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The role of osmoprotectants and antioxidant enzymes in the differential response of durum wheat genotypes to salinity

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Summary

This study aims to investigate the importance of accumulation of osmoprotectants and activities of some key antioxidant enzymes in genotypic variation (GV) observed among durum wheat genotypes in response to increasing NaCl salinity (0-200 mmol/L) at seedling stage. Germination and seedling growth traits of all the genotypes were significantly decreased by salinity. Mohamed Ben Bachir, the more salt-tolerant genotype, exhibited the lowest reduction in final germination percentage (FGP, <18%) and seedling growth (<60%, based on dry biomass), the lowest increase in proline (PRO) and water soluble carbohydrates contents but the highest increase in catalase (CAT) and ascorbate peroxidase (APX) activities. Correlation and principal components analysis revealed that the most important variables distinguishing salt tolerant vs. salt non-tolerant genotypes were root to shoot ratio (R/S, 36.1%), CAT (30.6%), APX (12.5%) and FGP (5.74%). Although PRO and WSC could play a key role in salt tolerance by mediating osmotic adjustment, these compounds do not seem to be significantly involved in genotypic variation (GV) for salinity tolerance in durum wheat.

Introduction

Salinity which affects more than 800 Mha of world's land area (FAO, 2008), is nowadays, one of the most damaging environmental factors that limit growth and productivity of crop plants (MUNNS and TESTER, 2008). In Algeria, for instance, more than 3.2 Mha of arable land already suffer from salinisation (BENMAHIOUL et al., 2009). Overall, salinity adversely affects plant growth and development not only through imposition of osmotic stress, ion toxicity, but also by secondary effects such as oxidative stress and hormonal imbalance (ASHRAF et al., 2012).

Even though durum wheat (*Triticum durum* Desf.) is regarded as one of the most widely cultivated crops, especially in the Mediterranean basin where about 75% of total durum wheat production is provided (HABASH et al., 2009), its production is often limited by poor seed germination and stand establishment, mainly due to drought or soil salinity (SAYAR et al., 2010). Hence, it is imperative to develop wheat genotypes with improved salt tolerance. This is of obvious importance in the case of tetraploid wheat, which lacks the D genome, making it less tolerant to salt stress (MUNNS et al., 2012).

Proper seed germination and seedling emergence are required traits to achieve a high grain yield in wheat (PAULSEN, 1987). Therefore, it has been established that under arid conditions, the most valuable cultivars are those, which are emerging rapidly because rainfall after sowing may result in a soil crust that prevents seedling emergence (TAHIR, 2010). In addition, the early emerging and the fast growing crops are able to take full advantage of the soil moisture leading to better seedling establishment and grain yield. Although the major variation in the coleoptile length is genetic, environmental conditions can significantly affect this trait (HAKIZIMANA et al., 2000). Accordingly, identifying the physiological and biochemical attributes associated with a more vigorous and rapidly growing seedling, particularly under stress conditions will be very meaningful (RASHID et al., 1999).

Seed germination and seedling emergence are well-regulated processes characterized by high metabolic activity mediated by reactive oxygen species (ROS) in the cell (BAILLY, 2004). Antioxidants act to scavenge ROS, and therefore play an important role in the regulation of growth processes that occur during germination such as radicle protrusion or coleoptile emergence (BAILLY, 2004). While superoxide dismutase (SOD) is the most important antioxidant enzyme synthesized in response to oxidative stress, its effectiveness was dependant on the H_2O_2-scavenging enzymes activity notably that of ascorbate peroxidase (APX) and catalase (CAT). The combined action of these two enzymes seems to play an important role in plant growth and production since seed filling was associated with a high potential of the H_2O_2-detoxification, often due to APX and CAT activities (COSTA et al., 2010).

In recent years, numerous studies have been conducted to investigate whether there are differences between several durum wheat genotypes in their response to salinity (MUNNS et al., 2012; RASHID et al., 1999; SAYAR et al., 2010). However, to the best of our knowledge no work has been done to investigate the difference in responses to salinity of wheat genotypes used in the present study, particularly with respect to their H_2O_2-scavanging capacity and osmoprotectants accumulation. Therefore, this study aimed to investigate the genotypic variation (GV) for salinity tolerance among three durum wheat landraces selected based on their good adaptation to drought-stressed Mediterranean environments.

Materials and methods

Experimental details and treatments

Germination assay and growth measurements: The present investigation was performed in a randomized complete design (RCD) with two factors and three replications. Certified seeds of durum wheat (*Triticum durum* Desf.) vars. Mohamed Ben Bachir (MBB), GTA-dur and Waha were surface sterilized with sodium hypochlorite (5% w/v) for 3 min, washed several times against sterile water and dried in oven. Thirty dried seeds were then placed in each Petri dish containing two layers of Whatman. No. 1 filter paper moistened with 10 mL of one of the prepared solutions (100 or 200 mmol/L NaCl) or distilled water (control). Seeds were germinated in darkness in a temperature-controlled chamber held at 22 ± 0.5 °C. The number of germinated seeds was counted every day until day 7, when there were no more germinated seeds. Final germination percentage (FGP) was calculated at the end of the germination period (7 days). Mean germination time (MGT) was calculated according to ELLIS and ROBERTS (1981):

$$MGT = \sum Dn / \sum n$$

Where n is the number of seeds germinated on day D, the number of days counted from the beginning of germination. Final length of shoot (coleoptile) and root of seedlings seven days post-sowing were measured and used for vigor index (VI) estimation according to ABDUL-BAKI and ANDERSON (1973):

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$$VI = FGP \times SL$$

where SL is seedling length (cm). The salt tolerance index (ST) was calculated according to CANO et al. (1998):

ST = (FW in NaCl-saline solution) × 100/(FW in NaCl-free solution)

where FW is fresh weight.

Physiological and biochemical changes

Physiological and biochemical measurements were in general based on shoot (coleoptile) samples of three 7-day-old seedlings of uniform size from each cultivar.

Relative water content: Shoot relative water content (RWC) was determined according to BARR and WEATHERLEY (1962):

$$RWC = (FW - DW) \times 100/(TW - DW)$$

where FW is fresh weight, DW is dry weight and TW is turgid weight.

Proline and water-soluble carbohydrate content:

Quantitative determination of free proline (PRO) content was performed according to BATES et al. (1973). The optical density of the solution is read on a spectrophotometer at a wavelength of 528nm. The proline concentration was determined using a calibration curve of known L-proline concentration. Water-soluble carbohydrates (WSC) content was determined according to DUBOIS et al. (1956). Absorbance was measured at 485nm. The WSC concentration was determined using a calibration curve of known D-glucose concentration.

Hydrogen peroxide and lipid peroxidation assays: H_2O_2 content was determined according to VELIKOVA et al. (2000). Approximately 100 mg of fresh shoot samples were homogenized in 0.1% 5 mL of trichloro-acetic acid (TCA), and centrifuged for 15 min at 12,000 rpm. A 0.5 mL of the supernatant is then mixed with 0.5 mL of buffer (Potassium phosphate 10 mM pH 7) and 1mL of 1 M KI. The absorbance reading was taken at 390 nm. Lipid peroxidation (LPO), however, was estimated as malondialdehyde (MDA) according to HEATH and PACKER (1968). Approximately 500 mg of fresh shoot samples were homogenized in 10 mL of 0.1% TCA and the homogenate was centrifuged for 15 min at 15,000 rpm. To a 1.0 mL aliquot of the supernatant, 4.0 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA were added. The mixture was heated at 95 °C for 30 min, and then cooled in an ice bath. After centrifugation for 10 min at 10,000 rpm at 4 °C, the absorbance of the supernatant was recorded at 532 nm on a spectrophotometer. The MDA content was calculated according to its molar extinction coefficient of 155 mM^{-1} cm^{-1}.

H_2O_2-scavenging enzymes extraction: About 200 mg of fresh shoot tissues were collected from stressed and control seedlings and quickly frozen in liquid nitrogen and ground to a fine powder using a prechilled mortar and pestle. The exact weight of each powdered sample was determined before it was thoroughly homogenized in 1.2 mL of 0.2 M potassium phosphate buffer (pH 7.8 with 0.1 mM EDTA). The samples were centrifuged for 20 min at 15,000 rpm at 4 °C. The supernatant was removed, the pellet resuspended in 0.8 mL of the same buffer, and the suspension centrifuged for another 15 min at 15,000 rpm at 4 °C. The combined supernatants were stored on ice and used to determine antioxidant enzyme activities (ELAVARTHI and MARTIN, 2010).

H_2O_2-scavenging enzymes activity assay: The activity of CAT (EC 1.11.1.6) was measured according to the method of CHANDLEE and SCANDALIOS (1984) with little modification. The assay mixture contained 2.6 mL of 50 mM potassium/phosphate buffer (pH 7.0), 0.4 mL of 15 mM H_2O_2 and 0.04 mL of enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed in units mg^{-1} protein (U = 1 mM of H_2O_2 reduction min^{-1} mg^{-1} protein). The extinction coefficient of H_2O_2 (40 mM^{-1} cm^{-1} at 240 nm) was used to calculate the enzyme activity that was expressed in terms of mM of H_2O_2 per minute per gram fresh weight.

APX (EC 1.11.1.11) activity was assayed using a modified method of NAKANO and ASADA (1981). APX activity was determined by measuring decreased absorbance at 290 nm due to ascorbate oxidation. The assay mixture (1 mL) contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.5 mM H_2O_2, and 10 µl of crude extract. H_2O_2 was added last to initiate the reaction, and the decrease in absorbance was recorded for 3 min (U = 1 mM of Ascorbic acid reduction min^{-1} mg^{-1} protein). The extinction coefficient of 2.8 mM^{-1} cm^{-1} for reduced ascorbate was used in calculating the enzyme activity that gram fresh weight. The enzyme protein was estimated by the method of BRADFORD (1976).

Statistical analysis

For statistical analysis, the data of germination percentage were transformed by arcsine transformation. Data were subjected to analysis of variance (ANOVA) procedures, while the least significant difference (LSD) test at $\alpha = 0.05$ level of significance was used to compare the differences among treatment means. Correlation analysis and principal component analysis (PCA) were carried out to examine the relationships between and among the factors and variables using the statistical software package STATISTICA 8.0 (HILL and LEWICKI, 2007).

Results

Germination assay: As shown in Fig. 1A, final germination percentage (FGP) was adversely affected by NaCl salinity. The decrease in FGP caused by moderate salinity (100 mM) was less than 9% in all the genotypes, whereas high salinity (200 mM) induced substantial reduction in FGP especially in the genotype Waha, which has lost nearly 70% of its germination capacity. Statistical analysis revealed a significant effects for both factors (salinity 'S' and genotype 'G') and their interaction ($P<0.001$) (Tab. 1), suggesting the existence of a genetic variation. Unlike the FGP, the mean germination time (MGT) was only marginally affected by moderate salinity, whereas high salinity resulted in a more pronounced increase of MGT, especially in the genotype Waha.

Growth Analysis

Shoot and Root lengths: NaCl salinity decreased more or less significantly shoot and root lengths in all genotypes in a concentration-dependent manner (Fig. 1B). With the exception of the decrease in root to shoot ratio (R/S) recorded by the genotype Waha at 200 mM NaCl (> 40%), salinity seems to increase significantly the R/S (Fig. 1C). It was noteworthy that the genotype MBB had the highest R/S.

Total dry weight and Vigor index: Among the tested wheat genotypes, the total dry weight (DW) was affected more or less to the same extent by salinity (Fig. 1C). This effect was more pronounced when the concentration of NaCl becomes higher. Whilst root growth (particularly in genotype MBB) was much less affected, which

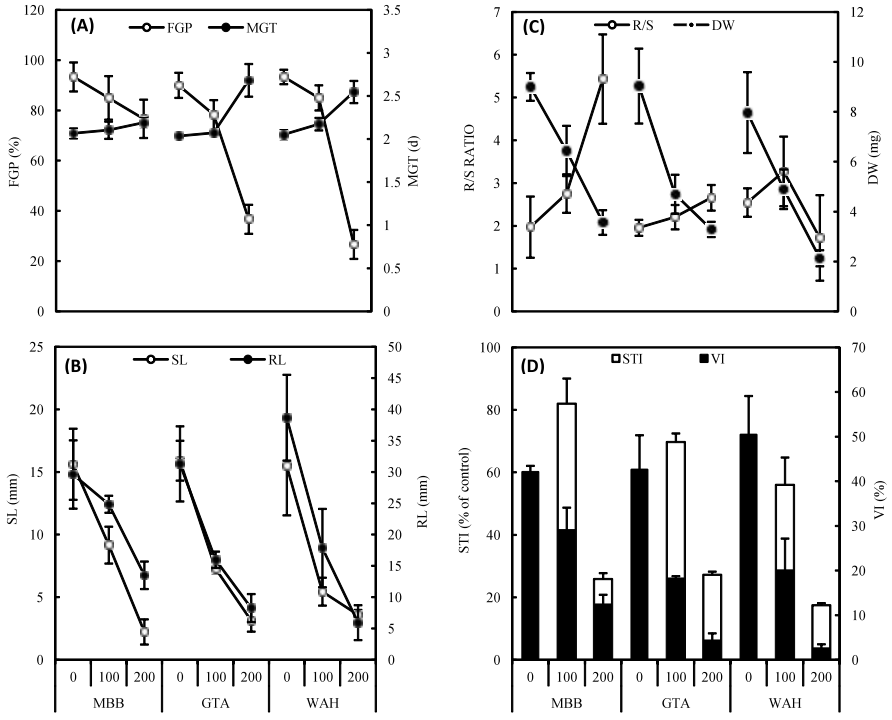

Fig. 1: Effect of salinity (mM) on (A) final germination percentage (FGP) and mean germination time (MGT); (B) shoot (SL) and root lengths (RL); (C) root**:**shoot ratio (R/S) and total dry weight (DW); (D) salt tolerance index (ST) and vigor index (VI) of durum wheat genotypes. Values are means of three replicates ± SE.

Tab. 1: F and LSD values of the effects of salinity (S), genotype (G) and the interaction between them (S×G) on the dependent variables.

Variation	F		LSD		
	Salt	Genotype	Interaction	Salt	Genotype
FGP	73.53***	12.03***	6.45***	0.16	0.19
MGT	55.07***	6.95**	8.40***	0.35	0.137
SL	107.8***	0.60 ns	1.35 ns	4.2	ns
RL	67.60***	2.01 ns	3.69*	10.32	ns
R/S	4.95*	5.50*	8.25***	1.11	0.87
DW	83.53***	4.57 *	0.88 ns	2.34	1.33
VI	136***	3.43 ns	3.29*	16.01	ns
ST	775.9***	18.0***	8.24***	30.75	7.83
RWC	22.63***	3.33 ns	2.49 ns	7.49	ns
PRO	1200***	8.27**	47.69***	67.6	8.76
WSC	210.3***	10.58 ns	3.41*	62.66	ns
MDA	16.33***	7.62**	0.43 ns	0.34	0.23
H_2O_2	25.92***	26.11***	1.58 ns	11.96	5.83
CAT	6.90**	5.93**	10.65***	0.99	0.93
APX	13.3***	6.55**	5.87**	8.57	6.18

*P< 0.05; **P< 0.01; ***P< 0.001; ns = not significant.

explains the high R/S, high salinity (200 mM) had reduced plant biomass by 50% to 70% compared to control groups. Increasing salinity induced a significant linear decline in seedling vigor index (VI) and salt tolerance index (ST) of all tested cultivars (Fig. 1D).

Physiological and biochemical parameters:

Relative Water Content: As shown in Fig. 2A, RWC of wheat seedlings, especially in genotype GTA (~84%) showed only slight and non-significant decrease with increasing NaCl concentration.

Proline and Water Soluble Carbohydrates contents: According to Fig. 2A, salinity induced a significant (P<0.001) increase of proline content of all genotypes. However, genotype GTA treated with 200 mM NaCl showed the highest amount of proline content (up to 3-folds compared to control) (Tab. 1). In all genotypes, NaCl induced a significant (P<0.001) accumulation of WSC in a concentration-dependent manner (Fig. 2A). While WSC content does not appear to differ significantly from one genotype to another, the existence of a significant interaction genotype × salt stress (Tab. 1) gives rise to conclude that these genotypes respond differently with increasing salt level.

Hydrogen peroxide content and Lipid peroxidation: From the Fig. 3A it is apparent that the amount of H_2O_2 increased in response to increasing salinity. Fig. 3A shows also that under both salinity levels, lipid peroxidation occurs in seedling tissues of all wheat cultivars.

Ascorbate Peroxidase and Catalase activity: Unexpectedly salt stress affected the activity of APX independently of NaCl concentration and differently from one genotype to another (Fig. 3B). Nevertheless, at 200 mM salt stress increased the activity of APX in the three genotypes with respect to their respective controls. On the other hand, salinity had not only affected differently the activity of CAT, but regardless of its concentration (Fig. 3B). However, at 200 mM, unlike genotype Waha CAT activity increased in the genotypes GTA and MBB.

Multifactorial analysis: Results of multifactorial comparison generalized over the entire set of data (432 entries) using the principal components analysis (PCA), were given in Fig. 4A and 4B. It is noteworthy that every single genotype was presented by 9 points (3 levels of salinity × 3 repetitions). PCA results revealed that the factor 1 (first principal component or PC1) explained 63.69% of the total data variation and had positive correlation with the growth/germination performance under both stress and non-stress environments (Fig. 4A and Fig. 1A-D). Thus this component was able to separate the genotypes according to their growth/germination potential under the three different stress treatments. The PC 2 explained 11.04% of the total data variation. The first two PCs accounted for 74.73% of total variation. Considering the projection of the variables, it appears that the mean values of proline and water-soluble carbohydrates decreased from the negative side of PC1 to its positive side (Fig. 4B). This reflects the highly significant negative relationships among proline, WSC and all the growth parameters (r over to - 0.8, P<0.001). PCA results also indicated that the indices could discriminate the tolerant genotypes are the R/S ratio (36.1% of contributions), CAT (30.6%), FGP (5.74%), which showed positive correlations with salt tolerance, and APX (12.5%), which showed a negative correlation with tolerance to salinity (Fig. 4B).

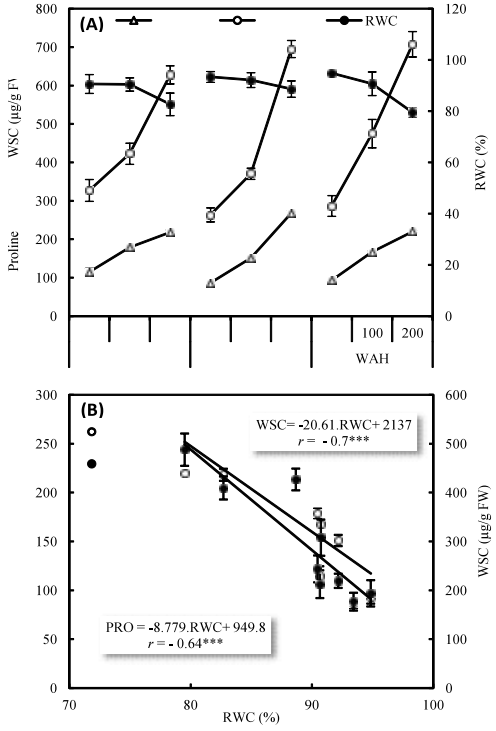

Fig. 2: Effect of salinity (mM) on (A) proline content (PRO), water soluble carbohydrates (WSC) and relative water content (RWC) of durum wheat genotypes; (B) Relationships of RWC with PRO and WSC contents. Values are means of three replicates ± SE.

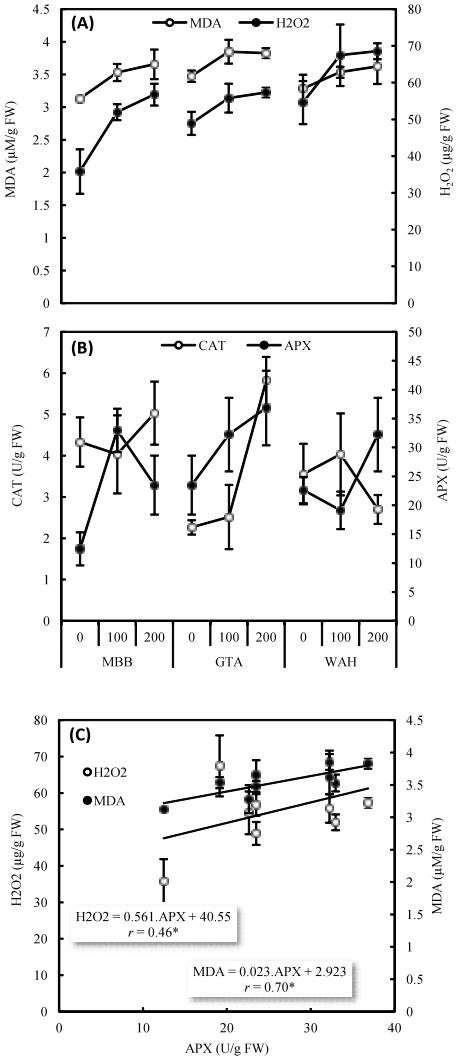

Fig. 3: Effect of salinity (mM) on (A) malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents; (B) catalase (CAT) and ascorbate peroxidase (APX) activities of durum wheat genotypes; (C) Relationships between the APX activity and H_2O_2 and MDA contents. Values are means of three replicates ± SE

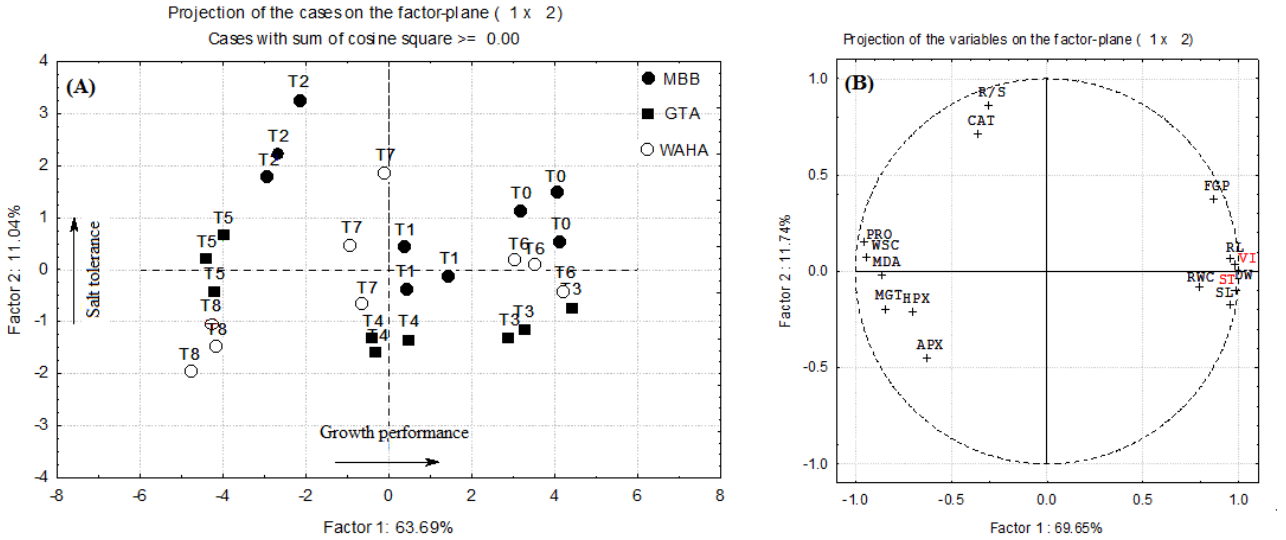

Fig. 4: Multifactorial comparison of the treatments and variables using PCA. Data are presented for each of three replicates for each treatment. The percent variance explained by each PC is provided.
Treatments: T0, T3, T6: control; T1, T4, T7: 100 mM NaCl; T2, T5, T8: 200 mM NaCl.
Variables codes: FGP: final germination percentage; MGT: mean germination time; DW: dry weight; RL: root length; SL: shoot length; R/S: root to shoot ratio; ST: salt tolerance index; VI: vigor index; PRO: proline; RWC: relative water content; WSC: water-soluble carbohydrates; APX: ascorbate peroxidase; CAT: catalase; HPX: hydrogen peroxide; MDA: malondialdehyde.

Discussion

It is evident from the results that NaCl salinity significantly decreases the percentage of germination (FGP) and delay the germination (MGT) in all the genotypes (Fig. 1A and Tab. 1). In this respect, Waha appears to be the most affected genotype. These results are in line with previous findings (Zhang et al., 2010). Salinity can affect germination of seeds either by imposing osmotic stress, which prevent water uptake, or by ion toxicity, which inhibits the metabolism of dividing and expanding cells, delaying germination and even leading to seed death (Zhang et al., 2010). The first visible evidence of germination is the protrusion of the radicle through the seed coat. This is the result of cell enlargement and, to a lesser extent, cell division (Haber and Luippold, 1960), both of which are very sensitive to dehydration, suggesting that salinity indirectly reduces growth of seminal roots by water privatization. Indeed, our results support this finding, since the decrease in germination performances was correlated to the decrease in RWC, particularly in Waha, and because a RWC of about 80% may trigger the accumulation of ABA that inhibit germination and radicle protrusion.

As on germination, salt stress decreases almost all of growth parameters (Fig. 1D) in all the wheat cultivars, which is may be attributed to either or both the osmotic and toxic effects of salinity as previously reported (Rashid et al., 1999; Sayar et al., 2010). In contrast to MBB, Waha appears to be the most affected by salinity.

Increasing salinity causes the reduction of cell turgor and the rate of shoot and root elongation (Munns and Tester, 2008). To maintain cell turgor by osmotic adjustment (OA), plants synthesize and accumulate several kinds of compatible osmolytes, such as PRO and WSC. Indeed, substantial increase in PRO content in all the genotypes was recorded under salt stress ($P<0.001$; Fig. 2A). However MBB, which exhibited the highest ST index, showed the lowest amount of PRO. On the other hand, the strong negative correlation found between PRO and RWC ($r = -0.64^{***}$; Fig. 2B), from one side, and since the genotype GTA which exhibited the highest RWC at 200 mM NaCl, showed the greatest PRO accumulation from the other side, suggests that PRO is involved in OA. Besides OA, PRO plays an important role in stabilization of enzymes/proteins and protection of membrane integrity (Ashraf and Harris, 2004; Ashraf et al., 2012). In line with these findings, correlation analysis revealed a strong positive relationship between H_2O_2 and PRO ($r = 0.49^{*}$; Fig. 3C) which corroborate recent suggestion that PRO could act as an antioxidant counteracting H_2O_2 (Ashraf et al., 2012).

Increase of WSC content has been considered as an adaptive response of plants to salt stress conditions (Parvaiz and Satyawati, 2008). WSC not only acts as an energy source, but it is also important to increase the biomass and provide the carbon backbones essential for the synthesis of numerous compounds that are involved in osmotic or anti-oxidative protection (Couee et al., 2006). In agreement with this, NaCl induced a significant ($P<0.001$) accumulation of WSC in all genotypes in a concentration-dependent manner (Fig. 2A). Moreover, WSC content showed a strong negative correlation with RWC ($r = -0.7^{***}$; Fig. 2B) and a positive correlation with H_2O_2 ($r = 0.53^{**}$).

Consistent with our results, salinity induces the accumulation of H_2O_2 in a concentration-dependant manner (Ashraf et al., 2012; Lee et al., 2001). Despite its role as a signaling molecule, H_2O_2 itself is toxic at high concentrations (Costa et al., 2010). In agreement with our results (Fig. 3A), salt-induced oxidative stress causes the lipid peroxidation (LPO) resulting at cellular level in membranes degradation, suggesting that salt-induced LPO occurs early in the seedling tissues of all wheat cultivars. Correlation analysis also revealed the significant positive relationships among MDA vs. PRO ($r = 0.58^{**}$) and MDA vs. WSC ($r = 0.43^{*}$), suggesting the involvement of PRO and WSC in anti-oxidative protection.

To withstand salt-induced oxidative stress, plants evolved enzymatic and non-enzymatic antioxidant defense systems. Among the ROS-scavenging enzymes, CAT and APX are primarily involved in the elimination of H_2O_2, and the most important antioxidant enzymes produced in different plant organelles (Costa et al., 2010). Our results support these aforementioned findings, showing that overall salt stress increases substantially the activity of APX in the three genotypes (Fig. 3B). Growing evidences suggests that ROS-scavenging enzymes are involved in the salt stress tolerance (Ashraf et al., 2012). While salt stress may result in a rapid increase in the activity of antioxidant enzymes, high concentration or long-term of salt stress inhibit the antioxidant enzyme activities (Ashraf

et al., 2012), which is partially in line with our results (Fig. 3B). Interestingly, LEE et al. (2001) showed that the overproduction of H_2O_2 promotes the induction of specific APX isoforms under catalase deactivation. Consistent with these findings, APX activity, but not CAT, was found positively correlated with the levels of both of H_2O_2 (r = 0.46*) and MDA (r = 0.70*) (Fig. 3C).

Based on PCA results it appears that the indices could discriminate the tolerant genotypes are the R/S (36.1% of contribution), CAT (30.6%), FGP (5.74%), which showed positive correlations with ST, and APX (12.5%), which showed a negative correlation with ST (Fig. 4B). This is in agreement with the fact that the most salt-tolerant cultivars are those with high germination rate, an important vigor index, able to maintain a vigorous root growth (high R/S) and showed relatively high antioxidant activity (ASHRAF et al., 2012; RASHID et al., 1999; SAYAR et al., 2010). In this case, MBB met the requirement.

Conclusion

Altogether, these results revealed that salt stress affects almost all studied aspects of germination and seedling growth in durum wheat in a concentration- and genotype-dependent manner. PCA results indicated that the most important variables that contributed to genotypic variation (GV) in salt tolerance among the tested genotypes were R/S ratio (36.1%), CAT (30.6%), APX (12.5%) and FGP (5.74%). While PRO and WSC have been shown to play a key role in salt tolerance by inducing osmotic adjustment (OA), these compounds do not appear to be involved in the GV for salinity tolerance among the tested genotypes. According to the response of wheat genotypes to high salinity (200 mM NaCl), it is suggested that MBB is the most tolerant genotype while Waha is the less tolerant one.

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