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Identification of Phenotypic and Physiological Markers of Salt Stress Tolerance in Durum Wheat (*Triticum durum* Desf.) through Integrated Analyses

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Abstract: Salinity is one of the most important stresses that reduces plant growth and productivity in several parts of the world. Nine Tunisian durum wheat genotypes grown under hydroponic conditions were subjected to two levels of salt stress (100 and 170 mM NaCl) for 21 days. An integrative analysis revealing the impact of salinity on key phenotypic and physiological marker traits was then conducted. Principal component analysis grouped these traits into three different clusters corresponding to the absence of salt stress and the two levels of salt stress. This analysis also allowed the identification of genotypes exhibiting various levels of tolerance to NaCl. Among the nine genotypes of *Triticum durum* Desf., cultivar Om Rabiaa was the most tolerant whereas cultivar Mahmoudi genotype was the most sensitive. Following the multivariate analysis of the examined phenotypic and physiological traits, we found that shoot length, shoot fresh weight, leaf area, the whole-plant stable isotope ratios of nitrogen ($\delta^{15}N$), shoot ammonium and proline contents, and shoot glutamine synthetase activity could be used as markers for the selection of salt-tolerant wheat genotypes.

Keywords: carbon; durum wheat; genotypes; salinity; markers; nitrogen

1. Introduction

Soil salinity is one of the most important abiotic stresses that has a detrimental impact on plant growth and development, thus significantly reducing agricultural productivity in several parts of the world [1,2]. It has been estimated that approximately 20% of the irrigated lands, corresponding to the production of approximately one third of the agricultural products necessary to feed the world population, are affected by salinity [3].

Saline soils contain excess soluble salts, especially sodium chloride (NaCl), which perturbs the physiology of plants by limiting the capacity of the roots to extract water from the soil (osmotic stress). In addition, the presence of elevated amounts of NaCl in the soil interferes with the uptake and assimilation of nutrients (ionic stress), including nitrogen (N), resulting in the inhibition of many physiological and biochemical processes such as photosynthetic carbon (C) assimilation [4–6].

Therefore, the ability of plants to cope with salinity stress is of major importance for maintaining the production of a number of major crops such as bred wheat and durum wheat.

Wheat, which is one the most widely grown crops in the world provides nearly 55% of the carbohydrate and 20% of the calories consumed by the world population [7–9]. Durum wheat, accounts for 8% of total wheat production around the world [10], approximately half of which originates in the Mediterranean basin [11]. In this area, salt accumulation can occur as the result of seawater intrusion into aquifers and irrigation with brackish water [12].

Therefore, many studies have been undertaken to improve our understanding on the physiological and molecular determinants that confer tolerance to saline environments in crops in general and wheat in particular [13–18]. For example, salt stress-induced alterations in carbohydrate metabolism have been investigated in several species [19,20] including wheat [20,21]. A decrease in starch has been observed, in rice [22], in barley and wheat [23], whereas an accumulation of soluble sugars occurred in rice [22,24] and wheat [25–27]. It was hypothesized that the changes in the contents of carbohydrates, which are molecules that can maintain cell turgor or act as respiratory substrates, could circumvent the negative impact of osmotic stress on plant growth and development [28,29].

Salinity not only alters plant N uptake [5], but also induces major changes in N metabolism notably by modifying the activities of key enzymes involved in the nitrate and ammonium assimilatory pathways such as nitrate reductase (NR) glutamine synthetase (GS) and glutamate dehydrogenase (GDH) [30,31]. In addition to phenotypic and physiological traits, ^{13}C or ^{15}N natural abundance is a useful indicator commonly employed to study the plant response to abiotic stresses such as salinity [30,32,33]. Salt stress modifies $\delta^{13}C$ via affecting the balance between stomatal conductance and carboxylation [30,34,35]. Similarly, ^{15}N natural abundance has been used to identify durum wheat genotypes that are more tolerant to a salt stress [30,33].

The aim of our study was to first reveal the phenotypic and physiological responses of nine durum wheat genotypes grown in the presence of increasing amounts of NaCl in an integrated manner, with emphasis on plant growth parameters and C and N metabolism. These nine genotypes were classified according to their level of resistance to the salt stress. Phenotypic and physiological marker traits that are representative of such resistance were identified in the early stage of plant development. The use of these markers for breeding strategies is discussed.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Tunisian wheat cultivars (*Triticum durum* Def.) including three landraces (Mahmoudi, INRAT 69, Chili) and the six most cultivated and commercially improved varieties (Ben Bechir, Karim, Razzak, Om Rabiaa, Nasr and Khiar) were selected for the study [36,37]. Seeds were supplied by the National Agronomic Research Institute of Tunisia (INRAT). The seeds were surface sterilized with 70% ethanol (v/v) for 2 min and then with a sodium hypochlorite solution containing 3% (w/v) active chlorine and were finally rinsed 10 times with distilled water. The seeds were germinated in the dark at 24 °C in Petri dishes on moistened filter paper. After three days, the Petri dishes were transferred to a growth chamber and incubated at 24 °C ± 1 °C; with 60% relative humidity during the day and at 19 °C ± 1 °C during the night under a 16/8 h (light/dark) light cycle with a light intensity of 200 µmoL m^{-2} s^{-1}. Nine-day-old seedlings of all cultivars were then transferred to a hydroponic culture system containing Hoagland's solution [38]. For the first seven days, the plants were grown on half-strength Hoagland nutrient solutions. Plants were acclimated to the hydroponic conditions for two days. Then, 100 mM or 170 mM NaCl was added to the growth medium. To avoid osmotic shock, NaCl was added twice daily in increments of 25 mM until the final concentrations were attained. A nutrient solution without NaCl was used as a control. Nine plants from each genotype and treatment were arranged in a completely randomized design (CRD) and used as replicates for the phenotypic and physiological trait measurements. The hydroponic solutions were aerated continuously and renewed twice a week.

The plants were harvested 21 days after the addition of NaCl, briefly washed with distilled water, separated into shoots and roots and weighed immediately to measure fresh weight (FW). The third leaf (F3) was scanned (Samsung EX2F Scanner, Seoul, South Korea) and ImageJ processing software [39] was used to measure leaf area. Plant shoot and root lengths were also measured on each of the individual plants. Finally, shoot and root samples were frozen in liquid N and stored at −80 °C. For dry weight determination, the shoots and roots were dried in an oven at 85 °C for 48 h.

2.2. Salt Stress Sensitivity Index Determination

The effect of salt stress on growth parameters (shoot and root length, shoot and root fresh weight, leaf area and whole-plant dry matter) was evaluated using a stress tolerance index (STI) calculated, as a percentage compared to the control, according to the following formula:

$$\text{STI (\%)} = [(M_{stressed})/M_{control}] \times 100 \quad (1)$$

where, $M_{control}$ and $M_{stressed}$ are the mean values of the growth parameters per plant in unstressed and stressed plants, respectively [14,40].

2.3. Metabolite Extraction and Biochemicals Analyses

To obtain a fine powder of homogeneous plant material, each shoot and root sample was ground for one minute in a chilled ball mill grinder (Retsch MM 400, Haan, Germany) cooled with liquid N and then stored at −80 °C. Shoot and root extracts were obtained by mixing 100 mg of fresh plant material with 1 mL of 80% ethanol/20% water (v/v) at 4 °C for 2 h under constant agitation (120 rpm) in an Orbital Shaker and then centrifuged at 12,000× *g* for 5 min. The supernatant was collected and the remaining pellet was reextracted with 1 mL of 60% ethanol/20% water (v/v) and finally with 1 mL of water. The three supernatants (3 mL) were pooled constituting the hydro-alcoholic extract. An aliquot of 2 mL was first evaporated using a CentriVap Concentrator (Labconco, Kansas City, Missouri, USA) and then resuspended in 440 µL of distilled water to obtain a concentrated extract. The hydro-alcoholic extracts and the remaining pellets were stored at −20 °C until further use for sugar, ammonium, amino acid and nitrate content determination and for starch content. Soluble sugars and starch contents were determined using an enzymatic D-Glucose kit from Boehringer Mannheim (R-Biopharm, Darmstadt, Germany) as described by Ni et al. [41] and Smith and Zeeman [42]. Total amino acids were determined using the Rosen colorimetric method [43]. For nitrate determination 10 µL of the hydro-alcoholic extract was added to 5% (w/v) salicylic acid dissolved in 96% sulfuric acid. The nitrate concentration was measured using the method of Cataldo et al. [44]. Ammonium content was determined using the phenol hypochlorite method described by Vega-mas et al. [45]. The colorimetric method of Carillo and Gibon [46] was used to measure proline content. This method allows the detection of proline concentrations in the nmol range [47].

2.4. Enzyme Extraction and Assay

Enzymes were extracted using plant material that had been previously stored at −80 °C. All extractions were performed at 4 °C. NR activity was measured according to the protocol described by Ferrario-Méry et al. [48]. Nitrate reductase (NR) activity measured in the presence of Mg^{2+} corresponds to the amount active enzyme (NRact), whereas NR activity measured in the presence of EDTA corresponds to the maximal enzyme activity (NRmax) [49]. Glutamine synthetase (GS) was measured according to the method of O'Neal and Joy [50]. Aminating NADH-dependent (NADH-GDH) and deaminating NAD-dependent (NADH-GDH) glutamate dehydrogenase activities were measured using the protocol of Turano et al. [51], except that the extraction buffer was the same as that used for GS. The soluble protein content was quantified via Bradford's method [52] using bovine serum albumin (BSA) as a standard.

2.5. Measurement of Carbon and Nitrogen Isotope Enrichment

N and C contents (% of total N or C on a dry matter basis) and $\delta^{13}C$ and $\delta^{15}N$ were measured using an elemental analyzer (FLASH EA 1112 series, Thermo Electron, Bremen, Germany) coupled to an isotope ratio mass spectrometer (DELTA V Advantage, Thermo Electron, Bremen, Germany). Powders of frozen plant samples stored at −80 °C were lyophilized at 4 °C for 48 h. Samples of approximately 6mg of lyophilized plant material and the internal standard were weighed in tin capsules (Courtage Analyse Service, Mont Saint Aignan, France) and sealed. Values for isotopic abundance are reported in the δ_{VPDB} notation relative to the standards Vienna Pee Dee Belemnite and air standards for C and N, respectively:

$$\delta_{sample} = 1000\ [(R_{sample}/R_{standard}) - 1] \quad (2)$$

$$R = {}^{13}C/{}^{12}C, \text{ or } R = {}^{15}N/{}^{14}N) \quad (3)$$

The overall analytical precision based on the standard deviation of an internal standard of a known isotopic signature calibrated according to international standards (International Atomic Energy Agency: IAEA-CH_6 et IeEA-NO_3), which was included every 10 samples and analyzed simultaneously with the shoot and root samples, was ±0.24% for $\delta^{13}C$ and ± 0.20% for $\delta^{15}N$.

2.6. Statistical Analyses

To evaluate the effect of the NaCl treatment, the genotype and their interactions on the various measured plant traits, a multivariate general linear model (mGLM) with Hotelling's Trace test statistic was run using SPSS Statistics v. 24 (SPSS Inc., Chicago, Illinois, USA). This analysis was chosen because of the non-independence of plant traits for each individual plant. Given the multidimensional nature of the dataset, trait variations between individual plants were analyzed by principal component analysis (PCA) [53] to identify potential morphological and physiological traits associated with the variation in NaCl concentration in the growth medium and in the different genotypes. This analysis was performed using PC-ORD v. 7.03 [54]. The distances between control and treated genotype centroids in the PCA diagram [55] were used to classify the genotypes according to their tolerance to salinity.

The significance of differences between mean values was determined by Tukey's test using GraphPad Prism software (version 6.0, San Diego, California, USA). Each data point was the mean of 9 independent replicates corresponding to 9 different plants (n = 9) ± standard error.

3. Results

In this study, 48 phenotypic and physiological traits were measured in order to analyze the impact of a salinity stress on nine durum wheat genotypes (including races and high-yielding commercial varieties) grown in the presence of 100 or 170 mM NaCl.

3.1. Analysis of Variance (ANOVA) of Phenotypic and Physiological Traits

The results of the multivariate analysis of variance (ANOVA, Table 1) showed that the effect of the two NaCl treatments on the examined phenotypic physiological marker traits was highly significant ($p < 0.001$). In addition, we observed that for all the measured traits, there was a highly significant genotypic effect ($p < 0.001$). The interaction between the genotype and the salt treatment was also highly significant ($p < 0.001$). Details of the statistical analysis performed for each phenotypic and physiological trait using multivariate analysis of variance are presented in Table 2. Table 2 defines all the abbreviations used for each trait in the subsequent analyses. Raw data are presented in Table S4.

Table 1. Multivariate general linear model analysis. The analysis shows the general impact of the NaCl treatments, the genotypic effect and their interaction on the phenotypic and physiological traits representative of plant growth and C and N assimilation. *F*: F-statistic of the Hotelling's trace test; *Df*: Degree of freedom.

	Value	*F*	*Df*	*Df* Error	*P*-Value
Model Constant	19,009.85	66,930.51	48	169	<0.001
Treatment	341.29	597.26	96	336	<0.001
Genotype	625.74	272.54	384	1338	<0.001
Treatment × Genotype	56.02	12.19	768	2674	<0.001

Table 2. Details of the results obtained through multivariate general linear model analysis. The analysis shows the impact of the NaCl treatments, the genotypic effect and their interaction on individual phenotypic and physiological traits representative of plant growth and C and N assimilation.

Plant Traits	Abbreviations	*P*-Value		
		Treatment [a]	Genotype [b]	Treatment × Genotype [c]
Root Amino Acids (nmol amino acids.mg^{-1} dry weight)	RAA	<0.001	<0.001	<0.001
Shoot Amino Acids (nmol amino acids.mg^{-1} dry weight)	SAA	<0.001	<0.001	<0.001
Whole-plant total Carbon (mg)	CPl	<0.001	<0.001	<0.001
Whole-Plant δ^{13}C (‰)	Plδ^{13}C	<0.001	<0.001	<0.001
Root δ^{13}C (‰)	Rδ^{13}C	<0.001	<0.001	<0.001
Shoot δ^{13}C (‰)	Sδ^{13}C	<0.001	<0.001	<0.001
Dry Matter Whole Plant (g)	DMPl	<0.001	<0.001	0.014
Dry Matter Stress Tolerance Index (%)	DMSTI	<0.001	0.993	1.000
Whole-Plant δ^{15}N (‰)	Plδ^{15}N	<0.001	<0.001	<0.001
Root δ^{15}N (‰)	Rδ^{15}N	<0.001	<0.001	<0.001
Shoot δ^{15}N (‰)	Sδ^{15}N	<0.001	<0.001	<0.001
Root Dry Weight (g)	RDW	<0.001	0.002	0.985
Shoot Dry Weight (g)	SDW	<0.001	<0.001	0.001
Root Fresh Weight/Root Dry Weight Ratio	RFW/DW	<0.001	<0.001	0.564
Shoot Fresh Weight/Shoot Dry Weight Ratio	SFW/DW	<0.001	<0.001	<0.001
Root Fresh Weight (g)	RFW	<0.001	<0.001	0.347
Shoot Fresh Weight (g)	SFW	<0.001	<0.001	<0.001
Root Fresh Weight Stress Tolerance Index (%)	RFWSTI	<0.001	0.533	0.983
Shoot Fresh Weight Stress Tolerance Index (%)	SFWSTI	<0.001	<0.001	0.006
Root Aminating GDH Activity (nmol. µg^{-1} protein.h^{-1})	RGDHAmi	<0.001	<0.001	<0.001
Shoot Aminating GDH Activity (nmol. µg^{-1} protein.h^{-1})	SGDHAmi	<0.001	<0.001	<0.001
Root Deaminating GDH Activity (nmol. µg^{-1} protein.h^{-1})	RGDHDea	<0.001	<0.001	<0.001
Shoot Deaminating GDH Activity (nmol. µg^{-1} protein.h^{-1})	SGDHDea	<0.001	<0.001	<0.001
Root Soluble Sugar (nmol D-glucose.mg^{-1} dry weight)	RSS	<0.001	<0.001	0.000
Shoot Soluble Sugar (nmol D-glucose.mg^{-1} dry weight)	SSS	<0.001	<0.001	<0.001

Table 2. *Cont.*

Plant Traits	Abbreviations	*P*-Value		
		Treatment [a]	Genotype [b]	Treatment × Genotype [c]
Root GS Activity (nmol γ-glutamyl hydroxamate. μg^{-1} protein.h^{-1})	RGS	<0.001	<0.001	<0.001
Shoot GS Activity (nmol γ-glutamyl hydroxamate μg^{-1} protein.h^{-1})	SGS	<0.001	<0.001	0.008
Leaf Area (mm^2)	LA	<0.001	<0.001	0.013
Leaf Area Stress Tolerance Index (%)	LASTI	<0.001	<0.001	<0.001
Root Length (cm)	RL	<0.001	<0.001	<0.001
Shoot Length (cm)	SL	<0.001	<0.001	<0.001
Root Length Stress Tolerance Index (%)	RLSTI	<0.001	<0.001	0.141
Shoot Length Stress Tolerance Index (%)	SLSTI	<0.001	<0.001	0.007
Root Ammonium (nmol ammonium.mg^{-1} dry weight)	RNH_4	<0.001	0.002	<0.001
Shoot Ammonium (nmol ammonium.mg^{-1} dry weight)	SNH_4	<0.001	<0.001	<0.001
Root Nitrate (μg nitrate.mg^{-1} dry weight)	RNO_3	<0.001	0.001	<0.001
Shoot Nitrate (μg nitrate.mg^{-1} dry weight)	SNO_3	<0.001	<0.001	<0.001
Whole-Plant Total Nitrogen (mg)	NPl	<0.001	<0.001	<0.001
Root NR-EDTA Activity (nmol nitrite. μg^{-1} protein.h^{-1})	RNREDTA	<0.001	<0.001	<0.001
Shoot NR-EDTA Activity (nmol nitrite. μg^{-1} protein.h^{-1})	SNREDTA	<0.001	<0.001	<0.001
Root NR activity (nmol nitrite. μg^{-1} protein.h^{-1})	RNR	<0.001	<0.001	<0.001
Shoot NR activity (nmol nitrite. μg^{-1} protein.h^{-1})	SNR	<0.001	<0.001	<0.001
Root Proline (nmol proline.mg^{-1} dry weight)	RPro	<0.001	<0.001	<0.001
Shoot Proline (nmol proline.mg^{-1} dry weight)	SPro	<0.001	<0.001	<0.001
Root Proteins (μg proteins.mg^{-1} dry weight)	RProt	<0.001	<0.001	<0.001
Shoot Proteins (μg proteins.mg^{-1} dry weight)	SProt	<0.001	<0.001	<0.001
Root Starch (nmol equivalent D-glucose.mg^{-1} dry weight)	RSt	<0.001	0.006	0.116
Shoot Starch (nmol equivalent D-glucose.mg^{-1} dry weight)	SSt	<0.001	<0.001	<0.001

[a] $Df = 2$, [b] $Df = 8$, [c] $Df = 16$, ×: interaction between explanatory variables of the model.

3.2. Genotype-Dependent Response to Salinity

Principal component analysis (PCA) was conducted using as variables the different phenotypic and physiological traits, the 9 genotypes and the 3 growth conditions including the control and the two treatments with NaCl (Figure 1).

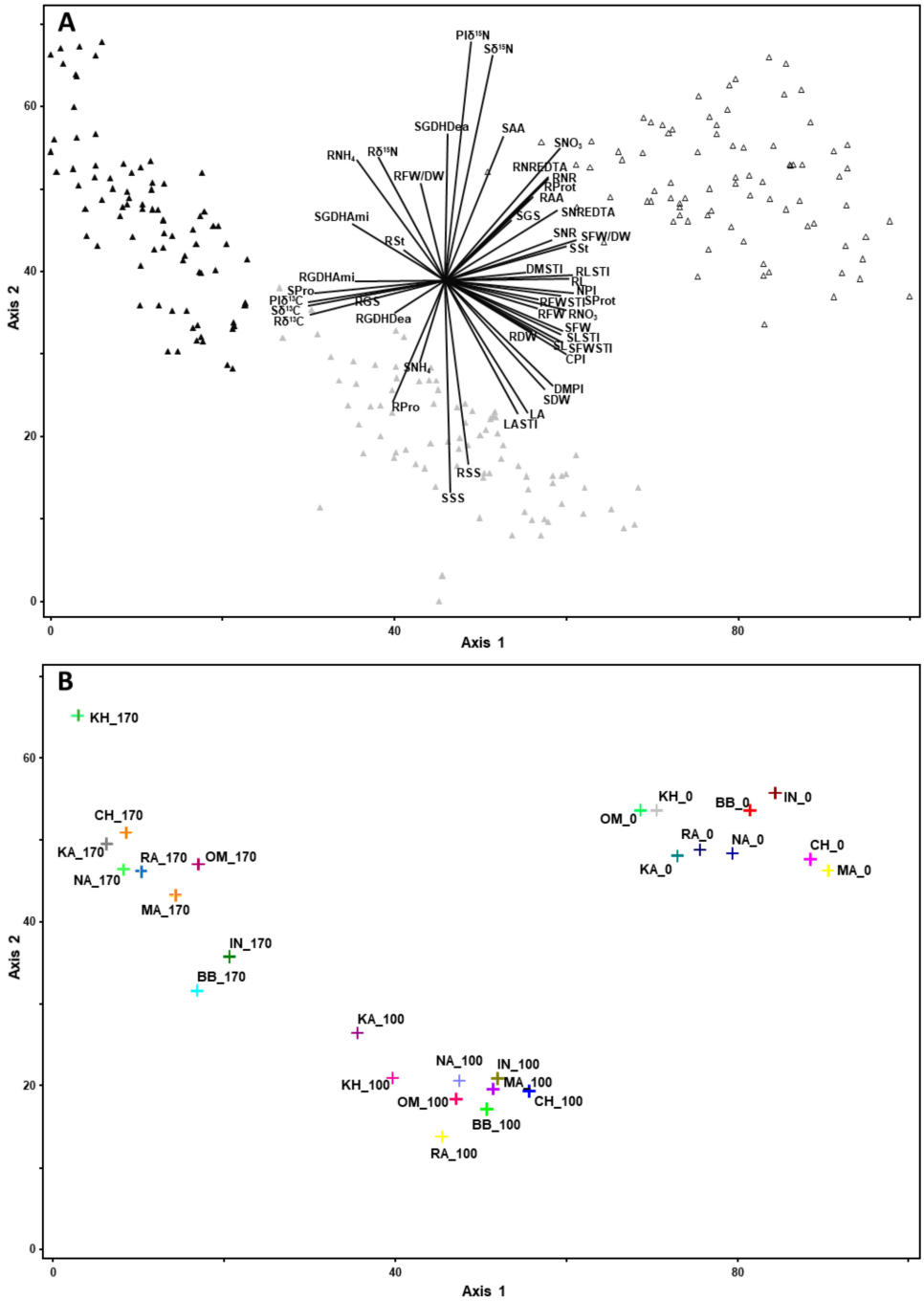

Figure 1. Ordination diagrams of the principal component analysis. The analysis was performed using the 243 plant samples and 48 phenotypic and physiological traits as variables. (**A**) Projection of samples and traits. White, grey and black triangles correspond to the three salinity treatments (0, 100 and 170 mM NaCl, respectively). (**B**) Projection of the centroids of the nine wheat genotypes for each salinity treatment. Percentage of variance: axis 1 = 45.307 %; axis 2 = 12.68 %. Ben Bechir (BB), Chili (CH), INRAT 69 (IN), Karim (KA), Khiar (KH), Mahmoudi (MA), Nasr (NA), Om Rabiaa (OM), Razzak (RA).

Along axis 1 (45.307% of explained variance), three groups of marker traits could be clearly identified. They correspond to the control (0 mM NaCl) and the two salt treatments (100 mM NaCl and 170 mM NaCl (Figure 1A). A biplot representation of the correlation between the different phenotypic and physiological traits showed that along axis 1, whole-plant total carbon (CPl), shoot fresh weight/shoot dry weight ratio (SFW/DW), shoot fresh weight (SFW), shoot fresh weight stress tolerance index (SFWSTI), root length (RL), root length stress tolerance index (RLSTI), shoot length

stress tolerance index (SLSTI), root nitrate (RNO_3), shoot nitrate (SNO_3), whole-plant total nitrogen (NPl), shoot proteins (SProt), and shoot starch (SSt) were positively correlated whereas whole-plant $\delta^{13}C$ ($Pl\delta^{13}C$), root $\delta^{13}C$ ($R\delta^{13}C$), shoot $\delta^{13}C$ ($S\delta^{13}C$) and shoot proline (SPro) were negatively correlated. In addition, the vectors of the biplot analysis showed that when the NaCl concentration was increased the values of traits such as the whole-plant $\delta^{13}C$ ($Pl\delta^{13}C$) and the shoot proline content (SPro) were much higher compared to those of the control plants (0 mM NaCl), (Figure 1A).

The second axis (12.684% of explained variance) showed that there was a high and positive correlation between the whole-plant $\delta^{15}N$ ($Pl\delta^{15}N$) and the shoot $\delta^{15}N$ ($S\delta^{15}N$), (see also Table S1). The different plant traits distributed along the two axes of the PCA analysis allowed us to classify the different genotypes according to their response to the salt treatment for each of the two NaCl concentrations (Figure 1B). The cumulative percentage of the two axes of the PCA explained 57,992% of the total variation between the different measured traits (Table S1).

Based on the scores obtained for axes 1 and 2 in the PCA analysis, the centroid distance values between the two salt stress treatments and the control were calculated. From this analysis a scatter plot was generated showing the ranking of the nine wheat genotypes in relation to the intensity of the salt stress (Figure 2). The commercial genotype Om Rabiaa (OM) which exhibited the lowest centroid distance in comparison to the untreated control plants, was found to be the most tolerant to NaCl. In contrast, the landrace Mahmoudi (MA) was classified as the most sensitive to salinity stress because the centroid distance from the control was greatest among all the other genotypes.

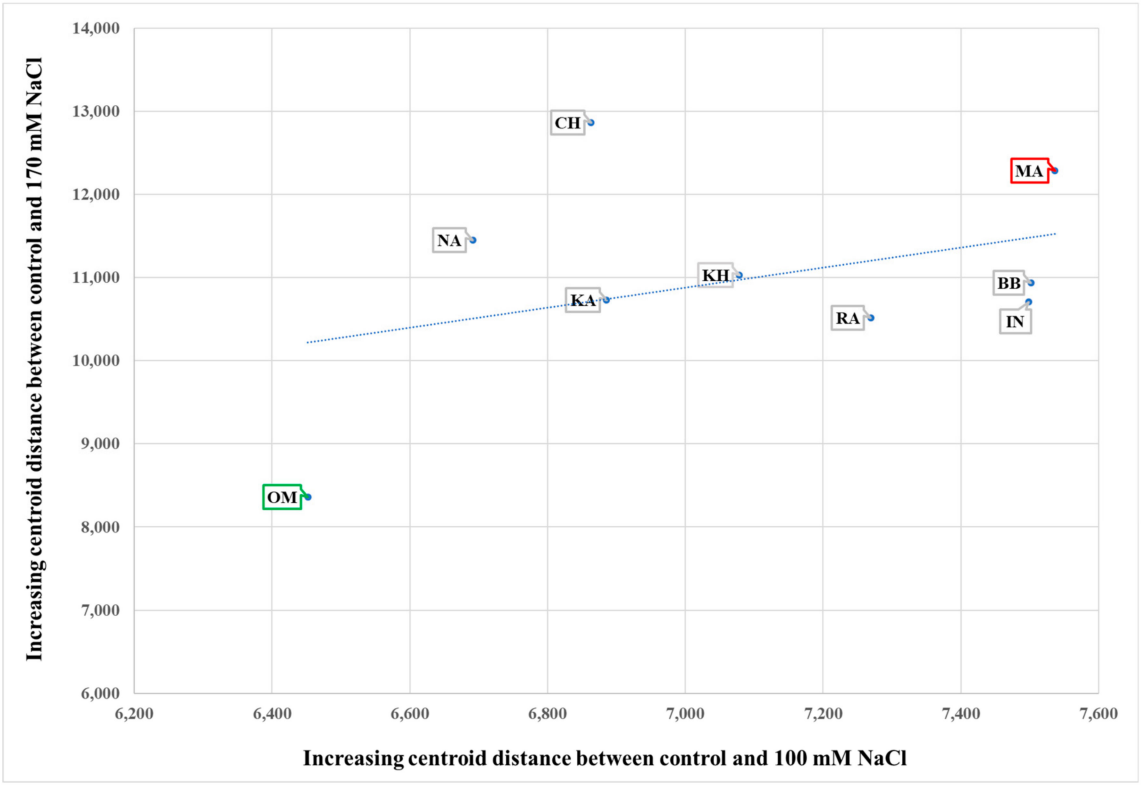

Figure 2. Ranking of genotypes according their resistance to salinity. Resistance to salinity was determined by the centroid distances between control and salt stress treatments (100 and 170 mM of NaCl). The most tolerant genotype Om Rabiaa (OM) is indicated in green font, whereas the most sensitive genotype Mahmoudi (MA) is indicated in red font. The other genotypes ranked between OM and MA are indicated in grey font. Inter-centroid distance = *Square root* [(control score axis 1 − salt score axis 1) ^{2} + (control score axis 2 − salt score axis 2) ^{2}]). The dotted line corresponds to the regression line for the 9 genotypes (linear regression equation: F(x) = 1,2058x + 2,4431; R-squared = 0.1404). Ben Bechir (BB), Chili (CH), INRAT 69 (IN), Karim (KA), Khiar (KH), Mahmoudi (MA), Nasr (NA), Om Rabiaa (OM), Razzak (RA).

3.3. Identification of Marker Traits Representative of a Tolerance to Salinity Stress

Statistical analyses, via the comparison of means, were first performed using the 36 markers traits, the nine genotypes and the three growth conditions (0 mM, 100 mM and 170 mM NaCl) as variables and are presented in Table S2. Then mGLM analyses allowed us to identify 15 traits including CPl, Plδ^{13}C, Plδ^{15}N, SFWSTI, RGDHAm, SGDHAmi, RGDHDea, RGS, SGS, LASTI, SLSTI, SNH_4, NPl, SPro and SSt. Based on the significant effect of the genotype and the two NaCl treatments, these 15 traits were chosen to discriminate between OM and MA with respect to their resistance to the two salt treatments (Table 3). Thus, only genotypes OM and MA were further used to compare the effect of a salt stress on the different phenotypic and physiological traits measured in the study via one-way ANOVA statistical analysis.

Table 3. Pairwise comparison between genotype Om Rabiaa (OM) and Mahmoudi (MA) showing the significant effect of the NaCl treatments. *P*-values were adjusted for the Tukey's multiple comparison test.

Plant Traits	Abbreviations	*P*-value	
		100 mM NaCl	170 mM NaCl
Whole-Plant Total Carbon (mg)	CPl	<0.0001	0.0377
Whole-Plant δ^{13}C (‰)	Plδ^{13}C	0.9986	0.0001
Whole-Plant δ^{15}N (‰)	Plδ^{15}N	0.0134	0.0404
Shoot Fresh Weight Stress Tolerance Index (%)	SFWSTI	<0.0001	0.204
Root Aminating GDH Activity (nmol μg^{-1} protein h^{-1})	RGDHAmi	0.1105	<0.0001
Shoot Aminating GDH Activity (nmol μg^{-1} protein h^{-1})	SGDHAmi	0.9996	0.0028
Root Deaminating GDH Activity (nmol μg^{-1} protein h^{-1})	RGDHDea	<0.0001	<0.0001
Root GS Activity (nmol γ-glutamyl hydroxamate μg^{-1} protein h^{-1})	RGS	0.1229	<0.0001
Shoot GS Activity (nmol γ-glutamyl hydroxamate μg^{-1} protein h^{-1})	SGS	0.4581	0.0035
Leaf Area Stress Tolerance Index (%)	LASTI	<0.0001	0.988
Shoot Length Stress Tolerance Index (%)	SLSTI	0.0037	<0.0001
Shoot Ammonium (nmol ammonium.mg^{-1} dry weight)	SNH_4	<0.0001	0.8938
Whole-Plant Total Nitrogen (mg)	NPl	<0.0001	0.0699
Shoot Proline (nmol proline. mg^{-1} dry weight)	SPro	0.0008	<0.0001
Shoot Starch (nmol equivalent D-glucose.mg^{-1} dry weight)	SSt	<0.0001	0.996

3.4. Impact of the Salt Stress on Traits Related to Growth and Development

Shoot length (SLSTI) was reduced only in MA in the presence of 100 mM (−11%) and 170 mM NaCl (−26%), (Figure 3A). In MA, shoot fresh weight (SFWSTI) was reduced by half or by 76% in the presence of 100 mM NaCl and 170 mM NaCl, respectively (Figure 3B). A two-fold reduction in SFWSTI was also observed in the salt resistant genotype OM only for the highest concentration of NaCl (170 mM). Interestingly, the salt stress led to an increase in the leaf area (LASTI) of OM (81%) only under 100 mM (Figure 3C). The leaf area index decreased in both MA and OM, in the presence of 170 mM NaCl, by 44% and 29%, respectively.

The shoot length stress tolerance index was highly and positively correlated with both the shoot fresh weight stress tolerance index ($r = 0.865$, $p < 0.01$) and the leaf area stress tolerance index ($r = 0.583$, $p < 0.01$) (Table S3).

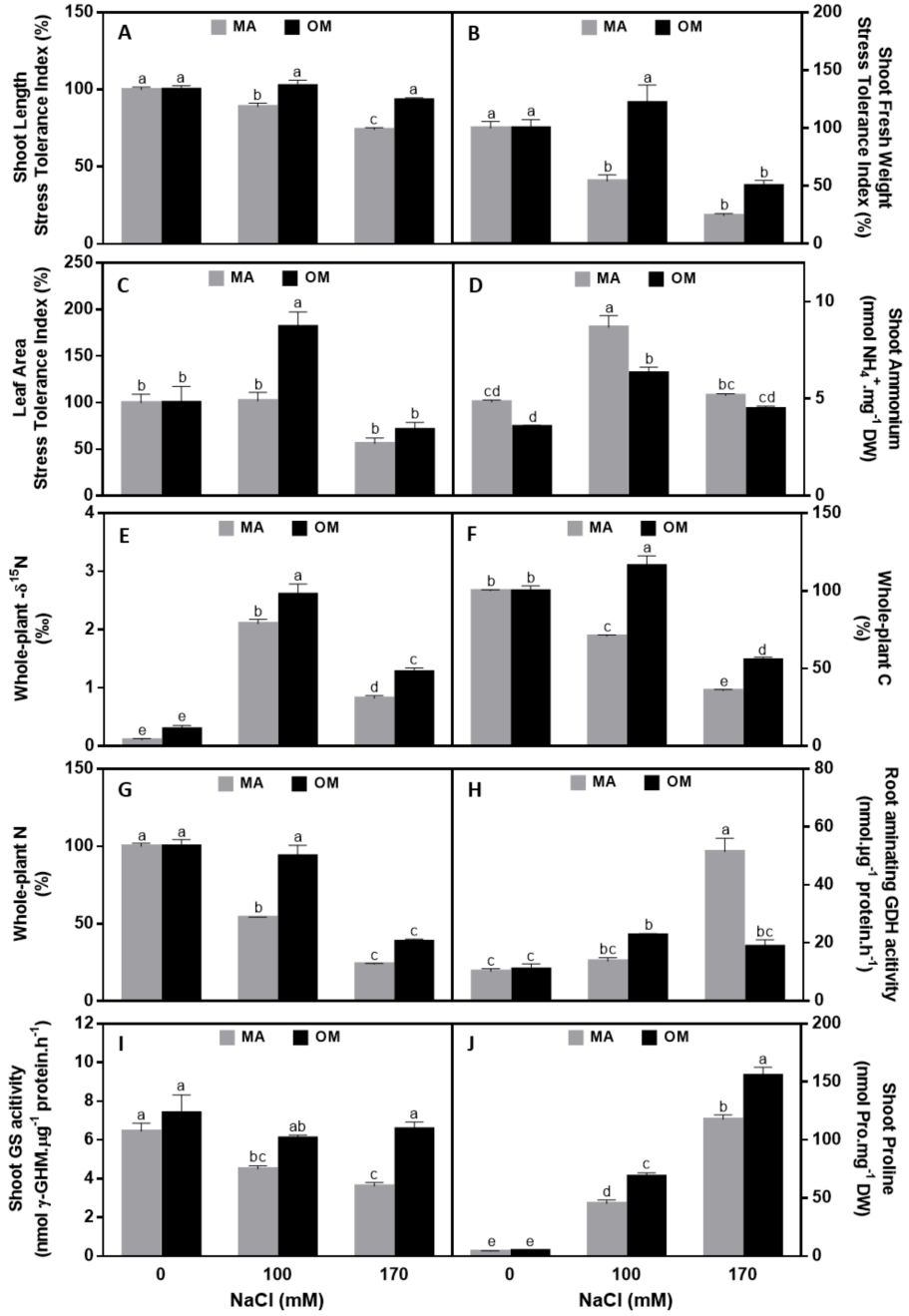

Figure 3. Main traits allowing the discrimination of MA and OM based on resistance to salinity. Grey bars are for MA and black bars for OM. Statistical analysis was performed using one-way analysis of variance (ANOVA). On top of each bar, lowercase letters indicate significant differences between the genotypes in the three salt treatments (Tukey's test at $p < 0.05$). DW: dry weight; γ-GHM: γ-glutamyl hydroxamate; Pro: proline.

3.5. Impact of the Salt Stress on Physiological Traits: Correlation Studies

In the presence of 100 mM NaCl, the shoot starch content (SSt) decreased by 50% in OM and by 18% in MA. A 60% decrease in SSt was also observed when OM and MA were grown in the presence of 170 mM NaCl (Table S2). SSt exhibited a positive correlation with SLSTI ($r = 0.638$, $p < 0.01$) and

with SFWSTI ($r = 0.595$, $p < 0.01$) (Table S3). Accumulation of ammonium (SNH_4) reaching at least 80% was observed in the shoots of OM and MA when 100 mM NaCl was present in the growth medium (Figure 3D).

Under saline stress, whole-plant ^{13}C isotopic abundance ($Pl\delta^{13}C$) was less negative compared to the untreated control plants. The changes in ($Pl\delta^{13}C$) were proportional to the NaCl concentration present in the growth medium (Table S2). In OM, $Pl\delta^{13}C$ increased by 5.40% whereas that of MA was 6.95% higher in the presence of 170 mM NaCl. In contrast, whole-plant ^{15}N-isotopic abundance ($Pl\delta^{15}N$) was markedly affected by the salt treatment, showing more negative values (−2%) in both OM and MA, particularly in the presence of 100 mM NaCl, (Figure 3E).

Only OM was able to maintain its C (CPl) and N (NPl) content at a level comparable to that in the control in the presence of 100 mM NaCl. At the higher concentration of NaCl (170 mM) plant C and N content were decreased by half both in MA and OM (Figure 3F,G). However, the C and N contents of OM remained 20% higher at this salt concentration. Irrespective of the salt treatment, CPl and NPl were highly correlated ($r = 0.964$, $p < 0.01$) and were correlated to a lower extent with SLSTI ($r = 0.655$, $p < 0.01$) and SFWSTI ($r = 0.625$, $p < 0.01$) (Table S3).

When the activity of two marker enzymes involved in ammonia assimilation, and stress resistance was examined, the most clear-cut results was a remarkable 3-fold increase in GDH aminating activity in the roots (RGDHAmi) of MA in the presence of 170 mM NaCl (Figure 3H). A 30–40% decrease in shoot GS activity (SGS) was observed only in MA irrespective of the intensity of the salt stress 100 and 170 mM NaCl (Figure 3I). In contrast, in the roots GS activity (RGS) was 175% higher in OM in the presence of 100 mM NaCl. The corresponding increase was only 98% in OM but was much higher in MA (+329%) when 170 mM NaCl was present in the growth medium (Table S2). RGDHAmi was negatively correlated with CPl ($r = -0.489$, $p < 0.01$) and NPl ($r = -0.524$, $p < 0.01$) (Table S3).

The increase in the shoot proline content (SPro) was proportional to the intensity of the salt stress in both OM and MA reaching a level that was approximately 29 to 34 times higher compared to that of the control (Figure 3J). The increase was slightly higher in OM under the two NaCl treatments. A highly positive correlation (Table S3) was found between SPro and RGDHAmi ($r = 0.627$, $p < 0.01$). Negative correlations were also found between SPro and SLSTI ($r = -0.673$, $p < 0.01$), SFWSTI ($r = -0.690$, $p < 0.01$), CPl ($r = -0.764$, $p < 0.01$) and NPl ($r = -0.816$, $p < 0.01$) (Table S3). The proline content (SPro) and GS activity (SGS) in the shoots were negatively correlated ($r = -0.429$, $p < 0.01$). In contrast, the correlation between the two traits was positive in the roots ($r = 0.223$, $p < 0.01$).

4. Discussion

Because salinity is a major environmental factor limiting plant growth by inhibiting biological processes such as nutrient uptake and assimilation [56], it is necessary to improve our knowledge of the physiological and molecular mechanisms involved, in order to select crops that remain highly productive even if there is an accumulation of NaCl in the soil [57,58]. Previous studies have been conducted to identify durum wheat genotypes that are more tolerant to a salt stress. Shoot length, root length, dry weight and shoot fresh weight, grain yield and expression of genes encoding GS traits were found to be good markers of the response to a salt stress in durum wheat [18,59–62]. A number of the genotypes used in our study have already been examined in field or controlled conditions in order to test their physiological and morphological responses in the presence of excess NaCl [37,63–68]. In the present investigation, a panel of genotypes including those previously tested by other research groups, was used in order to study the impact of a salt tress on a much larger portfolio of phenotypic and physiological traits representative of plant growth and development and of primary C and N metabolism. The aim of our study was to determine whether a larger number of phenotypic and physiological markers could be used to select ancient or modern durum wheat genotypes that are more resistant to salinity at the early stages of plant development.

Using these different traits measured in young developing plants grown under hydroponic conditions, a multivariate screening approach implemented via PCA was first applied in order to

identify genotypes exhibiting contrasting responses to increasing concentrations of NaCl. Such an approach is currently considered to be reliable tool both for cultivar evaluation and breeding [69,70]. Among the nine genotypes used in our study, we identified two genotypes exhibiting the most contrasting responses to salinity stress, we found that the commercial OM genotype was the most resistant, whereas the MA landrace was the most sensitive. Multivariate analysis has previously been performed using only yield-related traits, the shoot K^+/Na^+ ratio, osmotic potential and photosystem II activity to identify genotypes that are more resistant to a salinity stress [70] or a drought stress [69]. The originality of our work lies in the fact that the analysis was conducted in a representative panel of Tunisian genotypes and that screening was performed on the basis of a larger number of traits to identify more robust markers for NaCl resistance, notably by focusing the search on the most tolerant and most sensitive genotypes.

4.1. Traits Related to Growth and Development

Under our experimental conditions, salinity, induced a significant reduction in both shoot fresh weight and shoot length in the nine cultivars selected for the study except in OM grown in the presence of 100 mM NaCl. This result is in line with the finding that plant biomass and plant height are useful traits for screening of bread [71,72] and durum wheat [73] for salt tolerance. Leaf expansion is known to be sensitive to a water stress in many species [74]. Salinity stress also affects cell expansion in young leaves, generally causing a decrease in their area [56]. Genotype OM showed a significant increase in the leaf area index when 100 mM NaCl was present in the growth medium. Moreover, we observed that the shoot length stress tolerance index was positively correlated with that of shoot fresh weight ($r = 0.865$, $p < 0.01$) and the leaf area stress tolerance index ($r = 0.583$, $p < 0.01$). Therefore, shoot fresh weight, shoot length and leaf area can be used to screen durum wheat genotypes under moderate salt stress.

4.2. Traits Related to Carbon Metabolism

In the shoots, the decrease in starch was greater in OM compared to MA. In the presence of 100 mM NaCl, this decrease was accompanied by an increase in the total soluble sugar content. In several studies, it has been shown that the accumulation of soluble sugars is a way to provide energy to counteract the detrimental effects of salinity by increasing the osmotic potential of the cell. Cell turgor necessary for cell expansion to maintain plant growth [24,75]. In MA shoots, the accumulation of soluble sugars could be due to starch hydrolysis rather than a de novo synthesis, as the demand for carbohydrates must be lower, due to the reduced growth of the plant as previously reported by Lee et al. [76] and Hummel et al. [77]. In OM, starch degradation is probably much higher in order to provide C to the roots for an improved osmotic adjustment [28]. One can hypothesize that in OM such an adaptation of the roots to salt stress allows water and nutrient uptake to be maintained for optimal plant growth and development.

C isotope discrimination is one of the most useful techniques for investigating the efficiency of CO_2 assimilation, notably under stress conditions [30,78]. Significant differences in $\delta^{13}C$ were observed between OM and MA in the presence of 170 mM NaCl, and OM exhibited a smaller increase in $\delta^{13}C$ compared to MA. These findings are in agreement with other studies in which an increase in $\delta^{13}C$ was observed in durum wheat cultivars following salinity stress [33,73,79]. The increase in $\delta^{13}C$ mainly due to stomatal closure which restricts the supply of CO_2 to the enzyme Rubisco leading to an increase in the amount of ^{13}C in plant dry matter [33,80,81].

4.3. Traits Related to Nitrogen Metabolism

Soil salinity has a major impact on ammonium assimilation in plants by modifying the GS isoenzyme composition, notably the content of GS1 an enzyme involved in the synthesis of glutamine in the cytosol [82–85]. It has also been proposed that GDH, another enzyme found to be affected by certain physiological conditions such as salinity stress, is able to synthesize glutamate when there is

accumulation of ammonium [83,86,87]. Glutamate, which is one of the substrates of GS, is also an important amino acid that is a precursor for the biosynthesis of proline in plants, an amino acid that accumulates in response to various abiotic stresses [88–90].

In our study, we observed that under 100 mM NaCl, the increase in the shoot ammonium content was higher in MA compared to OM.

In the presence of 100 mM NaCl, the increase in the shoot ammonium content of MA could result from the decrease in GS activity which did not occur in OM. In contrast GDH activity was much higher in the stress-sensitive genotype MA when higher levels of NaCl were present in the nutrient solution. Such a finding is not surprising if we consider that the GS and GDH enzymes are differentially regulated under stress conditions [91]. Therefore, total shoot GS activity appears to be a reliable marker for the selection of salt-tolerant genotypes.

We observed an accumulation of proline in OM proportional to the level of the salinity stress. Proline accumulation in salt-tolerant genotypes has been observed in several plant species including wheat [92], barley [93], mulberry [94], sugar beets [95] and green gram [96]. We confirm that in several durum wheats genotypes the shoot proline content is a key marker representative of the plant tolerance to NaCl. It has been proposed that the preferential proline accumulation in shoots is a way to maintain the level of chlorophyll and the leaf cell turgor in order to maintain the photosynthetic activity of the plant under salinity stress conditions [97,98]. In roots, we observed that there was a positive correlation between GS activity and proline contents, whereas this correlation was negative in shoots. Therefore, it will be interesting to determine whether, in each organ, there is a regulatory mechanism mediated by the enzyme GS controlling the utilization of glutamate for proline production and transport via the vascular tissue [99].

In addition to metabolites and enzyme activities, $\delta^{15}N$ reflects the capacity of a plant to use N and allows monitoring its genotypic variability, as ^{15}N isotopic abundance is a key indicator of the balance between N uptake and N assimilation under stress conditions [30,35,100]. Under the two tested salt stress conditions, shoot $\delta^{15}N$ was more negative in both OM and MA. However, the $\delta^{15}N$ value was more negative in OM when 100 mM NaCl was present in the growth medium. In both durum wheat and barley, decreases in $\delta^{15}N$ have been reported when plants are subjected to salt stress [33,73] reflecting the capacity of the plants to store more N under these conditions. We also found that the salinity-tolerant OM genotype contained more N in the presence of 100 mM NaCl. Thus, we confirm that $\delta^{15}N$ is a key parameter for evaluating salt stress resistance in durum wheat.

5. Conclusions

Durum wheat genotypes exhibiting contrasting morphological and phenotypic responses to increasing concentrations of NaCl were used to identify marker traits representative of a salinity stress. Shoot length, shoot fresh weight, leaf area, whole-plant C, N, ammonium, and proline contents, whole-plant N isotopic composition, and shoot GS activity are key traits that can be used to screen for resistant genotypes at the early stage of plant development.

Supplementary Materials: The following materials are available online at http://www.mdpi.com/2073-4395/9/12/844/s1: Table S1: Eigenvectors of the principal components analysis (PCA). Table S2: Phenotypic and physiological traits measurements in the nine durum wheat genotypes. Table S3: Pearson correlation coefficients between phenotypic and physiological traits. Table S4: Raw Data.

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