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*in*

Porceddu E. (ed.), Damania A.B. (ed.), Qualset C.O. (ed.).  
Proceedings of the International Symposium on Genetics and breeding of durum wheat

**Bari : CIHEAM**

**Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 110**

**2014**

pages 371-377

Article available on line / Article disponible en ligne à l'adresse :

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To cite this article / Pour citer cet article

Chaabane R., Saidi A., Rouissi M., Ben Naceur E., Mejri C., Ben Naceur M. **Avenues for increasing salt tolerance of Tunisian durum wheat cultivars.** In : Porceddu E. (ed.), Damania A.B. (ed.), Qualset C.O. (ed.). *Proceedings of the International Symposium on Genetics and breeding of durum wheat.* Bari : CIHEAM, 2014. p. 371-377 (Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 110)



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# Avenues for increasing salt tolerance of Tunisian durum wheat cultivars

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**Abstract.** Salt-tolerant durum wheat cultivars offer great potential to grow them in marginal lands. To develop high-yielding, salt-tolerant cultivars for the various salt-affected areas of Tunisia we adopt two approaches. The first approach is the study of genetic variability for salt tolerance within Tunisian varieties using agro-physiological traits. We studied numerous agro-physiological traits but only few of them change very significantly and contribute to the salt tolerance mechanism. This allowed us to choose reliable screening traits and criteria useful for germplasm breeding programmes. The second approach is the introgression of *Nax* genes into elite Tunisian varieties by marker-assisted backcrossing (MAB). These *Nax* genes reduce leaf  $[Na^+]$  and increase durum wheat grain yield on saline soils.

**Keywords.** Durum wheat – Salt tolerance – Marginal land – *Nax* genes – Agro-physiological traits.

## ***Pistes pour augmenter la tolérance à la salinité des cultivars de blé dur tunisien***

**Résumé.** Les cultivars de blé dur tolérants au sel offrent un grand potentiel pour la culture sur des terres marginales. Afin de développer des cultivars à haut rendement, tolérants au sel pour les différentes régions de la Tunisie affectées par la salinité, nous avons adopté deux approches. La première approche est l'étude de la variabilité génétique pour la tolérance au sel chez des variétés tunisiennes sur la base des caractères agro-physiologiques. Nous avons étudié de nombreux caractères agro-physiologiques, mais nous avons observé que quelques-uns seulement varient de façon très significative et contribuent au mécanisme de la tolérance au sel. Cela nous a permis de choisir des caractères de sélection fiables et des critères utiles pour les programmes d'amélioration du matériel génétique. La seconde approche est l'introgression des gènes *Nax* dans les variétés élités tunisiennes par rétrocroisement assisté par marqueurs (MAB). Ces gènes *Nax* réduisent le  $[Na^+]$  de la feuille et augmentent le rendement en grain du blé dur sur des sols salins.

**Mots-clés.** Blé dur – Tolérance au sel – Terre marginale – Gènes *Nax* – Caractères agro-physiologiques.

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## **I – Introduction**

More than 3/4 of the Tunisian's arable land is located in arid or semi-arid areas. A significant proportion of these lands are affected by either drought and/or salinity. These areas are generally cultivated with cereals and results in lower yield when grown in salt-affected soils. Comparing to other cereals, the high price of durum wheat enhances farmers to cultivate more durum wheat than bread wheat or other cereals. Increase in salt tolerance of durum wheat is needed to sustain agriculture in these areas. Salt tolerance of wheat is known to change with growth stage. There are three avenues by which to introduce salt tolerance into durum wheat: traditional breeding techniques using physiologically-based phenotyping, marker-assisted selection, and through transformation of genes known to improve  $Na^+$  exclusion or tissue tolerance (Lindsay *et al.* 2004). Compared with conventional techniques that score and rank salt tolerance genotypes based on single trait, some success has already been realized by using multiple agronomic traits simultaneously at different growth stages (Zeng *et al.*, 2002). Identifying the multiple traits associated with salt tolerance during different growth stages is important for evaluating wheat genotypes and improving their salt tolerance (El-Hendawy *et al.*, 2005). Salt tolerance in wheat and many other species is associated with the ability to exclude  $Na^+$  so that high  $Na^+$

concentrations do not occur in leaves, particularly in the leaf blade (Munns, 2005). Durum wheat (*Triticum turgidum* L. subsp. *durum* [Desf.]) is particularly sensitive to salinity and has higher rates of Na<sup>+</sup> accumulation and poor K<sup>+</sup>/Na<sup>+</sup> discrimination and is less salt tolerant than bread wheat (Munns et al., 2006). A durum wheat Line 149, resulted from a cross between an old durum cultivar (Marrocos) and a *Triticum monococcum* accession was selected as having exceptionally low rates of Na<sup>+</sup> accumulation in leaves (Munns et al., 2000). The low Na<sup>+</sup> phenotype was found to be controlled by two dominant interacting genes of major effect (Munns et al., 2003). These genes, named *Nax1* and *Nax2*, enhance removal of Na<sup>+</sup> from the xylem, leading to low Na<sup>+</sup> concentrations in leaves (James et al. 2006). *Nax1* was mapped as a QTL to the long arm of chromosome 2A, tightly linked to flanking molecular markers, gwm312 and wmc 170 (Megan et al 2004). *Nax2* was located in the terminal 14% of chromosome 5AL, using telomeric deletion lines (Byrt et al. 2007). A tightly linked marker 'cslinkNax2' is used for selection of lines containing *Nax2*. These tightly linked markers can be used to introgress the *Nax* genes into elite varieties to improve their salt tolerance by means of marker-assisted selection. The objectives of this study were to identify the relative importance of morpho-physiological and molecular traits associated with salt tolerance, to screen Tunisian durum wheat genotypes and to develop salt-tolerant cultivars either by conventional or molecular approaches.

## II – Material and methods

### 1. Phenotyping

Six principal Tunisian varieties of durum wheat were used in this study (Karim, Khlar, Maali, Nasr, Razzek and Salim). These varieties were grown under semi-controlled conditions during the 2011/2012 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 studies (Chaabane et al, 2011) it was shown that 10g/l NaCl significantly affects the majority of agro-physiological characters in durum wheat. Similarly, El-Hendawy et al. (2011) reported that variations in salt tolerance indexes among spring wheat (*Triticum aestivum* L.) genotypes were reduced at high salinity (150mM 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 (150 mM NaCl) and a control (no NaCl). The salinity treatment was initiated at three-leaf stage. Agro-physiological measurements were conducted at different growth stages (60, 80, 100, 110, and 120 days after sowing and final harvest). Chlorophyll (Chl) content of the flag leaves was measured at 60, 80, 100, 110, and 120 days after sowing (DAS). Three different measurements were performed at the base, the middle 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. Heading date and flowering dates were also recorded. 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 (150 DAS). The ratio of harvested grain to total shoot dry matter known as harvest index was calculated. The data were also converted to salt tolerance index (STI) to allow comparisons among genotypes for salt sensitivity. A STI was defined as the observation at salinity divided by the average of the controls (El-Hendawy et al. 2005). Basing on STI the genotypes were grouped according to a one-way ANOVA, followed by Newman–Keuls' post hoc tests. Analyses of variance (ANOVA) (Tables 3, 4) were performed using Statistica 5.0 v. '98 Edition.

## 2. Genotyping

### A. 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%  $\beta$ -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 $\mu$ g/ml followed by a phenolic extraction: A treatment by equal volume Sigma-phenol:chloroform:Isoamyl alcohol 25:24:1, followed by a treatment by equal volume of Sigma-chloroform:isoamyl alcohol 24:1. DNA concentration was quantified by gel electrophoresis. The average DNA yield was 15  $\mu$ g DNA/g of tissue.

### B. Molecular analysis

PCR reactions were carried out in a 25- $\mu$ l reaction volume containing 1 U of taq polymerase, 50-100 of template DNA, 0.25  $\mu$ M of each primer, 0.2 mM of each dNTP, 2mM of MgCl<sub>2</sub> 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 2% 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.

## III – Results and discussion

### 1. Phenotyping

Salinity affected all of the considered traits at different growth stages. At the vegetative growth stages, tiller number, plant height, spikes per plant, spike-bearing tillers, shoot dry weight (DW), Chlorophyll content at 60, 80, 100, 110, and 120 DAS and heading date were significantly affected by salinity. At harvest, the shoot DW, the number of spikes per plant and the total grain yield were significantly affected by salinity.

At vegetative growth stages the heading date, the mean tiller number (Fig.1) and the plant height (Fig.2) were the most affected traits by salinity. Comparing to the control, the heading date of all varieties was earlier in the salinity treatments. The heading date at salinity treatment was one day (Karim) to five days (Khiar) before the control treatment. These results are in accordance with those obtained by Royo *et al.* (2003) who reported that salinity induced a heading date 6 days earlier for the different varieties in the most saline treatment. At salinity treatment, the plant high was reduced by 16,2% as compared with the control treatment. The tiller number for all varieties at salinity treatment was reduced by 43% as compared with the control treatment. These results confirm our previous results (Chaabane *et al.* 2011, 2012) and they are in accordance with those obtained by several authors: El-Hendawy *et al.* (2005) reported that tiller number was significantly more affected by salinity than leaf number and leaf area at the vegetative stage; Eugene *et al.* (1994) reported that salinity stress strongly influenced the distribution of spike-bearing tillers; Nicolas *et al.* (1994) found that salt stress during tiller emergence can inhibit their formation and can cause their abortion at later stages; Jones *et al.* (1977) reported that breeding genotypes with fewer, but less vulnerable tillers could substantially increase yields on salt-affected soils. The salt tolerance indexes of tiller number (Table 1) ranged from 0.46 (Khiar) to 0.74 (Maali). Therefore, for tiller number, Khiar was the most affected genotype by salinity and Maali was the least affected. Tiller number at salinity was decreased by 54% for Khiar and 26% for Maali, as

compared with the control. The average chlorophyll content of the flag leaf measured in Unit SPAD had a decreasing trend with time after the 60<sup>th</sup> DAS. Compared to the control treatment the average chlorophyll content at salinity treatment of the six varieties was increased by 4.78%, 4.6% and 8.0% respectively at 60, 80 and 100 DAS. At 110 and 120 DAS the average chlorophyll content of the 8 varieties was decreased by 37.8% and 54.0%. This reveals that senescence processes were promoted by salinity.

The most affected traits at harvest were the number of spikes per plant, the shoot dry weight (Fig.3) and the grain yield (Fig. 4). As compared with the control treatment, the number of spikes per plant was reduced by 37.3%, the shoot dry weight was reduced by 29.7% and the grain yield was reduced by 30.8%. However, some yield components (spikelets/spike, grains/spike) were much less affected by salinity. The number of spikelets per spike was reduced by 0.03% and the number of grains per spike was reduced by 1.4% as compared with the control treatment.

The salt tolerance indexes of all traits varied among varieties. The salt tolerance indexes (Table 1) for Chl (day 60), Chl (day 80), tiller number, spikes per plant, 1000-grain weight, and grain yield were significantly affected by salinity. These significantly affected traits can be used to compare the behaviour of the different analysed varieties in salt conditions. Maali and Salim were the less affected varieties (Table 1) for three traits (tiller number, spikes per plant, 1000-grain weight, and grain yield). Kerim and Khiar were the most affected varieties for the majority of traits.

Pearson's correlations were computed between salt tolerance indexes of different traits. Salt tolerance index of grain yield showed a very highly significant ( $P < 0.001$ ) positive correlation with salt tolerance index of shoot dry weight ( $r = 0.80$ ) and with harvest index ( $r = 0.67$ ). The STI of grain yield showed also a high correlation ( $P < 0.05$ ) with tiller number ( $r = 0.43$ ), spikelets per spike ( $r = 0.56$ ) and flowering date ( $r = 0.49$ ). These correlation studies showed that the grain yield sensibility to salt stress is highly correlated with the sensibility of shoot dry weight, tiller numbers, flowering date, spikelets per spike and harvest index. These traits are sensitive traits that affects final yield under salinity conditions. The significantly affected traits identified at early stages are not always correlated with that of harvest. 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).

**Table 1. Salt tolerance indexes.**

Variety	Chl. Day 60	Chl. Day 80	Tiller No.	Spikes/ plant	1000 GW	Grain yield
Karim	1,00 (a)	1,00 (a)	0,51 (ab)	0,55 (a)	0,86 (a)	0,64 (a)
Khlar	1,04 (ab)	1,06 (ab)	0,46 (a)	0,54 (a)	0,91 (ab)	0,68 (ab)
Maali	1,06 (ab)	0,99 (a)	0,74 (b)	0,71 (b)	1,19 (b)	0,72 (b)
Nasr	1,00 (a)	1,15 (b)	0,54 (ab)	0,65 (ab)	0,99 (ab)	0,71 (ab)
Razzek	1,10 (b)	1,00 (a)	0,59 (ab)	0,65 (ab)	0,87 (a)	0,67 (ab)
Salim	1,07 (ab)	1,07 (ab)	0,72 (b)	0,71 (b)	0,92 (ab)	0,74 (b)

(a), (ab), (b): Newman-Keuls comparison tests.

The STIs of previously reported sensitive traits are themselves correlated with STI of other significantly affected traits. Thus, these traits indirectly affect final yield at salinity conditions; therefore salinity effects on traits at early stages may affect directly or indirectly yield.

Finally, screening for salt tolerance should be done by studying and combining the maximum values of significantly salt affected agro-physiological traits evaluated at different growth stages and those of which STI are correlated with STI of final yield. All these traits could be used as simple, non-destructive criteria to target wheat genotypes in breeding programs for genetic improvement of the analysed varieties.



**Figure 5. Agarose (2%) gel electrophoresis of PCR products obtained using gwm312 and wmc170 primers. M:100 bp, 1:Karim, 2: Salim, 3: Khiar, 4:Nasr, 5: Razzak 6, Maâli.**

## IV – Conclusions

Salinity affected all of the considered traits. At all vegetative growth stages, tiller number, plant height, spikes per plant, spike-bearing tillers, shoot dry weight (DW), chlorophyll content (60, 80, 100, 110, and 120 DAS), and heading date were significantly affected by salinity. At harvest, the shoot DW, the number of spikes per plant, and the total grain yield were significantly affected by salinity. The different measured traits showed differential response to salt stress among the wheat cultivars. At vegetative growth stages the heading date, the plant height and the mean tiller number were the most affected traits by salinity. At harvest the most affected traits were the number of spikes per plant, the grain yield and the shoot dry weight. The correlation between the different STI showed that the grain yield sensitivity to salt stress is highly correlated with the sensitivity of shoot dry weight, tiller numbers, flowering date, spiklets per spike, and harvest index. The significantly affected traits identified at early stages were not always correlated with that of harvest. Therefore, screening for salt tolerance should be done by studying and combining the maximum values of significantly salt affected agro-physiological traits evaluated at different growth stages and those of which STI are correlated with STI of final yield. All these traits could be used as simple, non-destructive criteria to target wheat genotypes in breeding programs for genetic improvement of the analysed varieties. Finally, the present study showed that both conventional and molecular approaches are useful for improving salt tolerance of Tunisian durum wheat.

## Acknowledgments

We thank ICARDA especially Drs. Bari Abdallah and Inagaki Masanori for sustaining this work. The study was funded by Agricultural Research and Higher-Education Institute of Tunisia (IRESA) and the Ministry of Higher Education and Scientific Research of Tunisia.

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