may thus be concluded that these traits are the most important selection criteria, which would be dependable in defining the levels of salinity tolerance, and could also be associated with yield.

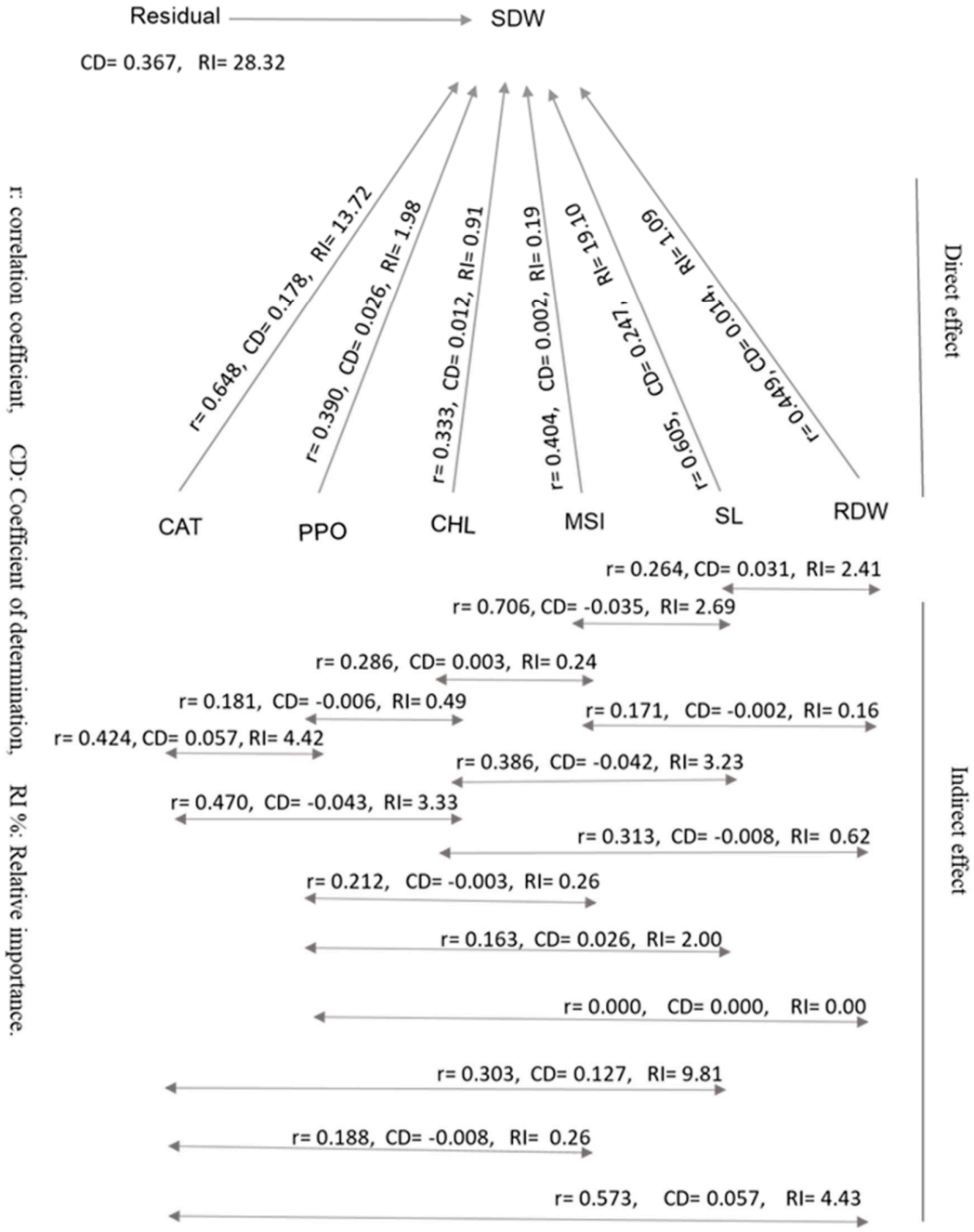

Figure 5. Path analysis (direct and indirect effects) to estimate six related attributes with shoot dry weight of wheat. Root dry weight (RDW), shoot length (SL), shoot dry weight (SDW, membrane stability index (MSI), chlorophyll content (CHL), polyphenol oxidase (PPO), and catalase (CAT).

3.7. Classification of Salt Tolerance of Sixteen Wheat Genotypes

Based on the outcome of the correlation and path analysis, SDW, SL, and CAT were chosen to classify the salinity tolerance of tested genotypes. We used these parameters to create a membership index for the phenotypic classification of wheat genotypes, to determine the extent of their salinity tolerance. The membership index was generated from scores computed from these three traits producing five major groups (Table 7). From the ranking of these groups, group I was assigned as highly tolerant (HT), with the highest score ($Fi \geq 0.8$). This group included the salt-tolerance check cultivar Sakha 93 and DHL21. Group II had scores of $0.6 \geq Fi < 0.8$ and was classed as tolerant (T). Group II included DHL25, DHL26, DHL2, DHL11, and DHL5. Group III was classified as moderately tolerant (I), obtaining scores of $0.4 \geq Fi < 0.6$, and which contained DHL23 and DHL12. Group IV was classified as sensitive (S) with scores of ($0.2 \geq Fi < 0.4$), and included DHL8, DHL7, DHL29, DHL14, and DHL15. The lowest scores ($Fi < 0.2$) were observed for group V, which was hence classified as highly sensitive (HS). Group V included DHL22 and DHL3. Some genotypes were continuously categorized in the same group, regardless of the trait used (Table 7).

The Fisher linear discriminant analysis (FLDA) is an approach similar to logistic regression, but the computation involved is more similar to MANOVA or canonical correlation. The procedure initially computes the Mahalanobis distance of each genotype to a group and then uses this distance to classify the genotype into the group to which it has the smallest generalized squared distance [52]. In FLDA, however, the test of homogeneity of covariance matrices was significant ($p < 0.0001$). Hence, we were prompted to use quadratic discriminant analysis (QDA) instead of FLDA. The QDA result indicated a 0.00% error rate, confirming that the membership index is a powerful method of classifying our genotypes (Table S3).

Table 7. Membership index for the 16 wheat genotypes based on three selected traits (shoot length, shoot dry weight, and catalase).

Genotypes	Shoot Length			Shoot Dry Weight			Catalase			Mean		
	Score	Rank	Class	Score	Rank	Class	Score	Rank	Class	Score	Rank	Class
DHL2	0.68	2	T	0.66	2	T	0.72	2	T	0.69	2	T
DHL3	0.00	5	HS	0.07	5	HS	0.43	3	I	0.17	5	HS
DHL5	0.86	1	HT	0.68	2	T	0.65	2	T	0.73	2	T
DHL7	0.55	3	I	0.18	5	HS	0.42	3	I	0.38	4	S
DHL8	0.43	3	I	0.30	4	S	0.47	3	I	0.40	4	S
DHL11	0.71	2	T	0.52	3	I	0.77	2	T	0.67	2	T
DHL12	0.47	3	I	0.54	3	I	0.63	2	T	0.55	3	I
DHL14	0.33	4	S	0.25	4	S	0.33	4	S	0.30	4	S
DHL15	0.35	4	S	0.09	5	HS	0.50	3	I	0.31	4	S
DHL21	0.73	2	T	1.00	1	HT	0.84	1	HT	0.86	1	HT
DHL22	0.31	4	S	0.19	5	HS	0.07	5	HS	0.19	5	HS
DHL23	0.48	3	I	0.30	4	S	0.52	3	I	0.43	3	I
DHL25	0.74	2	T	0.63	2	T	0.77	2	T	0.72	2	T
DHL26	0.97	1	HT	0.52	3	I	0.50	3	I	0.66	2	T
DHL29	0.50	3	I	0.30	4	S	0.11	5	HS	0.30	4	S
Sakha 93	0.93	1	HT	0.82	1	HT	1.00	1	HT	0.92	1	HT

Doubled haploid line (DHL), shoot length (SL), shoot dry weight (SDW), catalase (CAT), highly tolerant (HT), tolerant (T), intermediate (I), sensitive (S), and highly sensitive (HS).

3.8. Differentiation of Salinity Groups by Discriminant Function Analysis and MANOVA

Discriminant analysis was used to further understand the grouping and evaluate the extent of differences between groups of salinity. The three selected traits (SL, SDW, and CAT) had high and significant values in all statistics used by multivariate analysis, thus confirming the odds of prediction by group membership. Two-dimensional discriminant functions (five groups and three traits) were highly significantly correlated with the prediction of membership into salinity groupings for the genotypes used (Figure 6). Canonical discriminant function 1 (Can1) and canonical discriminant function 2 (Can2) accounted for 98% and 1.5% of the overall variance in traits, respectively (Table S4).

The loading of the variables to canonical discriminant functions showed that SL, SDW, and CAT were positive and highly correlated to Can1 (Table S4). From the variance explained by Can1 and the loading of trait variables, it appears that Can1 is a measure of the overall characteristics of salinity tolerance, encapsulated by the three parameters. In contrast, Can2 was positively correlated with SL but negatively correlated with SDW and CAT. Therefore, this result suggests that Can2 differentiates genotypes based on SL. In Can1, the maximum separation of group means was observed between HT and S (8.49 vs. −7.76), and the mean separation between S and T was −4.08 vs. 4.05. Examination of Can2 showed the separation of HT from the T group (−1.03 vs. 0.55) and separation of I from the S group (−0.47 vs. 0.41). Two groups with positive mean Can1 values had some salinity tolerance (HT and T). In contrast, HS, I, and S groups had negative mean Can1 values. In the plot of salinity groups against Can1 and Can2, the I group was placed in the center between the T and HS groups (Figure 6). HT had a positive Can1 mean (8.49) and a negative Can2 mean (−1.03), indicating that HT had high mean values for all traits, with the exception of a negative low SDW value. Group S was the opposite of HT, with negative and positive mean values in Can1 (−4.08) and Can2 (0.41), respectively. HS and I had both negative mean values to Can1 (−7.76 and −0.65) and Can2 (−0.91 and −0.047), respectively, indicating that such groups are like S, but have a higher SDW compared to S. T had positive mean values for Can1 (4.05) and Can2 (0.55), indicating higher mean values in all traits.

Further analysis using MANOVA for three traits across five groups indicated that the groups are significantly different. Moreover, least squared (LS) means comparisons between groups for each trait showed significant differences between HT and I, S and HS in all traits but was only significantly different from T group for SDW (Table S5, Figure 7). Group of T showed significant differences between S and HS in all traits but was only significantly different from I for SL. I showed significant differences between S and HS in SDW only. S showed a significant difference from HS in SL alone. Nonetheless, overall pairwise contrasts between groups were highly significant in all comparisons, indicating the complete separation of groups based on the three quantitative traits.

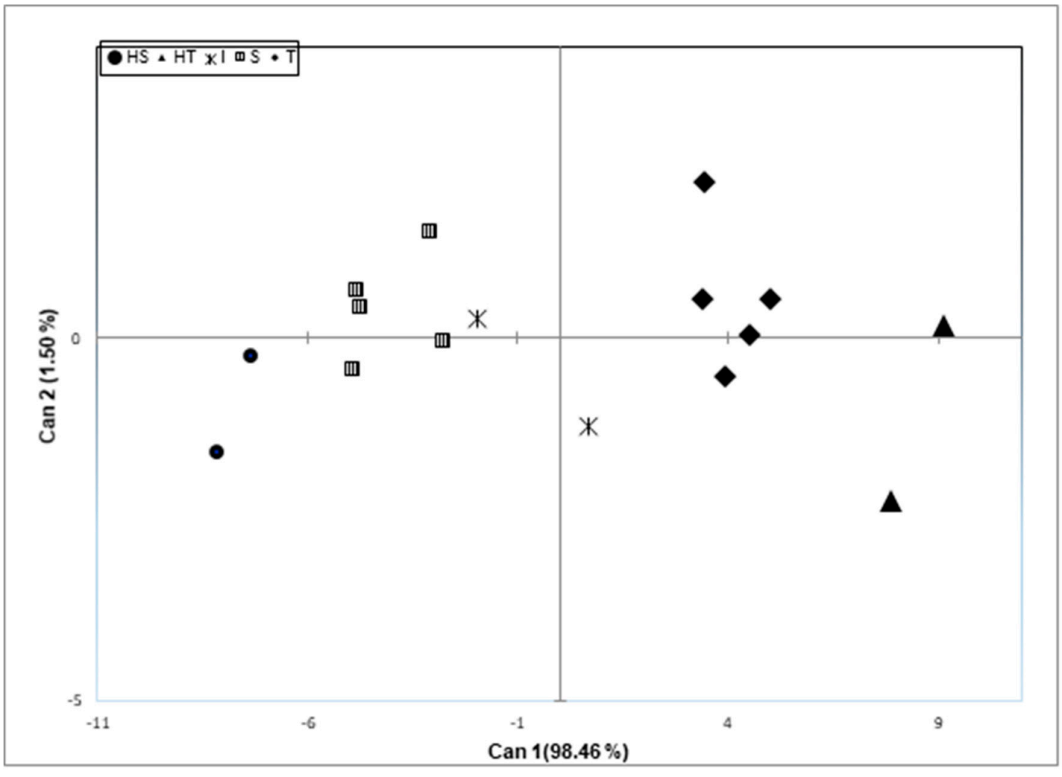

Figure 6. Distribution of 16 wheat genotypes by discriminant analysis of shoot length (SL), catalase (CAT), and shoot dry weight (RDW) traits responses to salt stress. Highly tolerant (HT), tolerant (T), intermediate (I), sensitive (S), and highly sensitive (HS).

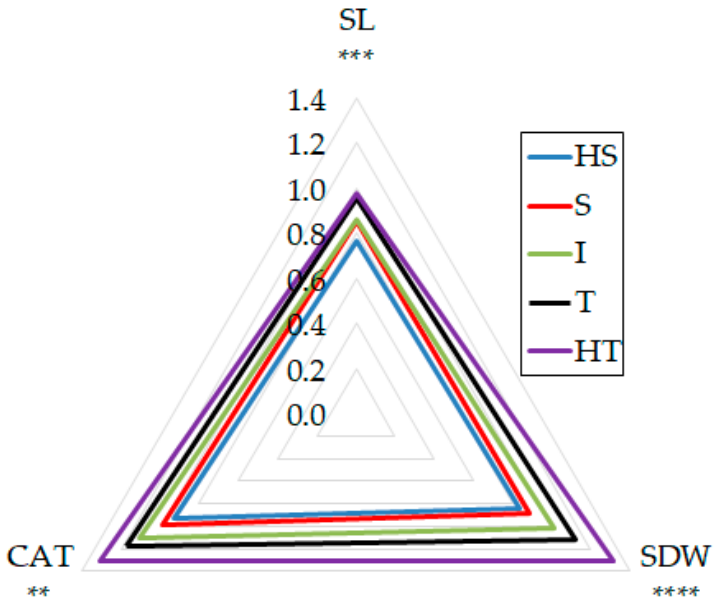

Figure 7. Radar charts comparing three traits of the five groups. Data were analyzed using the Least Squares (LS). **, ***, and **** indicate significance at $p < 0.05$, 0.001, and 0.0001, respectively. Shoot length (SL), catalase (CAT), shoot dry weight (RDW), highly tolerant (HT), tolerant (T), intermediate (I), sensitive (S), and highly sensitive (HS).

4. Discussion

Crop growth is strongly affected by salinity stress, especially throughout the early phases of seed germination and seedling growth stages [53]. Salinity stress causes negative physiological and biochemical variation as a result of complicated factors, including osmotic, ionic, and oxidative stresses [54]. Bread wheat grows well under moderate salinity (100 mM NaCl) conditions, although it is less salt tolerant than barley [55]. Increased salinity continues to have an adverse impact on yield. Simultaneously, low levels of salinity may not necessarily decrease yield, despite observations of decreased biomass, a number of leaves, and leaf area. This could explain why the increment of yield with salinity does not occur until a certain "threshold" of salinity is reached [56].

Crop breeding programs aim to produce new varieties of crops that are well-adapted to both abiotic and biotic stresses. In developing salt-tolerant cultivars, we evaluated the genetic diversity of 16 genotypes (15 DHLs and a salt-tolerant check cultivar) using different morphological, physiological, and biochemical traits, under varying levels of salinity stress. Genotypes varied significantly for all traits evaluated for salinity tolerance (Table 2). Different trait parameters indicated different rankings of genotypes in response to salinity tolerance, indicating genetic diversity among the 16 wheat genotypes used. Phenotypic variation of lines played a major role in dominant genotype × environment interactions. Only three traits were not submitted to a significant genotype × environment interaction (SLRL, SLSDW, SRL; Table 2). Some salt-sensitive lines, such as DHL3, DHL22, and DHL 29, exhibited high RN under a high salinity level (200 mM NaCl) compared with the control and moderate salinity treatments (Table 2; Figure 1). This may be due to the fact that stressful conditions like salt stress induce physiological drought, and plants tend to proliferate more roots at higher stress levels in order to absorb more water [57].

The significant differences among wheat genotypes in our study indicate the existence of variation for all measured traits. The improvement of salinity tolerance in crops like wheat will depend on the amount of genetic variability and heritability of the traits determining salinity tolerance. The broad sense heritability (h^2) provides information on the relative magnitude of genetic and environmental variation [58]. Whereas heritability estimates can be used to predict the reliability of the phenotypic value as a guide to breeding value [59], heritability alone is not enough to determine the selection. H^2, GCV, and genetic advance provide reliable estimates of the amount of genetic gain

to be expected through phenotypic selection [60]. The combination of high h^2 (>60.0%), GCV, genetic advance, and genetic gain (>20.0%) indicate that the variation in all traits investigated in this study is largely due to genetic factors, and selection would be effective for these traits (Table 3). Given the poor predicted response to selection based on SDW alone, determining the relationships between SDW and other measured traits could provide an indication of which of these measured traits could be indirectly selected for improved SDW under salinity stress. It could also help pinpoint which of the measured traits affect SDW, either positively or negatively, which, in turn, assists in deciding on an adequate selection or breeding strategy.

In the present study, most of the traits had high heritability coupled with high genetic gain, suggesting a preponderance of additive gene effects. Therefore, the traits with high h^2 and genetic gain can be used as reliable screening criteria for evaluating the salt tolerance of different genotypes [61]. Based on the results of heritability and genetic gain, all focal traits are potentially useful as screening criteria, with the exception of RWC. In addition, all measured traits represent nearly equal GCV and PCV, which is acceptable, although breeders wish to obtain higher GCV than PCV [62]. Many of the traits measured in this study, and their responses to salinity stress, have been used in breeding programs to evaluate the salinity tolerance of genotypes of cereal crops [63], which is desirable if the methods are easy, quick, and inexpensive [30,32]. PCA has been used to identify the most important traits, by using the first and second principal components. All influential traits (loadings ≥ 0.30) in PC1 and PC2 were seen as paramount (Table 4). The relationships between the attributes were investigated for eight traits out of 15, and these traits were used as efficient screening criteria (Table 4, Figure 1). These traits (RN, RL, SL, SDW, MSI, RDW, RWC, and CAT) had high values of heritability and genetic gain (Table 3).

The dendrogram based on these phenotypic traits allowed for the classification of the 16 genotypes into four main clusters (Table 5, Figure 2). The separation of genotypes was quite clear, and the results were compatible to an acceptable level (group HT with T or I, and the group I with S or SH) with the classification outcomes (Table 7). Our PCoA results are useful for comparing the merits of different wheat DHLs, and show which ones are capable of stability in comparison with the check salt-tolerant check cultivar across different salinity levels (Figure 3). [64] reported that PCoA could be a good method for the separation among genotypes when there are genotypes clearly separated from the majority of other genotypes. According to this investigation, PCoA seems to be necessary for an adequate description of the separation between genotypes [65].

The correlation between traits gives us a power of association between traits that describe salinity tolerance and will depend on the existence of genetic variation and identification of traits that are correlated to yield [66], instead of relying solely on visual score [67]. From the eight attributes selected based on the results of PCA analysis and genetic gains (RN, RL, SL, SDW, MSI, RDW, RWC, and CAT), the three traits (RN, RL, and RWC) were excluded, showing insignificant correlation with SDW (Table 6).

In this study, the seven parameters, including six independent variables (RDW, SL, MSI, CHL, PPO, and CAT), could be unbiased parameters for assessing salinity tolerance, given its contribution in the production of SDW as a dependent variable (Figure 5). The great contribution of two traits (SL and CAT) on SDW supported their importance as selection criteria in wheat at the seedling stage. Similar results were obtained by [68,69]. It could be concluded that the maximum direct effect upon SDW was exerted by SL and CAT, and their joint effects.

Taken together correlation and path analysis results indicate that the SDW and two traits (SL and CAT) are related, which may be used as evidence in screening and selection for evaluating the optimum genotypes for each salinity level. The use of a membership index was verified using discriminant analysis in order to precisely classify for salinity tolerance.

The highest degree of salinity tolerance was found in two genotypes: DHL21 and Sakha93 (Group HT, Table 7). The strong positive correlation between SL and CAT to SDW indicates that the photosynthetic ability of sensitive plants under salt stress became limited, which led to chlorosis and reduction in shoot growth [70–72]. These two genotypes had the least reduction in growth and highest relative CAT activity compared to the S and HS groups, which showed the exact opposite

pattern. In most breeding programs, simple visual salt injury scoring [9] is widely used for characterization because it reflects the plant's overall response to salt stress. However, although quantitative characteristics of salinity tolerance, such as Na-K selectivity [73], Na-Ca selectivity [74], and proline concentration [75], render evaluation more difficult, these studies still prefer to use these quantitative characters as a standard for classification of genotypes for salt tolerance.

In fact, it is natural for genotypes to be superior in at least one trait and inferior in other traits [38]. Instead of describing wheat genotypes for traits one by one, we used a multivariate path analysis using seven quantitative traits across 16 genotypes. Two out of six traits (SL and CAL) showed high contributions to SDW. Thus, they are fair estimates of the performance of the genotypes under salinity stress. Our findings demonstrated that the contributions were robust, and discriminant analysis was used to confirm the level of salinity tolerance. As indicated by MANOVA and discriminant functions, there were clear separations between salinity tolerance levels (Figure 6).

Based on morphological features, the HT group was less affected by salinity stress because of the high increase in CAT activity, CAT that detoxifies due to adjustments in leaf morphology, chlorophyll composition, heat dissipation by xanthophyll pigments, electron transfer to oxygen acceptors other than water, and the biochemical activities which prevent oxidative damage during photosynthesis [72,76]. This tolerance might also be due to genetic differences in salinity tolerance that are not necessarily due to differences in the ability to detoxify ROS. The differences in the level of expression or activity of antioxidant enzymes are associated with more tolerant genotypes, but conversely sometimes with more sensitive genotypes. We suggest that differences in antioxidant activity between genotypes may be due to genotypic differences in degrees of stomatal closure or in other responses that alter the rate of CO_2 fixation [72].

Both groups T and I had the same salt tolerance responses, but I had less ability to maintain CAT than T criteria (Table S5, Figure 7). The HT and T groups had considerably higher values for SL, CAT, and SDW compared to both groups S and HS. The I group was statistically superior to S, regarding SDW only, as well as HS for SL and SDW. Therefore, the genotypes in I offered a novel source of tolerance and an apparently distinct mechanism from those found in HT. Trait responses were not significantly different between S and HS, except in SL.

5. Conclusions

Our results confirmed the importance of SL, CAT, and SDW in objective genotype classification for salinity tolerance. Moreover, the results demonstrated the force of multivariate analyses (PCA, PCoA, clustering, path analysis, MANOVA, and canonical and linear discriminant analyses) to assert and demarcate tolerance levels.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: The pedigree, salt tolerance and response of another culture of the bread wheat genotypes used in this study. Table S2: Partitioning of correlation coefficient between shoot dry weight (SDW) with six related attributes; root dry weight (RDW), shoot length (SL), membrane stability index (MSI), chlorophyll content (CHL), polyphenol oxidase (PPO) and catalase (CAT). Table S3: Prior and posterior classification, membership probabilities in salinity groupings by linear discriminant analysis. Table S4: Total canonical structure of eigenvalue, canonical discriminant function and class means of salinity group to canonical discriminant function. Table S5: Summary (LS means) of all pairwise comparisons for Class (Fisher (LSD).

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