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Molecular and Agro-physiological Approaches for Parental Selection before Intercrossing in Salt Tolerance Breeding Programs of Durum Wheat

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ABSTRACT

A set of six Tunisian durum wheat varieties (*Triticum turgidum* L. subsp. durum Desf.) were used for developing and validating an approach to select parental genotypes before intercrossing them for salt tolerance breeding programs. This research was designed to study both molecular genetic diversity and differences in patterns of agro-physiological responses to salt stress. Different agro-physiological measurements were conducted for both the saline treatment (10 g/l NaCl) and the control. The data were converted to salt tolerance indices to allow comparisons among genotypes for salt sensibility. Genetic diversity among these varieties was determined by comparing their molecular traits. A dendrogram of genetic similarity was established using 46 polymorphic markers from eight SSRs and four RAPD primers. The results revealed that the most affected agro-physiological parameters were tiller number, chlorophyll content at 110 and 120 days, shoot dry weight, number of spikes/plant and total grain yield. For these parameters the most affected genotypes were 'Karim' and 'Nasr' and the least affected were 'Maali' and 'Razzek'. The dendrogram discriminated 'Karim' from the remaining analysed varieties. Except for 'Karim', the remaining varieties were tightly clustered in the dendrogram, which reflects a narrow genetic base and low genetic variability among those genotypes. The genetic diversity of Tunisian durum wheat varieties could be enlarged by combining desired traits from different local and wild populations of different geographical origins into the breeding lines. Finally, we conclude that both molecular and agro-physiological approaches are helpful for current and future breeding programs in order to select genetically distinct parents with different salt tolerance components.

Keywords: breeding programs, genetic diversity, molecular markers, RAPD, salt stress, SSR
Abbreviations: AFLP, amplified fragment length polymorphism; **PCR**, polymerase chain reaction; **PIC**, polymorphism information content; **QTL**, quantitative trait locus; **RAPD**, random amplified polymorphic DNA; **SSR**, simple sequence repeat

INTRODUCTION

Adverse environmental conditions impose extreme limitations to growth and development of plants, restricting their genetic potential, reflected by losses in yield (Victoria *et al.* 2011). Salinity is one of the major factors reducing plant growth and productivity worldwide (Flowers *et al.* 1997; Ruan and Teixeira da Silva 2011). Arid and semi-arid Mediterranean regions suffer from an ever increasing area of salt-affected land. In the Mediterranean area, with Tunisia as an example, and where fresh water resources for agricultural use are rather limited, an extension of irrigated agriculture is mainly possible by the use of saline water (Katerji *et al.* 2000). In Tunisia, 30% of the land area faces varying degrees of salinity resulting in low or no crop productivity, especially for durum wheat (*Triticum turgidum* L. subsp. durum Desf.), which is considered to be more salt sensitive than bread wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (Gorham *et al.* 1990; Rawson *et al.* 1988). Therefore, new genetic resources carrying salinity tolerance are needed for crops to be grown on salt-affected land. Improving the salt tolerance of crops requires efficient techniques for the evaluation of their salt-tolerance and genetic diversity.

Previous studies have screened different physiological and morphological characters for salt tolerance in wheat. Some authors studied the effects of NaCl on accumulation of minerals such as Na^+ and K^+ in *T. turgidum*. For example, Hadi *et al.* (2007) reported that durum wheat genotypes grown in tanks containing nutrient solutions died when irrigated with 200-300 mM NaCl and their growth was stunted with 150 mM NaCl. By increasing the K^+ concentration in shoots and decreasing Na^+ uptake or accumulation, they were able to increase salt tolerance. Rascio *et al.* (2001) identified a wheat mutant that accumulated more K^+ in the leaf tissue than the wild-type line. Munns (2003) found that bread wheat is a classical 'salt excluder', characterized by low rates of Na^+ transport to the shoot, thus keeping mesophyll cells as Na^+ free as possible. James *et al.* (2006) identified two genes (*Nax1* and *Nax2*) for Na^+ exclusion and for salt tolerance in durum wheat.

For wheat, some authors used yield in saline versus non-saline conditions as the main criterion for evaluating salt tolerance. Other authors used biomass production at high salinity relative to biomass in non-saline conditions (Kingsbury and Epstein 1984; Martin *et al.* 1994). This may not prove to be an effective method, since the efficacy of using a single complex parameter such as yield or biomass

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production as a criterion for selecting other complex traits such as salinity tolerance is clearly low. Compared with these conventional techniques that score and rank salt-tolerant genotypes based on single parameter, some success has already been achieved by using multiple agro-physiological parameters (Zeng *et al.* 2002; El-Hendawy *et al.* 2005; Chaabane *et al.* 2011). Such an evaluation procedure may facilitate the improvement of salt tolerance of tested genotypes in bread or durum wheat breeding programs.

Houshmand *et al.* (2005) reported *in vitro* screening to be more successful than field screening when searching for salt-tolerant durum wheat genotypes.

In breeding populations the potential responses from selection lie in genetic diversity (Rodríguez *et al.* 1999) and the assessment of genetic diversity of available germplasm is a crucial aspect of breeding for maximizing genetic improvement (Sudaric *et al.* 2008). To evaluate genetic diversity, there have been advances through morphological and physiological assessment, although further progress is needed to improve accuracy. Molecular markers have improved and facilitated our ability to characterize genetic variability. These markers, which directly assess polymorphism at the DNA level, are now frequently used in the analysis of genetic resources in plant breeding programs (Santoni *et al.* 2000). The first advantage of molecular techniques is their capacity to detect genetic diversity at a higher level of resolution than other methods. Furthermore, DNA-based assays are reliable, efficacious, rapid, and may be obtained from little amounts of plant material at any stage of development and are not affected by environmental conditions. Molecular characterization of germplasm prior to crosses of parental lines can increase genetic diversity among parental genotypes, maximize genetic variation present in breeding populations, and minimize the efforts in screening, for either direct selection in traditional breeding or indirect selection through quantitative trait loci, or QTLs (Zeng *et al.* 2004). The most widely used molecular markers in wheat are microsatellites or simple sequence repeats (SSRs), amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) (Najimi *et al.* 2003). SSR and RAPD are relatively simple, less expensive and reliable. The main reason for the success of RAPD analysis is the large number of genetic markers that require little DNA without the need for cloning, sequencing or any other form of molecular characterization of the genome of the species in question (Fevzi *et al.* 2001). SSRs have many desirable characteristics and are increasingly used for studies of genetic diversity in crops (Jernej *et al.* 2001).

The objectives of the present research were to develop and validate screening techniques for salt tolerance and selecting agro-physiological traits for conferring salinity tolerance to the main Tunisian varieties of durum wheat. This is useful for selecting salt-tolerant progeny in which agro-physiological characters for salinity tolerance should be introduced. In these progeny, genetic diversity should be enough to maintain the survival and proliferation of this population, despite selection pressures. Therefore, we propose a new approach to select salt-tolerant progeny by combining two methods: screening salt tolerance through agro-physiological traits and studying genetic diversity using molecular markers.

This study aimed to (i) compare the behaviour of the principle Tunisian durum wheat varieties under saline stress and selecting for agro-physiological traits related to salt tolerance; (ii) investigate the genetic diversity of these varieties using molecular markers; (iii) Evaluate the usefulness of combining these two approaches for selecting salt-tolerant progeny in which agro-physiological characters for salinity tolerance should be introduced.

MATERIALS AND METHODS

Six Tunisian durum wheat varieties ('Karim', 'Khiar', 'Maali', 'Nasr', 'Om Rabiaa', 'Razzek') were used for this study. These varieties were released by the National Agronomic Research Institute of Tunisia (INRAT).

Behaviour of plants under salt stress

The 6 varieties were grown under semi-controlled conditions during the 2009/2010 growing season in pots (4 plants/pot) filled by a loamy sand soil collected from the soil surface (0–15 cm) at the Ariana Experimental Station of INRAT. The soil was air-dried, ground, passed through a 5-mm mesh screen, and thoroughly mixed. The experiment was conducted in triplicate with a completely randomised design. In our previous study (Ben Naceur *et al.* 2001) it was shown that 10 g/l NaCl (171 mM) is the optimal concentration that significantly affects the majority of agro-physiological characters in durum wheat. Similarly, El-Hendawy *et al*. (2011) reported that variations in salt tolerance indices among spring wheat (*Triticum aestivum* L.) genotypes were reduced at low salinity (40 and 80 mM NaCl) than at high salinity (160 mM NaCl). This suggests that the selection criteria can be considered appropriate for screening wheat genotypes only when they are measured under high salinity. Therefore, two treatments were used, a saline treatment (10 g/l NaCl) and a control (no NaCl). The salinity treatment was initiated at the four-leaf stage. Agro-physiological measurements were conducted at different growth stages (60, 80, 100, 110, 120 days after sowing (DAS) and final harvest). The data were also converted to a salt tolerance index (STI) to allow comparisons among genotypes for salt sensitivity. STI was defined as the observation at salinity divided by the average of the controls (Zeng *et al*. 2002 quoted by El-Hendawy *et al.* 2005). Ranking numbers were assigned to each genotype for each parameter based on STI means, and were used to score genotypes. For each parameter, the genotypes were classified according to their corresponding STI from highest to lowest. Then, for each genotype and for each parameter, a score from 1 to 6 was assigned according to the position in the classification. For each agro-physiological parameter the total score is the sum of the different scores.

Chlorophyll (Chl) content of the flag leaves was measured at 60, 80, 100, 110 and 120 DAS. Three different measurements were performed at the base, the center and apex of the leaf using a portable Minolta SPAD 502 Meter. In this protocol the rate of Chl was estimated per unit SPAD. The height of the main shoot of each plant was measured with a ruler at 50, 60, 70, 80 and 90 DAS. Tiller number was recorded at 120 DAS. After harvesting, shoots were oven-dried at 70°C for 48 h to determine the dry weight (DW). The number of spikes/plant, the number of spikelets/spike, the grain number, the grain weight/spike and the 1000-grain weight were also determined at final harvest (130 DAS). Analysis of variance (ANOVA) (**Tables 3, 4**) was performed using Statistica 5.0 v. '98 Edition.

Genetic diversity analysis

1. DNA extraction

Total DNA was extracted from young leaves of a single plant per genotype. The extraction buffer (pH 8) was composed of 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 1.44 mM NaCl, 3% CTAB (w/v), 1% β-mercaptoethanol (v/v). All reagents were from Sigma-Aldrich (St. Louis, USA). DNA was purified by a treatment with RNase (10 mg/ml, Fermentas) at a final concentration of 10 µg/ml followed by a phenolic extraction (treatment with an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v/v), followed by treatment with an equal volume of chloroform: isoamyl alcohol (24: 1, v/v). DNA concentration was quantified by spectro-photometry and quality was checked by gel electrophoresis. The average DNA yield was 15 µg DNA/g of tissue.

2. SSR analysis

Twelve SSR primer pairs were selected on the basis of their chromosomal location. Their names and sequences are listed in **Table 1**. PCR reactions were carried out in a 25-µl reaction volume containing 1 U of *Taq* Polymerase, 50-100 ng of template DNA, 0.25 µM of each primer, 0.2 mM of each dNTP, 2 mM of

$MgCl_2$ and 1X PCR reaction buffer. Amplifications were performed in a DNA thermocycler (Biometra Thermocycler, Goettingen, Germany) programmed for one cycle of 95°C for 3 min and 35 consecutive cycles of [1 min denaturing at 94°C, 1 min annealing at 55°C and 2 min extension at 72°C] followed by 10 min at 72°C. Amplified PCR products were separated by electrophoresis using a 3% agarose 1X TBE gel, stained with 0.5 mg/ml ethidium bromide and visualized under UV light and photographed by a Gel Documentation System (GDS). A 100-bp DNA ladder (Promega, Ariana, Tunisia) was used as the molecular size standard.

3. RAPD analysis

A total of 12 10-mer random oligonucleotide primers were selected according to the number and consistency of amplified fragments (**Table 1**). PCR reactions were carried out in a 25-µl reaction volume containing 1 U of *Taq* Polymerase, 20 ng of template DNA, 0.2 µM of primer, 1 mM of each dNTP, 2 mM of $MgCl_2$ and 1X PCR reaction buffer. Amplifications were performed in a Biometra DNA thermocycler programmed for one cycle of 95°C for 3 min and 45 consecutive cycles each consisting of 30 sec at 94°C, 1 min at 37°C and 72°C for 2 min, followed by 10 min at 72°C. The amplification products were subjected to electrophoresis in 2% agarose gels in TBE buffer running at 150 V for 2 h. The gels were stained with ethidium bromide (1 µg/ml final concentration), viewed under UV light and photographed by the same GDS as used for SSR primer analysis. A 1-Kb DNA ladder (Promega) was used as the molecular size standard.

4. Cluster analysis

Amplified fragments were classified as present (represented by 1) or absent (represented by 0) for both RAPD and SSR analyses. A data matrix was prepared for the analysis. A pair-wise similarity matrix (**Table 2**) was generated with the software NTSYSpc-2.02j (NTSYS-Numerical Taxonomy and Multivariate Analysis; Rohlf 1998) using a simple matching coefficient (Sokal *et al.* 1958). A dendrogram was then constructed based on the similarity matrix data using UPGMA (Unweighted Pair-Group Method using Arithmetic Averages) cluster analysis of NTSYSpc-2.02j. Bootstrap analysis (Felsenstein 1985) was also carried out using the TREECON 1.3B program (Van de Peer *et al.* 1994). SSR data was bootstrapped by resampling 2000 times.

5. Degree of polymorphism

The data matrix was used to compute the diversity for each SSR and RAPD marker. This is equivalent to the polymorphism information content (PIC) at each SSR as described in the following equation by Anderson (1992):

$$PIC = 1 - \sum_{j=1}^{n} P_{ij}^2$$

where P_{ij} is the frequency of the j^{th} SSR pattern for marker i and the summation covers n patterns.

RESULTS

Polymorphism of SSR markers

Among the 12 SSR markers used in this study only 8 (WMC50, WMC16, WMC283, WMC48, WMC24, WMC14, WMC21, WMC27) generated clear polymorphic bands (**Fig. 1**), while two (WMC17, WMC23) generated a monomorphic profile and another two (WMC19, WMC22) produced ambiguous patterns (**Table 1**). The 8 polymorphic SSR markers revealed a total of 25 bands, 18 of which were polymorphic. The PIC/marker among the tested genotypes ranged from 0.27 to 0.67 with an average of 0.42 (**Table 1**).

Polymorphism of RAPD markers

Among the 12 RAPD markers used in this study, 4 (OPB-03, OPC-07, OPA-05, OPP-13) generated clear reproducible polymorphic bands (**Fig. 2**) and the remaining primers generated monomorphic profiles (**Table 1**). The 4 polymorphic RAPD markers revealed a total of 21 bands among which 19 were polymorphic. The PIC/marker among the tested genotypes ranged from 0.41 to 0.89 with an average

Table 1 PCR primers, their sequences and PIC values.

PCR primers	Sequences 5′-3′	PIC
WMC14F	ACC CgT CAC Cgg TTT ATg gAT g	0.50
WMC14R	TCC ACT TCA AgA Tgg Agg gCA g	
WMC16F	ACC gCC TgC ATT CTC ATC TAC A	0.28
WMC16R	gTg gCg CCA Tgg TAg AgA TTT g	
WMC17F	ACC TgC AAg AAA TTA ggA ACT C	--
WMC17R	CTA gTg TTT CAA ATA TgT Cgg A	
WMC19F	CTg ACA TgC ggC ATT CAC TTC C	--
WMC19R	Agg CTT AgA ACA CAC CgA CAC g	
WMC21F	CgC TgC CgT gTA ACT CAA AAT C	0.44
WMC21R	AgT TAA TTg ggC gCT CCA AgA A	
WMC22R	ATC ATT ggT TTC CTC TTC ACT T	--
WMC22R	gTg gAC TAT TTA ACA TCT TCA T	
WMC23F	ATT CgC TCA TAC gAT Agg gTT g	--
WMC23R	AgA ggC Tgg TgT AgT Tgg TTT g	
WMC24F	gTg AgC AAT TTT gAT TAT ACT g	0.27
WMC24R	TAC CCT gAT gCT gTA ATA TgT g	
WMC27F	AAT AgA AAC Agg TCA CCA TCC g	0.27
WMC27R	TAg AgC Tgg AgT Agg gCC AAA g	
WMC283F	CgT Tgg CTg ggT TAT ATC ATC T	0.67
WMC283R	gAC CCg CgT gTA AgT gAT Agg A	
WMC48F	gAg ggT TCT gAA ATg TTT TgC C	0.67
WMC48R	ACg TgC TAg ggA ggT ATC TTg C	
WMC50F	CTg CCg TCA ggC CAg gCT CAC A	0.28
WMC50R	CAA CCA gCT AgC TgC CgC CgA A	
OPA-05	AggggTCTTg	0.41
OPB-03	CATCCCCCTg	0.89
OPB-12	CCTTgACgCA	--
OPC-07	gTCCCgACgA	0.61
OPD-11	AgCgCCATTg	--
OPE-12	TTATCgCCCC	--
OPE-17	CTACTgCCgT	--
OPI-16	TCTCCgCCTT	--
OPK-07	AgCgAgCAAg	--
OPL-02	TgggCgTCAA	--
OPL-04	gATgCACAC	--
OPP- 13	ggAgTgCCTC	0.5

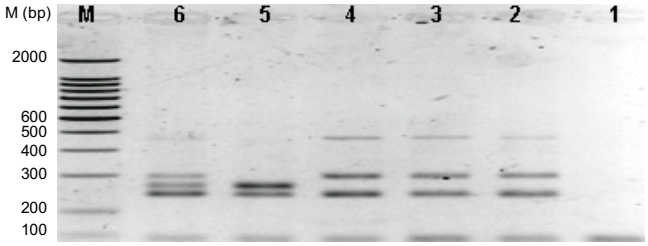

Fig. 1 Agarose gel showing the allelic segregation of the WMC48 SSR marker in six analysed varieties. Var. number: 1: Karim, 2: Khiar, 3: Maali, 4: Nasr, 5: Om Rabia, 6: Razzak. M: Molecular size standard (100 bp DNA ladder).

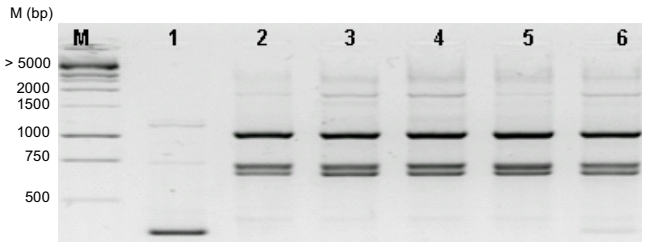

Fig. 2 Agarose gel showing the allelic segregation of the OPP13, RAPD marker in six analysed varieties. Var. number: 1: Karim, 2: Khiar, 3: Maali, 4: Nasr, 5: Om Rabia, 6: Razzak. M: Molecular size standard (1Kb DNA ladder).

Table 2 Similarity matrix among the analysed wheat genotypes.

	Karim	Khiar	Maali	Nasr	Om Rabiaa	Razzek
Karim	1.00					
Khiar	0.37	1.00				
Maali	0.35	0.85	1.00			
Nasr	0.35	0.85	0.87	1.00		
Om Rabiaa	0.35	0.80	0.78	0.83	1.00	
Razzek	0.35	0.80	0.83	0.78	0.78	1.00

of 0.6 (**Table 1**).

Cluster analysis

The 8 SRR and 4 RAPD markers were used to characterize and evaluate the genetic diversity of these varieties. A total of 46 alleles were detected and used to compute the genetic diversity among all of the analysed verities. The similarity matrix (**Table 2**) showed that the most closely related cultivars were 'Nasr' and 'Maali' with a highest similarity coefficient of 0.87. 'Karim' was the most distantly related variety with a low similarity coefficient of 0.35.

The dendrogram (**Fig. 3**) computed with the DNA marker similarity coefficients based on SSR and RAPD marker data showed four nodes. The bootstrap values reported on the dendrogram (**Fig. 3**) showed two nodes with bootstrap values of 100%, one node of 38% and one of 45%. Using a cut-off at a genetic similarity of 0.8, the dendrogram (**Fig. 3**) discriminates 'Karim' from the remainder of the varieties, all of which were grouped into one cluster. Using a cut-off at a genetic similarity of 0.85 the dendrogram discriminated 'Karim', 'Razzek' and 'Om-Rabiaa' from the rest of the varieties, which formed one sub-cluster.

Agro-physiological characterisation

Salinity affected all of the considered parameters at different growth stages. The tiller number and Chl contents of plants in the salinity treatment varied significantly compared to the control (**Table 3**).

Mean tiller number for all varieties in the salinity treatment was reduced by 27.46% compared with the control. The STIs of tiller number (**Table 4**) ranged from 0.6 ('Nasr') to 0.88 ('Razzek'). For tiller number, 'Nasr' was most affected by salinity and 'Razzek' was least affected. Salinity decreased tiller number by 40% for 'Nasr' and by 12.5% for 'Razzek' compared with the control. The average Chl content of flag leaves varied over time. The Chl content increased slowly at early vegetative stages reaching a maximum at advanced stages. In salinity treatments the average Chl content of the 6 varieties increased by 2.82, 4.24 and 6.47% at 60, 80 and 100 DAS. At 110 and 120 DAS the average Chl content of the 6 varieties decreased by 39.06 and 55.85%. This reveals that senescence was enhanced by salinity.

The average STIs (**Table 4**) of Chl content for all varieties ranged from 0.96 to 1.1, from 1.00 to 1.15, from 1.05 to 1.14, from 0.34 to 0.81 and from 0.19 to 0.63 at 60, 80, 100, 110 and 120 DAS, respectively. Plant height was least affected by salinity.

At final harvest the various yield components showed different responses to salinity. Different parameters (shoot DW, number of spikes/plant, 1000-grain weight and total grain yield) decreased significantly compared to the control (**Table 5**). The shoot DW was reduced by 29.51%, the number of spikes/plant by 24.35%, the 1000-grain weight by 7.23% and total grain yield by 32.01% compared with the control treatment. However, some yield components (spikelets/spike, grains/spike) were much less affected by salinity.

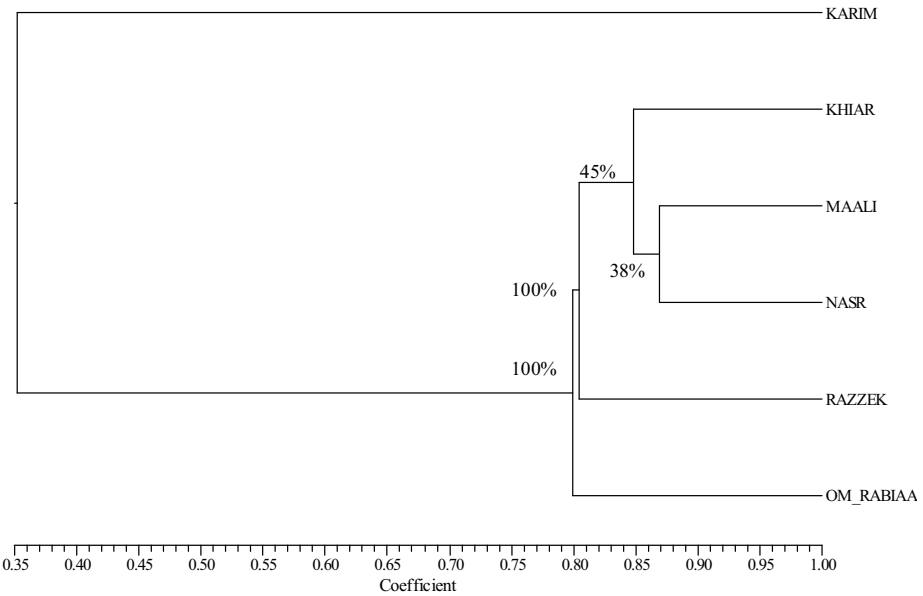

Fig. 3 Dendrogram showing similarity and clustering of six Tunisian durum wheat varieties.

Table 3 Variance analysis of tiller number and chlorophyll content.

Source of variation	d.f	MS	Fcal	P	MS	Fcal	P	MS	Fcal	P
		Tiller number			Chlorophyll content 60 DAS			Chlorophyll content 80 DAS		
Genotypes (Gen)	5	1.58	2.36	0.07	26.49	6.78	0.000***	21.87	5.38	0.001**
Treatments (Treat)	1	9	13.5	0.001**	13.72	3.51	0.07	42.37	10.42	0.003**
Gen x Treatments	5	0.8	1.2	0.34	6.40	1.64	0.18	5.50	1.35	0.27
Error	24	0.66			3.90			4.06		
		Chlorophyll content 100 DAS			Chlorophyll content 110 DAS			Chlorophyll content 120 DAS		
Genotypes (Gen)	5	48.99	9.26	0.000***	212.68	4.97	0.002**	187.65	3.52	0.015*
Treatments (Treat)	1	129.70	24.51	0.000***	1357.71	31.73	0.000***	2489.08	46.76	0.000***
Genx Treat	5	2.69	0.51	0.767	35.71	0.83	0.53	5.81	0.10	0.989
Error	24	5.29			42.78			53.22		

*,**,*** significant at 0.05, 0.01 and 0.001 levels, respectively by the *F*-test.

Table 4 Salt tolerance indices of different measured parameters in wheat genotypes at different growing stages.

Variety	Chl day 60	Chl day 80	Chl day 100	Chl day 110	Chl day 120	Tiller number	Final Shoot weight	Spikelets/ spike	1000-grain weight	Grain yield	Grains/ spike	Spikes/ plant
Karim	1.01	1.00	1.07	0.47	0.31	0.68	0.70	0.97	0.85	0.64	1.00	0.74
Khiar	1.07	1.07	1.07	0.58	0.39	0.74	0.70	0.97	0.89	0.68	0.92	0.77
Maali	1.04	1.06	1.12	0.69	0.49	0.81	0.74	1.00	0.90	0.72	0.98	0.81
Nasr	1.01	1.15	1.14	0.34	0.19	0.60	0.68	1.06	0.97	0.71	1.06	0.65
Om Rabiaa	0.96	1.06	1.11	0.81	0.41	0.72	0.66	0.97	0.93	0.67	0.97	0.75
Razzek	1.10	1.00	1.05	0.81	0.63	0.88	0.71	0.96	0.89	0.67	0.89	0.88

Chl = chlorophyll content

Table 5 Variance analysis of shoot dry weight. number of spikes/plant. grain yield and 1000-grain weight.

Source of variation	d.f	Shoot dry weight			Spikes/plant			Grain yield			1000-grain weight		
		MS	Fcal	P	MS	Fcal	P	MS	Fcal	P	MS	Fcal	P
Genotypes (Gen)	5	2.97	1.90	0.13	0.78	3.77	0.011*	1.30	2.31	0.07	177.56	8.29	0.00***
Treatments (Treat)	1	103.41	66.34	0.00***	3.83	18.56	0.00***	28.44	50.71	0.00***	221.67	10.35	0.003**
Gen x Treat	5	0.15	0.09	0.99	0.15	0.71	0.618	0.08	0.14	0.98	8.48	0.39	0.84
Error	24	1.56			0.21			0.56			21.40		

*,**,*** /significant at 0.05, 0.01 and 0.001 levels, respectively by the *F*-test.

Table 6 Rankings of genotypes for their relative salt tolerance (1 = high tolerance; 6 = low tolerance) in terms of agro-physiological parameters.

Variety	Chl (110 DAS)	Chl (120 DAS)	Tiller number	Spikes/plant	Grain yield	Shoot weight	Total score
Karim	5	5	5	5	6	3	29
Khiar	4	4	3	3	3	4	21
Maali	3	2	2	2	1	1	11
Nasr	6	6	6	6	2	5	31
Razzerk	1	1	1	1	4	2	10
Om Rabiaa	2	3	4	4	5	6	24

The number of spikelets/spike was reduced by 1.19% and the number of grains/spike was reduced by 3.51% compared with the control treatment. The number of spikelets/ spike and number of grains/spike were least sensitive to salinity whereas the number of spikes/plant and total grain yield were the most affected yield components.

The STIs (**Table 4**) of final harvest parameters varied among genotypes. STIs ranged from 0.66 ('Om Rabiaa') to 0.74 ('Maali') for shoot DW, from 0.64 ('Karim') to 0.72 ('Maali') for grain yield, from 0.85 ('Karim') to 0.97 ('Nasr') for 1000-grain weight, from 0.65 ('Nasr') to 0.88 ('Razzek') for number of spikes/plant, from 0.96 ('Razzek') to 1.06 ('Nasr') for number of spikelets/spike, and from 0.89 ('Razzek') to 1.06 ('Nasr') for number of grains/spike. 'Maali' was the most productive (i.e. yield) variety (4.16 g/plant), followed by 'Khiar' (3.99 g/plant), 'Razzek' (3.87 g/plant), 'Nasr' (3.83 g/plant), and 'Karim' (3.66). The least productive variety under salt stress was 'Om Rabiaa' (3 g/plant).

DISCUSSION

The various agro-physiological parameters showed different responses to salinity. At the vegetative growth stage, tiller number and Chl content at 110 and 120 DAS were the parameters most affected by salinity, whereas plant height was least affected. However, several other research studies (Royo *et al*. 2003; Dashti *et al*. 2010; Bai *et al*. 2011; Hossain *et al*. 2012a, 2012b) have shown that plant height was significantly affected by salinity.

At harvest, the shoot DW, the number of spikes/plant and the total grain yield were the parameters most affected by salinity whereas the number of spikelets/spike and number of grains/spike were least affected. The most affected agro-physiological parameters (i.e. tiller number; Chl content at 110 and 120 days; shoot DW; number of spikes/plant and total grain yield) can be used as simple and non-destructive measurements to target wheat genotypes in breeding programs for genetic improvement of the analysed varieties. For these parameters the varieties showed different reactions to salt stress and were classified into three groups (**Table 6**): 'Maali' and 'Razzek' ranked as the least affected varieties (total score 10-11); 'Khiar' and 'Om Rabiaa' ranked as moderately affected varieties (total score 21-24); 'Karim' and 'Nasr' ranked as most affected varieties (total score 29-31).

In a previous study (Chaabane *et al*. 2011), it was shown that the sensitivity of grain yield to salt stress is highly correlated with the sensitivity of shoot DW, and number of tillers and spikelets/spike. Salt tolerance index of grain yield showed a very highly significant positive correlation ($r = 0.90$, $P < 0.001$) with STI of shoot dry weight and a highly correlation ($r = 0.43$, $P < 0.05$) with tiller number and spikelets/spike. In other words, these three parameters contributed most of the variation to grain yield among the parameters investigated. These parameters could be used as simple, non-destructive and rapid criteria to screen for salt tolerance. However, salt tolerance at early growth stages does not always correlate with that at ensuing growth stages (Zeng *et al.* 2002; El-Hendawy *et al*. 2011). Screening for salt tolerance should be done by studying and combining the maximum values of significantly salt affected agro-physiological parameters evaluated at different growth stages.

Except for 'Karim', the remainder of the varieties were tightly clustered in the dendrogram (**Fig. 3**), which reflects a narrow genetic base and low genetic variability among the principle Tunisian varieties. Therefore, the genetic diversity of Tunisian durum wheat varieties should be broadened by combining desired traits from different local and wild populations of different geographical origins into the breeding lines. Increased genetic diversity will undoubtedly increase the probability of identifying desirable recombinant genotypes during screening for salt tolerance (Zeng *et al.* 2004). Some authors (El-Maghraby *et al.* 2005; Sudaric *et al.* 2008) reported that crosses between more diverse elite parents resulted in a population with greater genetic variation for important agronomic traits than crosses between similar parents.

In the dendrogram (**Fig. 3**), 'Khiar', 'Maali' and 'Nasr' were grouped into the same SSR cluster, indicating that the genetic backgrounds of these genotypes are similar. However, differential responses to salt stress were observed among these tightly clustered genotypes. The varieties used in this study could be characterized by 8 SSR and 4 RAPD loci. These loci were selected randomly on the durum wheat genome and most likely do not include all salt tolerance genes. Therefore, it is obvious to have some sensitive geno-

types mixing with tolerant ones in the same cluster. To have more agreement between the two screening methods (molecular and phenotypical) under salt stress, an attempt can be made to use previously identified molecular markers associated with salt tolerance QTLs, as those identified by James *et al.* (2006). These authors identified two novel genes for excluding Na^+ from leaf blades, named *Nax1* and *Nax2*. The use of such markers for studying genetic diversity has been demonstrated as a good method in rice (Mohammadi-Nejad *et al.* 2008) and will be useful for both genetic diversity studies of germplasm with different levels of tolerance to saline stress and for testing the usefulness of the identified QTLs for marker-assisted selection. These QTLs should enhance the recovery rate of the isogenic recurrent genome after hybridization and facilitate the introgression of QTLs necessary to increase stress tolerance in breeding programs (Hussain *et al*. 2010).

Salt tolerance breeding programs can be achieved by selecting parental genotypes before intercrossing based on their genetic distances estimated by variations in molecular markers. The identification of the genetic distances among breeding lines or cultivars will be important to maximize their use in breeding for salt tolerance (Zeng *et al.* 2004). Intercrossing genetically distant genotypes maximizes genetic variation present in breeding populations, which is essential for genetic improving programs for multigenic characters like salt tolerance in durum wheat. For such programs, closely related varieties like 'Khiar', 'Maali' and 'Nasr' should not be intercrossed because this limits the success of selection in the segregating population. However, 'Karim' could be improved for salt tolerance by intercrossing with the 'Maali' or 'Razzek' because of the considerable genetic distance and the significant differences in physiological characters under saline stress making 'Karim' distinct from 'Maali' or 'Razzek'.

As mentioned above the most affected genotypes by salinity are 'Nasr' and 'Karim'. Unlike 'Karim', 'Nasr' could not be improved by intercrossing with the least affected varieties ('Maali' and 'Razzek') because of the small genetic distance separating 'Nasr' and these varieties.

Finally, in combinations with more genetically distant parents most of the target traits could be combined in the derived lines and breeding aims could be achieved.

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