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RESEARCH ARTICLE

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Comparison of an antioxidant system in tolerant and susceptible wheat seedlings in response to salt stress

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Abstract

It has been demonstrated previously that the physiological and molecular analysis of seedlings of the tolerant (Om Rabia3) and susceptible (Mahmoudi) Tunisian wheat genotypes were different at short and long-term response to salinity. In this study, we examined the antioxidant defence system in seedlings of these two cultivars at short-term response to different NaCl concentrations. The findings showed that high salinity tolerance of cv. Om Rabia3, as manifested by lower decrease in its dry biomass, was associated with lower malondialdehyde and hydrogen peroxide contents, lower accumulation of the superoxide (O_2^-) in the roots and the shoots, and also lower decrease in ascorbate content than those in cv. Mahmoudi. Moreover, the expression of some genes coding for antioxidant enzymes such as the catalase, the superoxide dismutase and the peroxidase were enhanced by NaCl stress especially in the salt-tolerant cultivar. In parallel, their activities were increased in response to the same condition of stress and especially in the cv. Om Rabia3. Taken together, these data suggested that the capacity to limit oxidative damage is important for NaCl tolerance of durum wheat.

Additional keywords: antioxidant; oxidant; reactive oxygen species; salinity; *Triticum durum*.

Abbreviations used: APX (ascorbate peroxidase); AsA (ascorbic acid); CAT (catalase); DW (dry weight); GPX (glutathione peroxidase); GR (glutathione reductase); GSH (glutathione); MDA (malondialdehyde); NBT (nitroblue tetrazolium); POD (peroxidase); PMSF (phenylmethylsulfonyl fluoride); ROS (reactive oxygen species) SOD (superoxide dismutase); TCA (trichloroacetic acid).

Authors' contributions: Conceived and designed the experiments, statistical analysis and data interpretation: KF and FB. Performed the laboratory experiments: KF and ST. Wrote the paper: KF. Supervised and coordinated the work: FB.

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Introduction

In the developing and under developed world, about 2.5 billion people survive on wheat as an element source of energy (http://faostat.fao.org). In many arid and semi-arid regions in the world, wheat production yield is limited by the availability of water resources, the use of poor water quality and also when soil drainage is poor. Salinity is one of the major abiotic stress issues worldwide affecting plant cultivation and productivity, and its adverse impacts are getting more serious problem in regions where saline water is used for irrigation (Türkan & Demiral, 2009). The sodium exclusion from the leaves is the major mechanism that confers salt tolerance in wheat (Gorham *et al.*, 1990; Husain *et al.*, 2003). In general, the hexaploid bread wheat (*Triticum aestivum*: AABBDD) has a better capacity to exclude Na^+ than durum wheat (*Triticum durum*: AABB), which is linked to the *Kna1* locus on chromosome 4D (Dvorák *et al.*, 1994). High salinity causes a primary effect like hyperosmotic stress and ion disequilibrium producing secondary effects, such as ion toxicity, oxidative stress, hormonal imbalances and nutrient disturbances (Ashraf, 2009; Türkan & Demiral, 2009). In plant cells, salinity induces the production of reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-). ROS are continuously generated during normal metabolic processes in the mitochondria, peroxisomes and cytoplasm. But, at high level they are highly cytotoxic and can react with vital biomolecules and consequently causing peroxidation, protein denaturation and DNA mutation (Choudhury *et al.*, 2013; Hossain *et al.*, 2015).

In plant cells, a complex defense system is implicated to maintain the redox homeostasis, which comprise of enzymatic and non-enzymatic components. In plants, many genes encode for different ROS-detoxifying or ROS-producing enzymes forming well organized ROS gene web (Mittler *et al.*, 2004). Several enzymes are involved in the detoxification of ROS like superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), catalase (CAT; E.C 1.11.1.6), glutathione reductase (GR; EC 1.6.4.2) and glutathione peroxidase (GPX; EC 1.11.1.9). SOD is the first enzyme implicated in the removal of ROS, and it catalyzes the conversion of O_2^- to H_2O_2, and it occurs ubiquitously in every plant cells (Breusegem *et al.*, 1999; Ashraf, 2009). H_2O_2 is a toxic ROS and has deleterious effects in plant tissue. It is scavenged by CAT and different classes of peroxidase (POD) (Mhamdi *et al.*, 2010). Other enzymes that are very important in the ROS scavenging system and function in the ascorbate-glutathione cycle are glutathione reductase (GR), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and dehydroascorbate reductase (DHAR; EC 1.8.5.1) (Foyer & Noctor, 2011). Several genes encoding for plant antioxidant enzymes have been cloned, characterized, and used in the construction of transgenic lines. Transgenic plants overexpressing SOD (Bowler *et al.*, 1991; Van Camp *et al.*, 1996; Breusegem *et al.*, 1999; Alscher *et al.*, 2002; Feki *et al.*, 2016), APX (Wang *et al.*, 1999), GR (Foyer *et al.*, 1995), GPX (Roxas *et al.*, 1997, 2000) and CAT (Feki *et al.*, 2015) showed tolerance to various abiotic stresses.

It has been demonstrated that NaCl tolerance is closely correlated with the antioxidant capacity in numerous plants, such as bread wheat (Sairam *et al.*, 2002, 2005; Mandhania *et al.*, 2006; Choudhury *et al.*, 2013; Hossain *et al.*, 2015). However, it has been showed that the increase of the activity of some antioxidant enzymes is concomitantly with salt sensitivity. For example, in the leaves of the salt-sensitive rice plants, NaCl stress preferentially enhanced the activities of some antioxidant enzymes like SOD and APX (Lee *et al.*, 2001). In addition, it has been reported that the responses of plant antioxidant enzymes to salinity show varying activity patterns according to the species and analyzed tissues (Gossett *et al.*, 1994).

The non-enzymatic antioxidant system includes ascorbic acid (AsA), glutathione (GSH), α-tocopherols (vitamin E), flavonoids, anthocyanines, polyphenolic compounds and carotenoids (Schafer *et al.*, 2002; Suzuki *et al.*, 2012). The most abundant soluble antioxidants in plants are AsA and GSH, which play a key role in plant defense against oxidative stress (Foyer & Noctor, 2011; Venkatesh & Park, 2014). α-tocopherol, an abundant vitamin E compound, is a lipid soluble antioxidant found in chloroplasts where it counteracts lipid peroxidation through scavenging of lipid peroxyl radicals and detoxifies singlet oxygen and hydroxyl radicals (Munné-Bosch, 2005). Oxidative stress activates the expression of genes responsible for the synthesis of tocopherols in higher plants, generating its accumulation in plants (Shao *et al.*, 2007; Wu *et al.*, 2007).

In a previous work, the physiological analysis of the two Tunisian durum wheat genotypes showed differential tolerance to salinity (Brini *et al.*, 2009). However, data concerning the effects of salinity on ROS production and antioxidant defense system in these two durum wheat cultivars are still lacking. In this work, we aimed to provide an insight view to antioxidant enzyme system in two Tunisian durum wheat treated with different NaCl stress conditions, in order to understand the mechanisms relevant in salt tolerance.

Material and methods

Plant material, stress conditions and dry weight determination

Two Tunisian cultivars of durum wheat (*Triticum turgidum* L. subsp. *durum*), Mahmoudi (salt sensitive) and Om Rabia3 (salt tolerant) were sterilized and then germinated on Petri dishes as described by Brini *et al.* (2009). Three-day-old seedlings were transferred to containers with modified half-strength Hoagland's solution (Epstein, 1972). After 4 days, they were transferred to the same medium containing or not different NaCl concentrations (0, 50, 100 and 200 mM) and kept in stress for 3 days. All seedlings were grown in a glasshouse at 25 ± 5°C, 16 h photoperiod and 60 ± 10% relative humidity. After three days of NaCl exposure, five plants per each salt treatment were used for the determination of the dry weight (DW), malonyldialdehyde (MDA) and H_2O_2 contents, the antioxidative enzyme assays, and ascorbate content. All these experiments were repeated at least five times.

To determine the DW, shoots (leaf and sheath) were separated from roots, dried at 70°C and then weighed. Besides, fresh shoots samples from each plant were immediately frozen in liquid nitrogen and stored at 80°C, until performing the biochemical analysis.

Lipid peroxidation

The extent of lipid peroxidation was estimated by determining the amount of MDA in the leaves by the method of Ben Amor *et al.* (2005). Fresh shoots (0.15 g) were homogenized in 1.5 mL of 0.1% (w/v) trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 15,000 × g for 30 min. To 0.5 mL aliquot of the supernatant, 1.0 mL of 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) TCA solution was added. The mixture was heated at 90°C for 30 min, and then cooled on ice. The MDA equivalent was calculated by measuring absorbance at 532 and 600 nm in reference to a MDA standard curve.

Quantitative H_2O_2 measurement

The concentration of H_2O_2 was appraised following Velikova *et al.* (2000). Fresh shoots tissue (0.5 g) was homogenized with 5 mL of 0.1% (w/v) TCA. This homogenate was then centrifuged at 12,000 × g for 15 min. To 0.5 mL of the supernatant, 10 mM phosphate buffer (pH 7.0) and 1 M potassium iodide were added. The mixture was then vortexed and its absorbance was read at 390 nm, and H_2O_2 content was calculated using a standard curve with concentration ranging from 0.05 to 0.1 mM.

Superoxide radical staining assay

In order to detect O_2^-, we performed a staining assay based on nitroblue tetrazolium (NBT) (Jabs *et al.*, 1996), in different parts of the two Tunisian wheat cultivars treated or not with 200 mM NaCl for 3 days. The reduction of NBT to insoluble blue formazan was used as a probe for O_2^- generation. The samples were placed in the NBT solution (0.1 mM NBT, 25 mM HEPES pH 7.6) and subjected to vacuum infiltration for 5 min. Then, the samples were incubated under dark conditions for 2 h. Finally, they were treated with 80% ethanol and photographed. This staining assay was repeated three times using three to six different plants from each cultivar.

Ascorbate content

Ascorbate (AsA) was estimated following the method described by Mukherjee & Choudhuri (1983). Shoots material (0.25 g) was extracted with 10 mL of 6% (w/v) TCA solution. An aliquot of the extract was mixed with 2% dinitrophenyl hydrazine (in acidic medium) followed by the addition of one drop of thio-urea (in 70% ethanol). The mixture was boiled for 15 min in a water bath and after cooling at room temperature, 5 mL of 80% (v/v) H_2SO_4 were added to the mixture at 0°C. The absorbance was read at 530 nm. The levels of pure AsA were calculated from a standard curve plotted with varying known concentrations of pure AsA (Sigma-Aldrich).

RNA extraction and semi-quantitative RT-PCR

Total RNA from shoots treated or not with NaCl (200 mM NaCl) for 3 days was extracted using the TRIZOL method (Invitrogen). To remove contaminating DNA, total RNA (10 µg) of each treatment was treated with RNase-free DNaseI (Promega) at 37°C for 15 min and further incubated at 65°C for 10 min. Then, the cDNA was synthesized using 0.5 µg of DNase treated RNA samples, M-MLV reverse transcriptase (Invitrogen) and the oligo-dT (18 mer) primer. One microliter of each cDNA was used for PCR amplification with the corresponding primers and the wheat's actin gene was used as an internal control for gene expression (Table 1). The products were separated by electrophoresis on 1.5% agarose gel and quantified using the Gel DocXR Gel Documentation System (Bio-Rad). This software was used to calculate average band density which was recorded and used in graphic analyses. Band density was determined by this software and was given in arbitrary units and graphed using Microsoft Excel. The error bar was determined from three separate biological replicates. Each of three biological replicates consisted of pooled plants subjected or not to different stress conditions.

Protein extraction

Aliquots of frozen fresh shoot material (0.5 g) were ground to a fine powder with liquid nitrogen and homogenized in 1.5 mL of a cold solution containing

Table 1. Sequences of primers used for semi-quantitative RT-PCR analyses of *CAT*, *MnSOD* and *APX* genes and their accession numbers

Primer	Nucleotide sequence (5'-3')	Annealing site	Accession No.
ACTF	GTGCCCATTTACGAAGGATA	Actin	AY663392
ACTR	GAAGACTCCATGCCGATCAT		
CATF	CGACTTCGACCCGCTGGACGTGAC	CAT	GU984379
CATR	GCGTCGATCCATCTGTTGATGAATC		
SODF	GAAGCACCACGCCACCTACGTCGC	MnSOD	EF392662
SODR	TCACGCAAGCACTTTTTCATACTCT		
APXF	ATGGCGGCTCCGGTGGTGGACGCC	APX	EF555121
APXR	TACAATATCTTTGTCTGTTAACCCCA		

100 mM Tris-HCl buffer (pH 8.0), 10 mM EDTA, 50 mM KCl, 20 mM $MgCl_2$, 0.5 mM PMSF, and 2% (w/v) PVP. The homogenate was centrifuged at 14,000 × g for 30 min at 4°C, and the supernatant was used for determination of the antioxidative enzyme activities. Protein concentration was determined according to Bradford (1976).

Enzyme assays

The activity of SOD was monitored by the inhibition of photochemical reduction of NBT at 560 nm. The activity of SOD was determined by adding 50 µL of the enzymatic extract to a solution containing 50 µM NBT, 1.3 µM riboflavin, 13 mM methionine, 75 mM EDTA and 50 mM phosphate buffer (pH 7.8) (Giannopolitis & Ries, 1977).

CAT activity was determined according to Aebi (1984), by monitoring the disappearance of H_2O_2. Crude enzyme extract (0.1 mL) was added to 3 mL reaction mixtures containing 50 mM phosphate buffer (pH 7), 30 mM H_2O_2. Changes in optical density (OD) of the reaction solution at 240 nm were recorded every 20 s.

The activity of POD was determined according to Maehly & Chance (1954) by the guaiacol oxidation method. The final volume (3 mL) of the reaction mixture for POD contained 0.1 mL enzyme extract as well as 50 mM phosphate buffer (pH 7), 20 mM guaiacol, and 40 mM H_2O_2. Changes in OD of the reacted samples at 470 nm were recorded every 20 s.

APX activity was appraised following the method of Nakano & Asada (1981) with minor modifications. An aliquot of 0.2 mL enzyme extract was added to a mixture containing 0.8 mL of 50 mM potassium phosphate buffer (pH 7), 0.5 mM AsA, and 0.1 mM H_2O_2. The decrease in absorbance at 290 nm was recorded for 1 min. The enzyme activity of SOD, CAT, POD and APX was expressed as units/mg protein. One unit of SOD was defined as the enzyme quantity required causing 50% inhibition of the rate of NBT reduction at 560 nm in comparison with tubes lacking the plant extract. One unit of CAT and APX was defined as µmol/mL H_2O_2 decomposed per minute. One enzyme unit of POD is defined as change in 1 unit of absorbance per minute.

Electrophoresis and enzyme activity staining

Native-PAGE was carried out using 10% resolving gel at 4°C. POD isoforms were visualized by incubating the gel for 30 min in a 0.1 M sodium acetate buffer (pH 4.0) containing 1% (v/v) guaiacol. Then, gel was placed in a solution containing 4.7 mM 3-amino-9-ethylcarbazole, 38 mM N,N-dimethyl formamide, 0.1 M sodium acetate buffer (pH 5.0), 0.1 M $CaCl_2$ and 30% (v/v) H_2O_2. POD isoforms were appeared with brown bands after 10 to 20 min at 4°C (Vallejos, 1983). This experiment was repeated three times with similar results.

Statistics analysis

Data were analyzed using one-way ANOVA implemented in the SPSS software 13. Treatment mean separations were performed using Duncan's multiple range tests.

Results

Effect of NaCl concentration on shoots and roots growth

NaCl stress significantly reduced shoots and roots dry biomass of both durum wheat cultivars. However, this decrease was more pronounced in the salt-sensitive cultivar (Mahmoudi) than in the tolerant one (Om Rabia3). Indeed, the root growth of the sensitive cultivar was extensively inhibited at NaCl concentration up to 100 mM. In contrast, in the presence of low Na^+ in the medium (50 mM NaCl), shoots growth of these two wheat cultivars decreased significantly (Fig. 1).

Oxidative stress evaluation

In the present study, MDA content in the shoots of the two Tunisian durum wheat cultivars correlated with growth inhibition produced by NaCl stress. Moreover, MDA content increased significantly in cv. Mahmoudi shoots as compared to the other cultivar with the increase of NaCl concentration in the medium (Fig. 2).

The increase in the salt level of the medium caused a consistent increase in H_2O_2 in these wheat cultivars. Accumulation of H_2O_2 was superior in salt sensitive cultivar. Indeed, in the comparison with the non-treated plants, the presence of high NaCl concentration in the medium (200 mM NaCl) caused about three-fold increase on H_2O_2 content in cv. Mahmoudi. However, this increase was lower in cv. Om Rabia3 (Fig. 2).

Under standard conditions, a weak NBT staining was observed in the different part of these two wheat cultivars. After salt stress and in contrast to the cv. Om Rabia3, an intense NBT staining was observed on cv. Mahmoudi leaves and roots, indicating an increase in the amount of O_2^- (Fig. 3).

Ascorbate accumulation

In this study, shoots ascorbate content in both durum wheat cultivars was reduced by NaCl treatment.

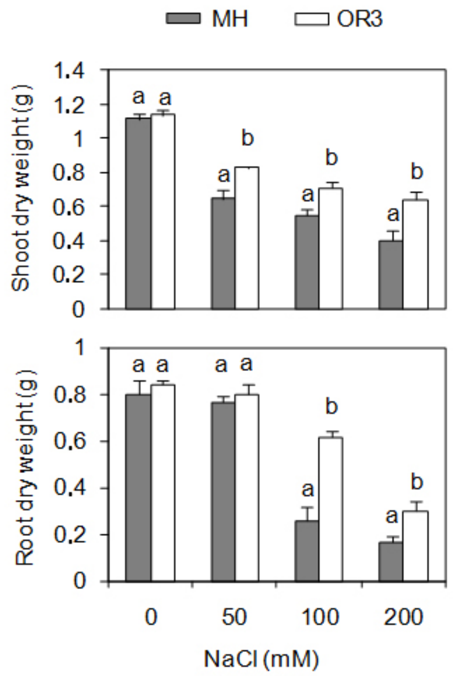

Figure 1. NaCl effects on dry weight (DW) of the two durum wheat cultivars Mahmoudi (MH) and Om Rabia3 (OR3). Average and standard deviation of five independent experiments are plotted. The means ± SE were calculated from five replicates (n = 5). Values having the same letter in each NaCl concentration are not significantly different at $p < 0.05$.

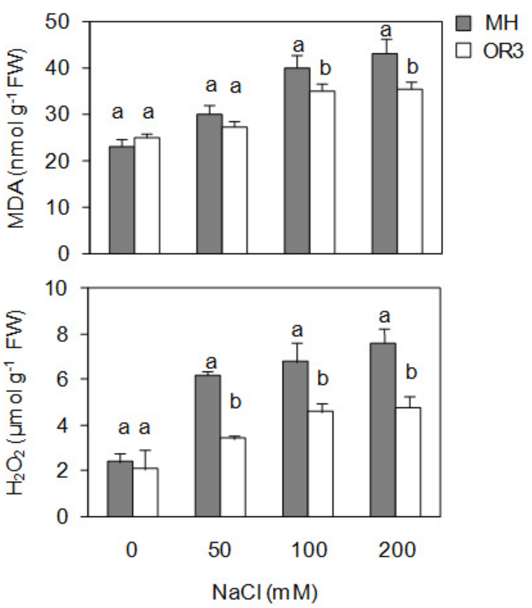

Figure 2. Determination of malondialdehyde (MDA) and hydrogen peroxide concentrations in the shoots of Mahmoudi (MH) and Om Rabia3 (OR3) treated or not with different salt concentrations. Values are means of 5 replicates ± SD (n = 5). Values having the same letter in each NaCl concentration are not significantly different at $p < 0.05$.

However, this decrease was significant in cv. Mahmoudi as compared to that in cv. Om Rabia3. Highest NaCl concentration in the medium caused a maximal decrease in ascorbate content in cv. Mahmoudi as compared to the other salt levels. Nevertheless, concerning the cv. Om Rabia3 the ascorbate content was stable at this high NaCl concentration (Fig. 4).

Expression analysis of *CAT*, *MnSOD* and *APX* genes

To monitor the expression profile of the three genes *CAT, MnSOD* and *APX* under salt stress, we performed RT-PCR analysis on the shoots of these two cultivars treated or not with NaCl for 3 days. Basal expression levels of these three genes were detected in the leaves of the non treated plants. After NaCl treatment, a significant increase in the level of these genes, about two to four times, was observed in the case of these two wheat cultivars. However, in the cv. Om Rabia3 the transcript accumulation of these genes was higher than in the cv. Mahmoudi. In addition, *CAT* and *MnSOD* transcripts were significantly accumulated under NaCl treatment compared to *APX* transcript in the cv. Om Rabia3 (Fig. 5).

Correlation between salinity and the activity of some antioxidant enzymes

In the present study, we observed a differential response in SOD activity in the two Tunisian wheat cultivars. Indeed, SOD activity of the salt tolerant cultivar (Om Rabia3) increased markedly with the increase in the external NaCl concentration, to attain a final activity about 5 times relative to unstressed plants. By contrast, SOD activity in the cv. Mahmoudi increased slightly and reached only about 3 times relative to the control plants.

Like SOD activity, the increase of CAT activity in these cultivars was different, and the CAT activity of cv. Om Rabia3 was higher than that in the cv. Mahmoudi in the presence of the three NaCl concentrations (Fig. 6).

In the two wheat cultivars, POD and APX activities increased significantly under salt stress compared to the non-treated plants. Moreover, this increase was higher in cv. Om Rabia3 than in cv. Mahmoudi. In the presence of high NaCl concentration in the medium (200 mM NaCl), POD activity of the salt-tolerant cultivar was about the double relative to the control plants and higher than that in cv. Mahmoudi. However, APX activity decreased in these two cultivars, compared to the treatment with medium NaCl concentration (100 mM NaCl). In addition, this decrease was significant in

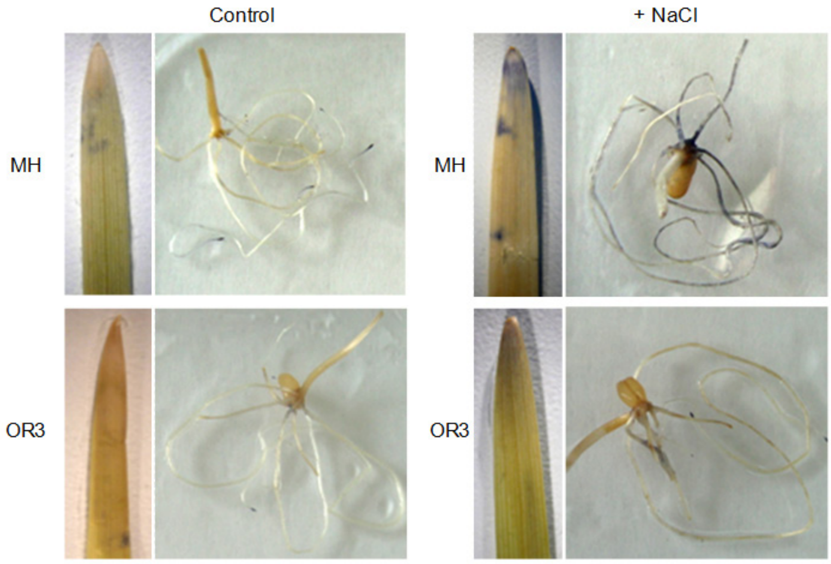

Figure 3. Detection of superoxide radical by NBT staining. Staining assay on the leaves, sheaths and roots based on NBT of the two durum wheat cultivars Mahmoudi (MH) and Om Rabia3 (OR3) treated or not with salt.

the cv. Mahmoudi compared to the other cultivar (Fig. 6).

Peroxidase isoform visualization

The isoenzyme composition of POD in these durum wheat cultivars treated or not with 200 mM NaCl was studied using a native PAGE. In normal condition, three main POD isoforms were revealed in these two cultivars. In the presence of 200 mM NaCl, an enhanced intensity of POD 3 isoform occurred in the two cultivars. However and compared to cv. Mahmoudi, an intense staining of POD 2 isoform was detected in the case of cv. Om Rabia3 treated with NaCl treatment (Fig. 7).

Discussion

Salinization of cropland in the Mediterranean region is a major limitation to crop yields. The ability to limit the accumulation of Na^+ in leaves may be an important mechanism in salt tolerance because the excessive accumulation of Na^+ causes the premature senescence of leaves (Pardo, 2010). In a previous work, it has been demonstrated that the response to NaCl treatment is different between the two Tunisian wheat cultivars (Om Rabia3 and Mahmoudi) at short and long-term. In this study, these two Tunisian wheat genotypes were grown for seven days and then subjected to NaCl treatment for short period (3 days). Significant differences of shoots and roots DW were detected between the non-treated and the stressed plants. Moreover, the salt-tolerant cultivar (Om Rabia3) presented higher roots and shoots DW than the salt-sensitive cultivar (Mahmoudi) under high NaCl concentration (Fig. 1). Similar to our findings, dry weight was less affected in salt tolerant sesame, sugar beet and moderately salt tolerant cotton (Greenway & Munns, 1980; Koca *et al.*, 2007).

Salt stress induces oxidative damage in many plants like pea, rice and tomato (Gómez *et al.*, 1999; Hernández *et al.*, 2001; Uchida *et al.*, 2002; Mittova *et al.*, 2003). The MDA content is considered as an indicator of the level of lipid peroxidation. Many studies

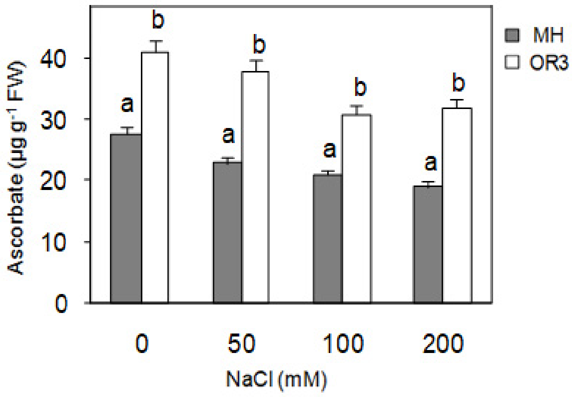

Figure 4. Effect of increasing NaCl concentration on ascorbate content in the two durum wheat cultivars Mahmoudi (MH) and Om Rabia3 (OR3). Average and standard deviation of three independent experiments are plotted. Values are means of 5 replicates ± SD (n = 5). Values having the same letter in each NaCl concentration are not significantly different at $p < 0.05$.

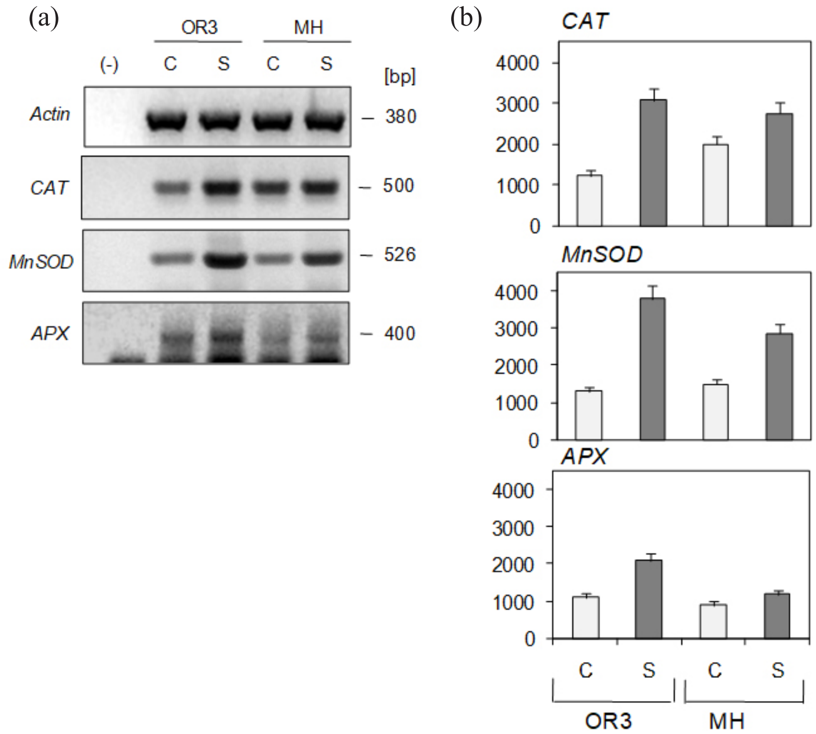

Figure 5. (a) Expression analysis of the *CAT, MnSOD* and *APX* genes in the shoot of the two durum wheat culitvars treated (S) or not (C) with salt for 3 days. MH, Mahmoudi; OR3, Om Rabia3. Actin gene was used as the positive control; (-) negative control without cDNA. (b) The histograms correspond to the band densities in the gels which are expressed in arbitrary units calculated by the analysis Gel DocXR software. The standard error was determined from three independent biologic replicates.

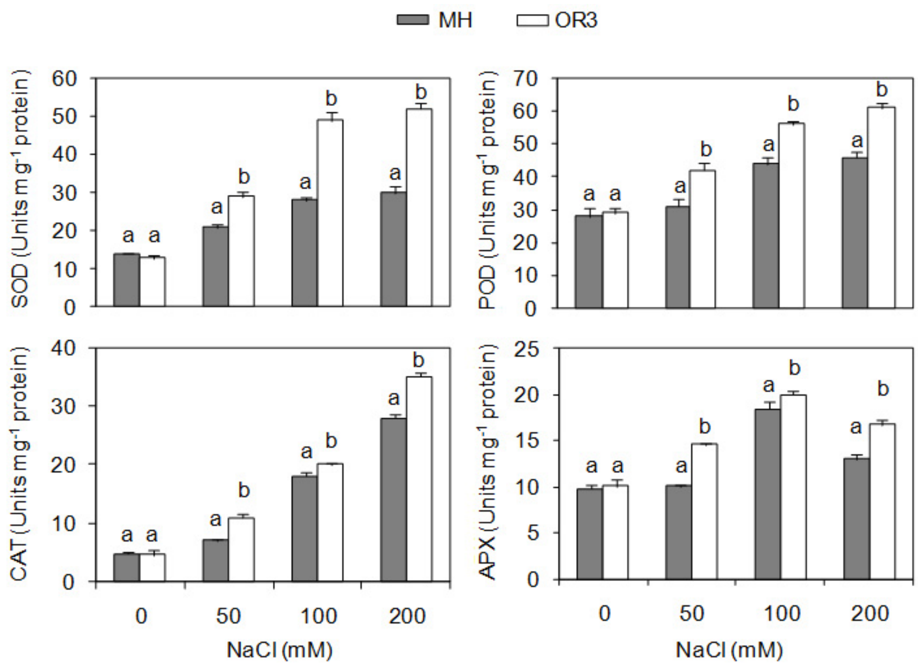

Figure 6. Antioxidant enzyme responses to NaCl treatments in durum wheat cultivars (MH, Mahmoudi; OR3, Om Rabia3). Values are means of 5 replicates ± SD (n = 5). Values having the same letter in each NaCl concentration are not significantly different at $p < 0.05$.

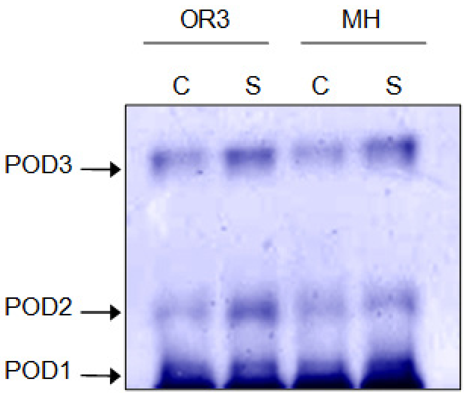

Figure 7. Activity staining of POD isozymes after a native PAGE of the cv. Mahmoudi (MH) and the cv. Om Rabia3 (OR3) leaves treated (S) or not (C) with NaCl.

showed that abiotic stresses lead to lipid peroxidation and consequently produce an accumulation of the MDA (Chaoui *et al.*, 1997; Mandhania *et al.*, 2006; Distelbarth *et al.*, 2012). In agreement with these observations, our results showed that salinity induced oxidative stress in these two Tunisian durum wheat leaves, manifested by an accumulation of O_2^- and an increase in MDA and H_2O_2 contents (Figs. 2, 3). Nevertheless, this increase was different between these two cultivars. Indeed, under NaCl treatment the level of lipid peroxidation and H_2O_2 content were higher in the cv. Mahmoudi than in the other cultivar. These data suggest that the cv. Om Rabia3 was better protected against oxidative damage under NaCl stress. Parallel to our results, low MDA content is important in terms of salt tolerance as represented in different studies. Salt tolerant barley (Liang *et al.*, 2003), tobacco (Ruiz *et al.*, 2005) and wheat cultivars (Sairam *et al.*, 2002, 2005; Mandhania *et al.*, 2006) also showed low level of lipid peroxidation which is important sign of higher oxidative damage limiting capacity under salinity.

AsA is the major antioxidant in the plant cell. As the generation of ROS is increased under stress conditions, AsA is believed to contribute actively in enhancing tolerance to various environmental stresses (Noctor & Foyer, 1998). It is clear that high level of endogenous AsA is essential effectively to maintain the antioxidant system that protects plants from oxidative damage due to abiotic and biotic stresses (Shigeoka *et al.*, 2002). Salt induces a decrease in AsA content in wheat at the vegetative stage (Sairam *et al.*, 2005) and at the reproductive stage (Athar *et al.*, 2008). In agreement with this observation, our study showed that salinity induces a decrease in AsA in the two durum wheat cultivars particularly in the cv. Mahmoudi (Fig. 4). This larger AsA decrease was also found in salt sensitive pea cultivar (Hernández *et al.*, 2001).

In *Arabidopsis* cells exposed to oxidative stress, some gene showed changes in expression levels. These genes encoded for proteins with antioxidant functions or were associated with defense responses or other stresses (Desikan *et al.*, 2001). For example, there are many reports on the changes in the activity of various SOD isoenzymes and corresponding mRNA under osmotic stresses (Zhu & Scandalios, 1994). Our results showed that higher NaCl concentration produces an elevate expression of *CAT, MnSOD* and *APX* genes (Fig. 5). Interestingly, the higher expression of these three genes was observed in the cv. Om Rabia3. *APX* gene is induced by various stress conditions suggesting his crucial role in many stress tolerance like drought and heat shock (Mittler & Zilinskas, 1992). Concerning the antioxidative enzymes activities, NaCl stress caused up-regulation of the activities in these wheat cultivars. Comparing the enzymatic activity in the two Tunisian durum wheat cultivars, the higher activities were obtained in the salt tolerant cultivar (Om Rabia3) than in the sensitive one (cv. Mahmoudi). This result suggested that the cv. Om Rabia3 presents higher capacity for scavenging ROS than the cv. Mahmoudi. Thus, the decrease in the content of H_2O_2 in cv. Om Rabia3 is the result of SOD reaction which is accompanied by an increased enzymatic capacity to decompose it. This was particularly clear in cv. Om Rabia3, in which a greater and parallel increase in SOD, CAT, POD, and APX activities occurred under low or high salinity (Fig. 6). The correlation between salinity tolerance and the increase of SOD activity has been demonstrated in many works (Hernández *et al.*, 2001; Sreenivasulu *et al.*, 2000; Ben Amor *et al.*, 2005; Mandhania *et al.*, 2006; Koca *et al.*, 2007; Ellouzi *et al.*, 2011). Increased SOD activity in the cv. Om Rabia3 at higher NaCl level probably coped with injuring effects of O_2^-. This suggestion was confirmed by the low amount of O_2^- in the different part of this cultivar. APX utilizes AsA as its specific electron donor to reduce H_2O_2 to water with the generation of monodehydroascorbate (Shigeoka *et al.*, 2002), and plays a crucial role in the management of ROS during stress (Ahmad *et al.*, 2008). It has been reported previously that salinity increases APX activity in many salt tolerant cultivars (Hernández *et al.*, 2001; Sairam *et al.*, 2005; Koca *et al.*, 2007). The susceptibility of the cv. Mahmoudi to salinity could be due to the inhibition of APX activity at higher salinity level. This inhibition was also observed previously in two bread wheat cultivars HD 2009 and HD 2687 (Sairam *et al.*, 2005). Concerning POD, our results showed that salt stress produces an enhancement of POD activity in the two durum wheat cultivars. Nevertheless, POD activity remained unchanged in the salt sensitive cv.

Mahmoudi treated with high NaCl concentration. Interestingly, the increase of POD activity in cv. Om Rabia3 is concomitant with the enhanced intensity of POD2 isoform, which probably produces more activity in this cultivar than in the cv. Mahmoudi. CAT is the main scavenger of strong oxidant H_2O_2 in peroxisomes and it converts H_2O_2 to water and molecular oxygen (Willekens *et al.*, 1995). Higher activities of CAT and APX decrease H_2O_2 level in cell and increase the stability of membranes. In both cultivars, CAT activity and salt stress effect increased in parallel. In the presence of high salinity level (200 mM NaCl), higher CAT activity was observed in the cv. Om Rabia3 resulting a better cope with reactive oxygen species. Similar to our results, higher CAT activity was found in many salt tolerant cultivars such as sesame, maize and wheat (Azevedo Neto *et al.*, 2006; Koca *et al.*, 2007). It is worth to note that the enzyme POD increased more its activity in the tolerant cultivar in response to salt stress. Consequently, the low amount of H_2O_2 in the cv. Om Rabia3 under high salt stress condition could be attributed to an increased detoxification capacity mediated by the POD2 isoform activity (Fig. 7). Taken together, it seems that the salt tolerance phenotype of the cv. Om Rabia3 could be due to a low production of ROS or to its better ability to counteracting ROS than the salt sensitive cultivar.

In this study, we show that the two Tunisian durum wheat cultivars respond differently to salt stress. Salt leads to oxidative stress and it is an abiotic elicitor of antioxidative defenses. The better development of the cv. Om Rabia3 plant results from the greater efficiency of the antioxidative response under NaCl stress conditions. In fact, in this cultivar the antioxydant enzyme system is enhanced more efficiently compared to the salt sensitive cultivar with higher capacity to accumulate ascorbate. Consequently, under saline conditions, the growth inhibition of this cultivar is retarded with low lipid damage. Thus, it is important to understand the genetics of detoxification of ROS in order to enhance crop salt tolerance.

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