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Osmoprotectants and Antioxidative Enzymes as Screening Tools for Salinity Tolerance in Radish (*Raphanus sativus*)

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A B S T R A C T

The research addresses the identification of a screening methodology for salt stress tolerance in radish cultivars. In the first experiment, two different radish cultivars (long white and round red) were compared in their morphological and physiological responses to different salinity levels. Round red radish showed better morphological and physiological responses to incremental salinity in terms of yield and better adaptation of overall water relations. In the second experiment, the most tolerant genotype from the first experiment was used as a control against other seven round red radish genotypes ranked by their salinity tolerance according to morphological, physiological and biochemical indices. Salt stress did not significantly affect malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content, and ascorbate peroxidase (APX) activity in the studied cultivars. Nonetheless, the relatively salt tolerant cultivar SAXA2 showed higher ability to accumulate compatible solutes (e.g. proline and proteins) and maintain osmotic adjustment. In addition, cultivar SAXA2 also showed considerable increase in glutathione reductase (GR) activity. Our results supported that accumulation of proline and higher GR activity are associated with radish salt tolerance, whereas no relationship with salinity was observed in superoxide dismutase (SOD), MDA and H_2O_2 content.

Keywords: radish; salt stress; oxidative stress; secondary metabolite; antioxidant enzyme

1. Introduction

Soil salinity is one of the major abiotic stresses that adversely affect plant productivity and quality (Ayyub et al., 2016). It was estimated that up to 20% of irrigated lands in the world are affected by different levels of salinity and sodium content (Orsini et al., 2011). Crop salinity sensitivity varies with species, genotypes, and growth stages (Pujari and Chanda, 2002). Consistently, genotypic diversity may provide useful traits for improving salt tolerance (Maggio et al., 2005). Salt stress induces various biochemical and physiological responses in plants and affects almost all plant processes like photosynthesis, nitrogen metabolism, ion homoeostasis (Ashraf, 2009), proline metabolism and osmolytes accumulation (Kilani et al., 2014). Salt stress causes stomatal closure, which reduces the CO_2/O_2 ratio inside leaf tissues and inhibits CO_2 fixation. Consequently, an over reduction of the photosynthetic electron transport chain occurs, which causes the generation of Reactive Oxygen Species (ROS) (Asada, 2006). In addition, excess of Na^+ and Cl^- ions may lead to changes in the protein structure, while osmotic stress leads to turgor loss and cell volume change (Errabii et al., 2007). To achieve salt tolerance, plant cells have evolved several biochemical and physiological pathways such as the exclusion of Na^+ ions and their compartmentation into vacuoles as well as accumulation of compatible solutes such as proline, glycinebetaine, and polyols (Errabii et al., 2007). Plants are equipped with an array of enzymatic and non-enzymatic antioxidant molecules to alleviate cellular damage caused by ROS (Ozyigit et al., 2016). Several known antioxidant enzymes are involved in the scavenging of ROS. Superoxide dismutase (SOD) reacts with the superoxide radical to produce H_2O_2 (Xing et al., 2015). Catalase (CAT) has been found predominantly in leaf peroxisomes where it functions chiefly to

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remove H_2O_2 formed in photorespiration or in β-oxidation of fatty acids in the glyoxysomes (Dat et al., 2000). Ascorbate peroxidase (APX), which uses ascorbic acid as a reductant in the first step of the ascorbate glutathione cycle, and glutathione peroxidase (GPX) that uses glutathione as electron donors, are the most important peroxidases involved in H_2O_2 detoxification and increase the stability of membranes and CO_2 fixation (Asada, 2006). Glutathione reductase (GR) is responsible for the reduction of oxidized glutathione, for the chain reactions of scavenging H_2O_2 by APX and GPX to be completed and continued (Ozyigit et al., 2016).

It is generally assumed that salt-tolerant genotypes of most plant species have higher antioxidant enzyme activities than those of salt-sensitive ones (Abd Elgawad et al., 2016) although in some cases the reverse is also true (Munns and Tester, 2008). Many authors proved that the antioxidant enzyme systems are altered under abiotic stresses, including salinity, and the changes are often related to the levels of plant resistance to the stress. In rice, salt-tolerance has been evidenced in varieties that have higher SOD activity and lower lipid peroxidation as compared with salt-sensitive ones (Hazman et al., 2015). Similarly, in tomato and citrus, salt-tolerance has been attributed to increased activities of SOD, APX, and CAT (Mittova et al., 2004).

To date, most of the available research on radish (*Raphanus sativus* L.) addressed the identification of phytochemical compounds associated with beneficial health effects such as phenolics and anthocyanins (Sgherri et al., 2003; Wang et al., 2010). However, there is lack of studies about the effect of salt stress on physiological and metabolic features, as well as enzymatic activities of radish. The aim of present work was to define to which extent antioxidant enzymes are involved in functional salt stress response in radish based on the presence of intraspecific variation, and to propose their use for rapid population screening and salt tolerance breeding.

2. Materials and methods

2.1. Plant materials and growth conditions

Two experiments were conducted in an experimental glasshouse at the Department of Agricultural Sciences (DipSA) of the University of Bologna (Italy) under controlled conditions (T_{max} 23 °C; T_{min} 13 °C; RH 60%). In both experiments, seeds were provided by Esasem seed company (Verona, Italy). Experiment 1 included two cultivars of radish named: cv. SAXA2 (Round red radish) and cv. Candela di ghiaccio (Long white radish). In Experiment 2, seven round red radish cultivars were used: cv. SAXA2 (control, the most tolerant cultivar from Experiment 1); cv. Tondo Precocissimo (TP); cv. SAXA; cv. Lungo a Punta Bianca (LPB); cv. Lungo Rosso a Punta Bianca (LRPB); cv. Tondo a Punta Bianca (TPB); and cv. Tondo a Piccola Punta Bianca (TPPB). The first experiment lasted for 52 days while the second one lasted 42 days. Seeds were sown in polyethylene trays filled with peat moss. Seven days after germination seedlings were transplanted onto small plastic pots filled with a mix of perlite and vermiculite (1:1, v:v). Salt application started 4 days after transplanting (DAT) in both experiments by applying three salt concentrations: 0 (2.68 $dS \cdot m^{-1}$), 100 (7.68 $dS \cdot m^{-1}$) and 200 (8.72 $dS \cdot m^{-1}$) $mmol \cdot L^{-1}$ NaCl dissolved in the nutrient solution. The growing system was an open cycle hydroponic with nutrient solution, whose chemical composition was as follows: 16.5 $mmol \cdot L^{-1}$ NO_3^-, 1 $mmol \cdot L^{-1}$ NH_4^+, 1.50 $mmol \cdot L^{-1}$ $H_2PO_4^-$, 1.50 $mmol \cdot L^{-1}$ SO_4^{2-}, 7.0 $mmol \cdot L^{-1}$ K^+, 5.0 $mmol \cdot L^{-1}$ Ca^{2+}, 1.5 $mmol \cdot L^{-1}$ Mg^{2+}, 15 $\mu mol \cdot L^{-1}$ Fe^{2+}, 10 $\mu mol \cdot L^{-1}$ Mn^{2+}, 25 $\mu mol \cdot L^{-1}$ B^{3+}, 5.0 $\mu mol \cdot L^{-1}$ Zn^{2+}, 0.5 $\mu mol \cdot L^{-1}$ Cu^{2+}, 0.5 $\mu mol \cdot L^{-1}$ Mo^{2+}. Harvest was performed at 40 and 30 days after salt stress application (DAS) in Experiment 1 and Experiment 2 respectively.

2.2. Growth measurements and leaf water relations

At harvest, nine plants from each treatment (three per replicate) in both experiments were sampled to determine shoots and roots fresh weight (FW). Leaf area (LA) was measured on digital images by Image J Bundled processing software (Orsini et al., 2011). Plant growth was defined by both the Relative Growth Rate (RGR) and the Net Assimilation Rate (NAR), calculated using the equations:

$$RGR = (\ln DM_2 - \ln DM_1)/(t_2 - t_1) \qquad (g \cdot g^{-1} \cdot d^{-1})$$

$$NAR = [(DM_2 - DM_1)/(LA_2 - LA_1)] \times [(\ln LA_2 - \ln LA_1)/(t_2 - t_1)] \qquad (g \cdot m^{-2} \cdot d^{-1})$$

Where DM_1 was the initial (15 DAS) total (shoot + root) dry mass, DM_2 the final (30 DAS) total dry mass, LA_1 the initial leaf area, LA_2 the final leaf area, and ($t_2 - t_1$) the difference in time interval between the two samplings (15 d). Leaf water potential (Ψ_w) was determined by using a dew-point psychrometer (WP4, Decagon Devices, Washington, WA) on freshly harvested leaf discs (Sanoubar et al., 2015). The osmotic potential (Ψ_π) was measured with the same instrument on frozen/thawed leaf samples. Turgor potential (Ψ_p) was defined as the difference between water (Ψ_w) and osmotic (Ψ_π) potentials. Leaf gas exchange measurements such as net photosynthetic rate (*A*), stomatal conductance (G_s), and transpiration (*E*) were assessed by CIRAS-2 infrared gas analyser (PP-system Hitchin, UK). Punctual water use efficiency (WUE) was calculated as the ratio of *A* to *E*. Water loss (WL) determinations were performed at 15 DAS. Three plant pots for each treatment were sealed with a plastic film to prevent water loss from the soil surface, leaving the shoot protruding from the film. Before sealing, plants were re-watered to pot capacity with water (control) or water plus 100 or 200 $mmol \cdot L^{-1}$ NaCl. Each plant was then placed on an electronic balance and the weight loss was measured after 24 h. WL values were normalized to the whole plant dry weights taken at the end of the measurements.

2.3. Enzyme extraction and assays

Only in Experiment 2, analysis of enzymatic response to salinity was performed on plants' root tissues. For protein and antioxidant enzyme extraction, 10 g of fresh roots were homogenized in 10 mL of 200 $mmol \cdot L^{-1}$ chilled potassium-phosphate buffer (pH 7.5) containing 1% (v:v) insoluble polyvinylpolypyrrolidone (PVPP) and 0.1% (v:v) Triton X-100 placed in an ice bath. The homogenate was filtered through a layer of muslin cloth and centrifuged at 10 000 × *g* for 20 min at 4 °C. The supernatant was collected and eluted through Sephadex G-25 gel column (NAP-25, Amersham Biosciences, California, USA), then re-suspended in 10 $mmol \cdot L^{-1}$ *sodium-potassium phosphate buffer* (pH 7.0) and

used for the determination of the antioxidant enzymes. All enzymatic activities were assayed spectrophotometrically. The analysis was performed in triplicate and the results were normalized to plant fresh weight. The soluble proteins concentration of the extract was estimated according to Bradford's method using bovine serum albumin as a standard (Bradford, 1976).

2.3.1. *Malondialdehyde (MDA)*

The level of lipid peroxidation was determined by measuring malondialdehyde (MDA) formation using the thiobarbituric acid-reactive substance (TBA) method (Heath and Packer, 1968). For MDA extraction, 100 μL enzyme extract was mixed with 900 μL thiobarbituric acid solution containing 0.5% (w:v) 2-thiobarbituric acid and 0.5 mol·L^{-1} orthophosphoric acid. The mixture was heated in a water bath at 100 °C for 30 min then the reaction was quickly stopped by cooling the tubes in an ice water bath. Afterward, the mixture was centrifuged for 1 min at 13 000 × g to remove the unspecific turbidity. The absorbance of the supernatant was measured at 532 nm using spectrophotometer Cary-1 (Varian, California, USA). Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The amount of MDA-TBA complex (red pigment) was calculated from the difference of the two wavelengths based on a standard curve of MDA.

2.3.2. *Hydrogen peroxide (H_2O_2)*

The H_2O_2 content was determined by measuring the colorimetric reaction of xylenol orange with Fe (III) generated after the oxidation of Fe(II) by H_2O_2 (Sanoubar et al., 2015). Fresh root tissue was homogenised with 4 mL 36 mmol·L^{-1} H_2SO_4 + 1% PVPP and the homogenate was centrifuged at 10 000 × *g* for 20 min at 4 °C. The Fe-xylenol orange reagent (FOX) contained 100 μmol·L^{-1} xylenol orange, 250 μmol·L^{-1} $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 100 mmol·L^{-1} sorbitol solved in 25 mmol·L^{-1} H_2SO_4. For H_2O_2 extraction, 50 μL plant sample extract was mixed with 950 μL of FOX reagent and the mixture was incubated for 30 min at least. The absorbance of the supernatant was measured at 560 nm. The amount of H_2O_2 was calculated based on standard curve.

2.3.3. *Ascorbate peroxidase (APX)*

Ascorbate peroxidase activity was determined using an ascorbate reaction solution contained 50 mmol·L^{-1} sodium-potassium phosphate buffer (pH 7.0), 1 mmol·L^{-1} ascorbic acid, 0.5 mmol·L^{-1} hydrogen peroxide and 100 μL enzyme extract in a final assay volume of 1 mL (Chen et al., 2007). Ascorbate oxidation was followed at 290 nm. The concentration of oxidized ascorbate was calculated using an extinction coefficient $\varepsilon = 2.8$ L·mmol^{-1}·cm^{-1}. One unit of APX is defined as the enzyme activity catalysing the oxidation of 1 μmol ascorbic acid per minute.

2.3.4. *Catalase activity (CAT)*

Catalase activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Havir and McHale, 1987). Catalase reaction solution contained 50 mmol·L^{-1} sodium-potassium phosphate buffer (pH 7.0), 10 mmol·L^{-1} H_2O_2 and 20 μL enzyme extract in a final assay volume of 1 mL. The reaction was initiated by adding the enzyme extract and the decrease in H_2O_2 was measured following the changes in the absorbance of the reaction solution at 240 nm. The concentration of CAT was calculated using an extinction coefficient $\varepsilon = 0.036$ L·mmol^{-1}·cm^{-1}. One unit of CAT is defined as the enzymatic activity that catalyses the degradation of 1 μmol H_2O_2 per minute.

2.3.5. *Glutathione reductase (GR)*

Glutathione reductase activity was determined using a reaction solution contained 50 mmol·L^{-1} sodium-phosphate buffer (pH 7.5), 5 mmol·L^{-1} EDTA, 1 mmol·L^{-1} NADPH, 1 mmol·L^{-1} oxidized glutathione (GSSG) and 300 μL enzyme extract in a final assay volume of 1 mL. NADPH oxidation was determined at 340 nm (Foyer et al., 1991). Activity was calculated using an extinction coefficient $\varepsilon = 6.22$ L·mmol^{-1}·cm^{-1} for NADPH. One unit of GR is defined as the enzyme activity that oxidizes 1 μmol NADPH per min.

2.3.6. *Superoxide dismutase (SOD)*

Superoxide dismutase activity was evaluated by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) to blue formazan by flavins under illumination (Masia, 1998). Superoxide dismutase reaction solution contained 50 mmol·L^{-1} sodium-potassium phosphate buffer (pH 8.0), 300 μmol·L^{-1} methionine, 1.5 mmol·L^{-1} NBT, 120 μmol·L^{-1} riboflavin, 100 mmol·L^{-1} Na_2·EDTA, 300 μmol·L^{-1} potassium cyanide and 100 μL enzyme extract in a final assay volume of 1 mL. The riboflavin was added last. The reaction was started by illuminating the test tubes under 4 fluorescent lamps for 10 min. The absorbance of illuminated solution was measured by spectrophotometry at 560 nm. One unit of SOD activity is defined as the amount of enzyme that inhibited 50% of NBT photoreduction versus a blank cell containing no enzymatic extract.

2.3.7. *Proline level*

Free proline content was measured using a reaction solution contained 3 mmol·L^{-1} ninhydrin in 60% (v:v) acetic acid (Bates et al. 1973). The samples were heated at 100 °C for 1 h in water bath and then cooled in tap water to stop the reaction. The mixture was extracted with toluene and the absorbance of toluene fraction aspired from liquid phase was read at 520 nm. Proline concentration was determined using a calibration curve and expressed as μmol·g^{-1} FW.

2.4. *Statistical analysis*

Randomized block design, with three replications and 9 plants per treatment, was used in both experiments. A two-way analysis of variance (ANOVA) was performed using Co-Stat-ANOVA software program. The means and calculated standard errors are reported. The significant differences were defined using Student–Newman–Keuls at 5% significance.

3. Results

3.1. *Growth parameters in Experiment 1*

No genotypic differences were observed between the red and white cultivars in term of yield, RGR, and NAR under control conditions (Fig. 1, A, C and D). Red plants exposed to 200 mmol·L^{-1} NaCl showed a lower reduction of yield and plant LA (respectively −71% and −81% from control) compared to the white cultivar (respectively −91% and −94% from control) (Fig. 1, A and B). Furthermore, significant reduction in RGR was observed in the

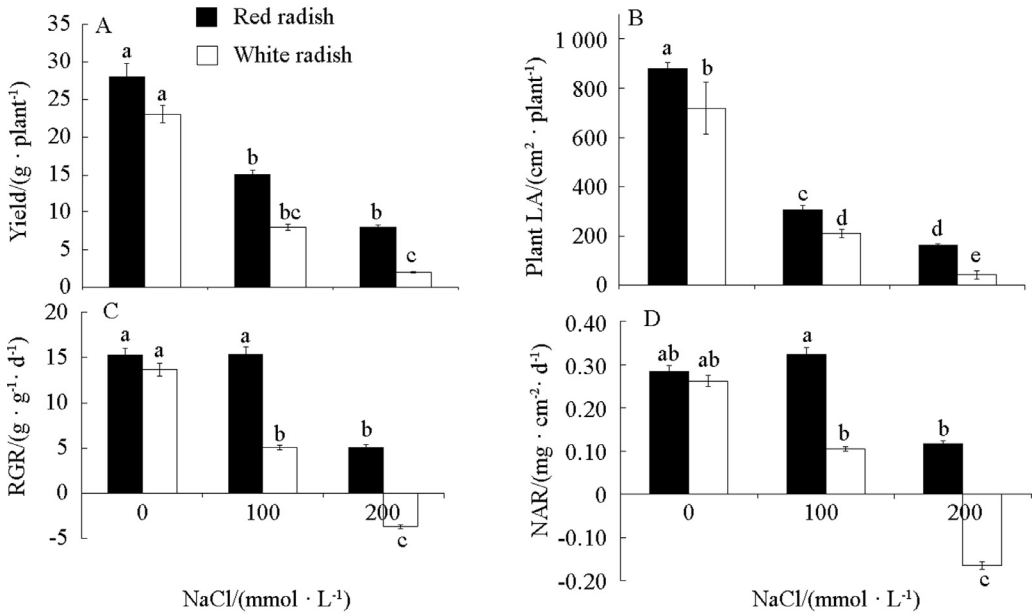

Fig. 1 Effect of different NaCl concentrations on yield (at 40 days after salt stress), leaf area, RGR and NAR (at 30 days after salt stress) in red and white radish seedlings

Data are expressed as mean values ± *SE* of three replications ($n = 27$). Different letters indicate significant differences among treatments at $P \leq 0.05$.

white radish plants upon 100 mmol·L^{-1} NaCl (−63% from control), while red plants showed no change (Fig. 1, C). However, under 200 mmol·L^{-1} NaCl, higher reduction of RGR value was found in white radish compared to the red one (−127% versus −67% from control) (Fig. 1, C). Similarly, NAR was reduced up to −60% in white radish exposed to 100 mmol·L^{-1} NaCl, whereas no salinity effects on this parameter was visible in the red cultivar (Fig. 1, D). 200 mmol·L^{-1} NaCl caused higher reduction in NAR in white radish compared to red one (−163% versus −59% from control) (Fig. 1, D).

3.2. *Plant water relations in experiment 1*

Transpiration rate (*E*), stomatal conductance (G_s) and net photosynthetic rate (A) showed a significant reduction at 100 mmol·L^{-1} NaCl (−79%, −87% and −45%, respectively) as compared to control treatments (Fig. 2, A, B and C). Transpiration rate and stomatal conductance did not present differences between moderate (100 mmol·L^{-1} NaCl) and high (200 mmol·L^{-1} NaCl) salt stress treatments in both cultivars (Fig. 2, A and B), while net photosynthetic rate of plants exposed to 200 mmol·L^{-1} NaCl presented lower values (−76%) as compared to those exposed to 100 mol·L^{-1} NaCl for both cultivars (Fig. 2, C). Water use efficiency (WUE) increased substantially (275%) in the salt-exposed red cultivar (comparing control value to the mean value of 100 and 200 mmol·L^{-1} NaCl), while it showed a sharp reduction (−72%) in the white one at 200 mmol·L^{-1} NaCl as compared to control conditions (Fig. 2, D). While water and osmotic potential reduction (Ψ_w and Ψ_π) were not affected by genotype variation at 100 mmol·L^{-1} NaCl (Fig. 2, E and F), 200 mmol·L^{-1} NaCl application produced a more dramatic reduction in both Ψ_w and Ψ_π in the white cultivar (−75% and −21%, respectively) as compared to the red ones. Consistently, red cultivar maintained higher turgor potential (Ψ_p, 248%) than white cultivar at 200 mmol·L^{-1} NaCl (Fig. 2, G). On the other hand, water loss values in white radish were 2-fold higher than those observed in the red ones under control condition (Fig. 2, H). Upon salt stress, however, a greater reduction in water loss was experienced in the white cultivar as compared to the red one (−67% and −57%, respectively, mean value of the salt treatments as compared to control; Fig. 2, H).

3.3. *Growth parameters in experiment 2*

Genotypic variability in shoot fresh weight (FW) among the round red radish cultivars was observed in non-treated plants, particularly, with TP cultivar that presented the highest shoot FW values (25 g·plant^{-1}), followed by SAXA2 (17 g·plant^{-1}, Fig. 3, A). At 100 mmol·L^{-1}, all cultivars responded to salinity with a significant reduction in shoot FW compared to control plants. Though more marked effect on shoot FW values were recorded in 200 mmol·L^{-1} NaCl, the highest FW values were observed in TP and SAXA2 cultivars (mean value 5.5 g·plant^{-1}) versus in all other cultivars (2.4 g·plant^{-1}).

A similar trend was observed in yield data. The highest yield under control conditions were in both SAXA2 and TP cultivars (mean value 22.5 g·plant^{-1}), followed by SAXA and LRPB (mean value 19.5 g·plant^{-1}), TPPB (18 g·plant^{-1}), LPB (16 g·plant^{-1}) and TPB (14 g·plant^{-1}, Fig. 3, B). Upon 100 mmol·L^{-1} salinity, higher yield values were obtained in both TP and SAXA2 (7 and 6 g·plant^{-1}, respectively), whereas yield was dramatically reduced in all other cultivars (mean value 2.8 g·plant^{-1}). Finally, significantly lower reductions in yield were in both TP and SAXA2 cultivars (4 g·plant^{-1}) versus in all cultivars (mean value 2.5 g·plant^{-1}) at 200 mmol·L^{-1} NaCl .

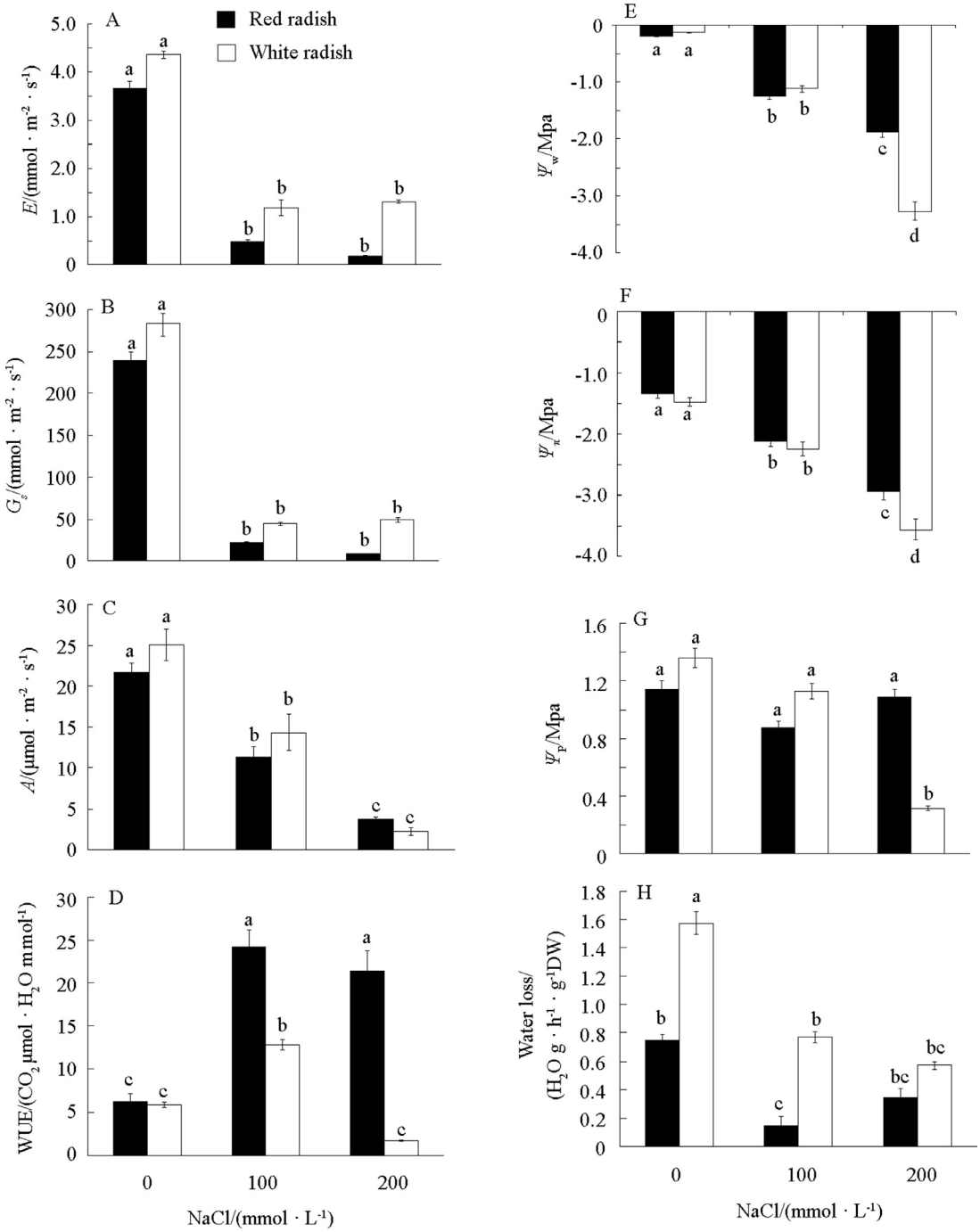

Fig. 2 Effect of different NaCl concentrations on transpiration rate (*E*), stomatal conductance (G_s), net photosynthetic rate (*A*), water use efficiency (*WUE*), water potential (Ψ_w), osmotic potential (Ψ_p), turgor potential (Ψ_p) and water loss (WL) in red and white radish seedlings at 40 DAS

Data are expressed as mean values ± *SE* of three replications ($n = 27$). Different letters indicate significant differences among treatments at $P \leq 0.05$.

3.4. *Plant water relations in experiment 2*

Under control conditions, net assimilation rate (*A*) did not show significant differences between the studied cultivars (mean value of 22 $\mu mol \cdot m^{-2} \cdot s^{-1}$) (Fig. 4, A). Upon 100 and 200 $mmol \cdot L^{-1}$ NaCl application, there was a dramatic reduction in average *A* values. However, the differences in *A* value between the two salt treatments were only significant in LPB and LRPB. A significant interaction between cultivar and salinity was observed in WUE (Fig. 4, B). While salinity did not affect WUE in TP, SAXA and LRPB cultivars, 200 $mmol \cdot L^{-1}$ NaCl resulted in lower WUE values in LPB, TPB and TPPB. Furthermore, SAXA2 cultivar increased WUE in 100

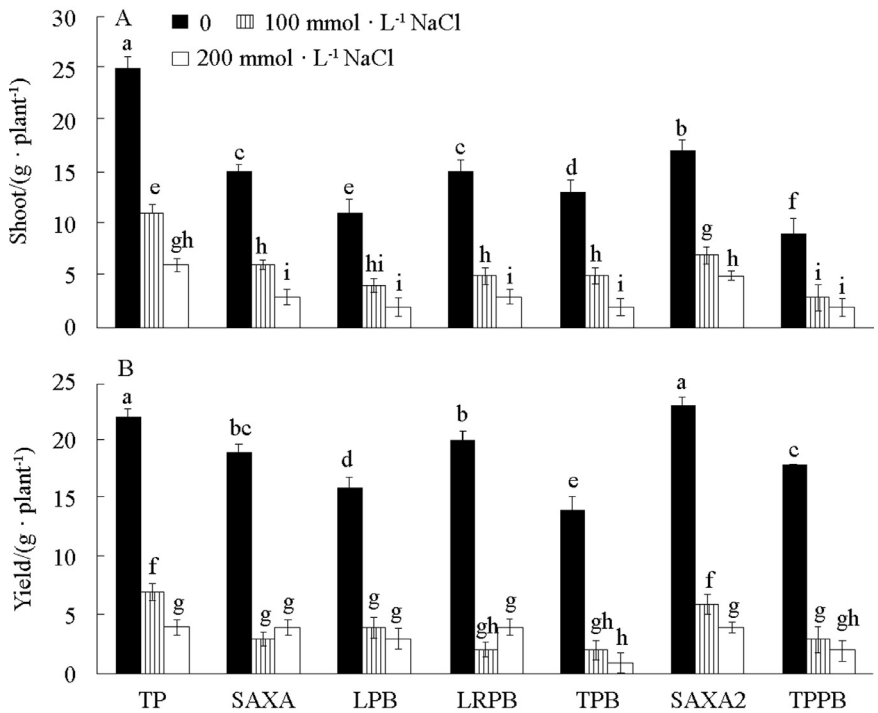

Fig. 3 Effect of different NaCl concentrations on shoot fresh weight (FW) and yield of seven round red radish seedlings
Data are expressed as mean values ± *SE* of three replications ($n = 27$). Different letters indicate significant differences among treatments at $P \leq 0.05$.

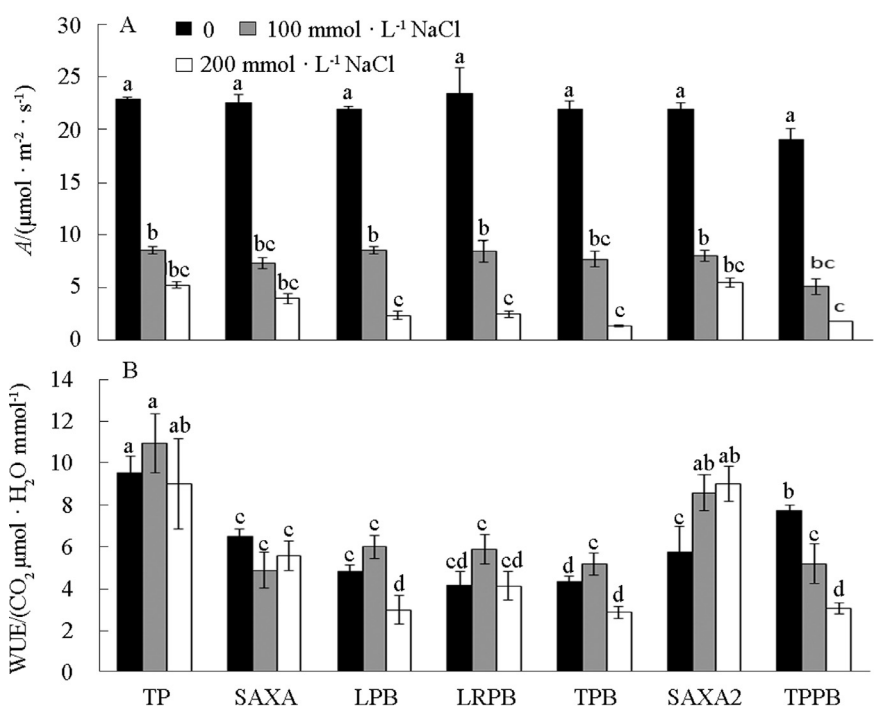

Fig. 4 Effect of different NaCl concentrations on net assimilation rate (*A*) and water use efficiency (WUE) of seven round red radish seedlings
Data are expressed as mean values ± *SE* of three replications ($n = 27$). Different letters indicate significant differences among treatments at $P \leq 0.05$.

and 200 mmol·L^{-1} NaCl compared to control treatment. Both water (Ψ_w) and osmotic (Ψ_π) potentials were reduced by salinity in all studied cultivars (data not shown), similarly to what observed in Experiment 1.

3.5. *Root biochemical composition in experiment 2*

Total soluble proteins content was significantly different in SAXA2 cultivar in 200 mmol·L^{-1} NaCl, about 2-fold increase than the other measured values (Fig. 5, A). All control plants showed constitutively comparable amount of proline (Fig. 5, B). TP and SAXA2 cultivars showed the highest value of proline accumulation (14.1 μmol g^{-1} FW) in 200 mmol·L^{-1} NaCl and significant differences between the two salt treatments. Contrarily, no significant differences in proline content between the salt treatments were observed in SAXA, LRPB, TPB and TPPB (Fig. 5, B). MDA and H_2O_2 levels were not influenced by genotype or salinity. Similarly, salinity and cultivars did not affect APX concentration (data not shown).

Control plants of cultivars TP and SAXA2 constitutively showed the highest CAT activity, followed by TPB, LPB, TPPB, LRPB, and SAXA (Fig. 6, A). Saline levels significantly reduced CAT activity in all cultivars compared with control treatments with the exception of SAXA. However, SAXA2 in 200 mmol·L^{-1} NaCl showed less reduction in CAT activity (52%) as compared to control plant while this reduction was higher in all other cultivars (83%, as a mean value). On the contrary, salinity caused an appreciable increase in GR activity in all plants and particularly in SAXA2 and TP cultivars (Fig. 6, B). SOD activity increased from control conditions in all cultivars in 100 mmol·L^{-1} NaCl (mean value of 91 U·g^{-1} FW), while a decrease down to control conditions or below in all cultivars was observed when 200 mmol·L^{-1} NaCl was supplied (Fig. 6, C).

3.6. *Correlation matrix of experiment 2*

Correlation analyses were conducted between different salt concentrations and some biochemical indices (Table 1). Correlation matrix revealed a slight positive correlation of salt stress with protein and relatively high correlation with proline content as well as GR activity, whereas negative relationships were presented amongst CAT, APX, SOD, MDA, and H_2O_2 concentrations.

Table 1 Correlation matrix for biochemical traits of red radish plants grown in salt stress condition

	Salt	Protein	Proline	CAT	GR	APX	SOD	MDA
Protein	0.11							
Proline	0.88	0.22						
CAT	−0.68	0.26	−0.62					
GR	0.54	0.31	0.78	−0.36				
APX	−0.43	−0.03	−0.33	0.00	−0.26			
SOD	−0.16	−0.17	0.14	−0.30	0.45	0.13		
MDA	−0.16	0.47	0.09	0.29	0.23	0.21	−0.01	
H_2O_2	−0.15	−0.01	0.03	−0.09	0.07	0.32	0.34	0.34

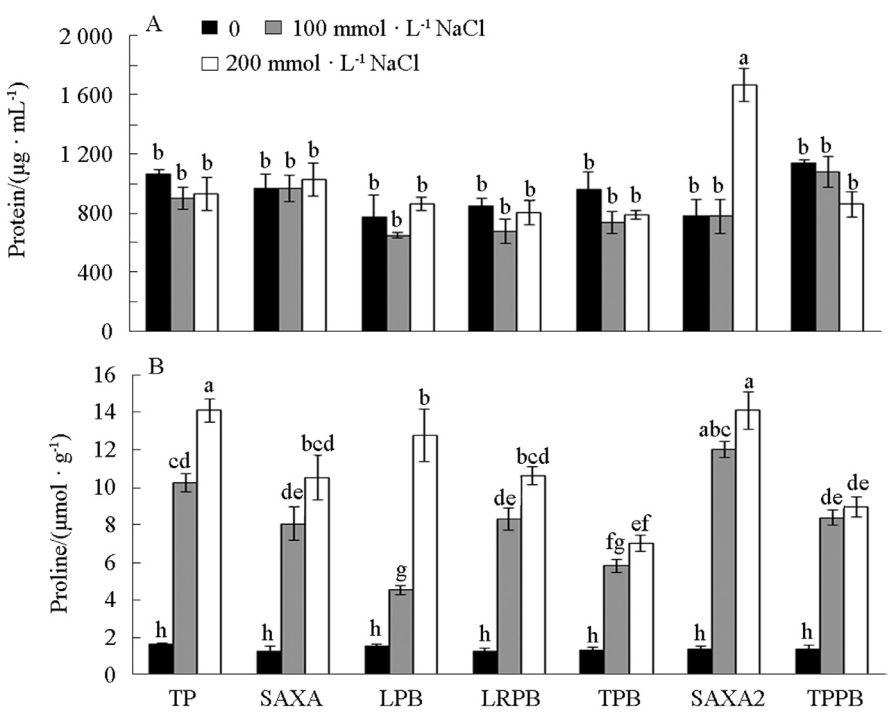

Fig. 5 Effect of different NaCl concentrations on proteins and proline contents of seven round red radish seedlings
Data are expressed as mean values ± *SE* of three replications ($n = 27$). Different letters indicate significant differences among treatments at $P \leq 0.05$.

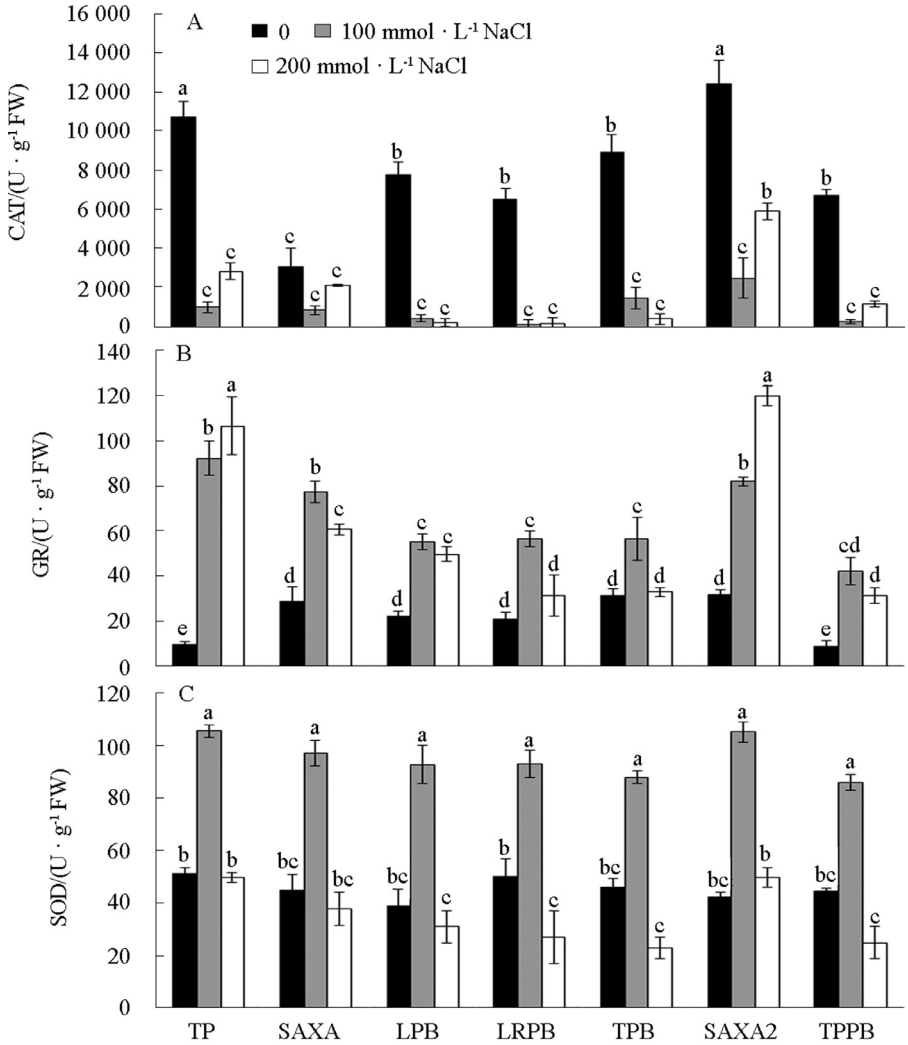

Fig. 6 Effect of different NaCl concentrations on CAT, GR, and SOD activities of seven round red radish root
Dara are expressed as mean values ± *SE* of three replications ($n = 27$). Different letters indicate significant differences among treatments at $P \leq 0.05$.

4. Discussion

4.1. *Vegetative growth in response to salinity*

A previous study indicated that radish could tolerate salt stress up to 12.7 dS·m^{-1} (Yildrim et al., 2008). Upon salinity, the nutrient's availability to plants is hampered (Munns and Tester, 2008; Ashraf, 2009) and this leads to diminished plant development (Chen et al., 2007). In Experiment 1, greater reduction in yield and leaf area were observed in the white cultivar as compared with the red one upon 200 mmol·L^{-1} NaCl, these were the most commonly adopted agronomic selection parameters (Ashraf, 2009). The photosynthetic capacity in crop plants is vital for dry matter production (Ulfat et al., 2007), where the final economic yield can be improved either by increasing the net photosynthetic rate or by optimizing assimilate partitioning (Orsini et al., 2011). All data in Experiment 1, based on biomass production and yield, showed a better performance of the red cultivar compared to white one under saline condition. Accordingly, a greater salt tolerance might be presented in red plants comparing with the white one. In Experiment 2, the salt tolerant genotype identified in Experiment 1 (SAXA2) was assessed against other six red radish cultivars to evaluate the genotypic variation in term of morphological, physiological and biochemical plant responses. Lower shoot fresh weight reduction was illustrated with TP and SAXA2 cultivars at both salt levels compared to control plant. We might suggest that both cultivars are relatively the most tolerant plants in this study. This result is overall confirming that crop salinity sensitivity varies with genotype (Pujari and Chanda, 2002) within *Raphanus sativus* species and suggesting that intraspecific genotypic variation could be addressed for future screening (Ashraf, 2009).

4.2. *Salinity tolerance in radish and plant water relations*

Plant defends itself from water deficit caused by salt in the growth medium by closing the stomata to avoid fast dehydration (Orsini et al., 2011). During Experiment 1, gas exchange parameters (E, G_s, and A) were similarly affected by salinity in both red and white cultivars. Similarly, in Experiment 2, the net photosyn-

thesis of all radish cultivars showed consistent significant reduction upon both salt treatments, as compared to control plants. Accordingly, salt avoidance mechanism in relatively salt tolerant cultivars (SAXA2 and TP) might be ascribed to different defence techniques adopted by plant to cope with a salt challenging environment.

The ability of plant to control transpiration water flux under salinized condition is a critical tolerance determinant (Orsini et al., 2011; Barbieri et al., 2012). Indeed, the red cultivar more efficiently regulated WUE in response to salinity, resulting in 13-fold higher WUE value under 200 mmol·L^{-1} NaCl, as compared to the white genotype in Experiment 1. In Experiment 2, the highest WUE values were recorded in both TP and SAXA2 cultivars.

A critical high accumulation of solutes beyond the limits of cytoplasm regulation will lead to cell toxicity, which will cause plant growth impairment (Munns and Tester, 2008). In Experiment 1, at 200 mmol·L^{-1} NaCl, the most decreases in water and osmotic potential were recorded in the white cultivar which might be associated with greater Na^+ and Cl^-ions accumulation that possibly exceeded the need for the osmotic adjustment. Consequently, this process accelerated tissue dehydration causing injury to metabolic systems (Munns and Tester, 2008). Furthermore, the plant's ability to adjust osmotically under saline environments was related to higher leaf turgor potential value (Maggio et al., 2005; Munns and Tester, 2008). Consistently, during Experiment 1, the red cultivar showed three times higher Ψp than the white one at 200 mmol·L^{-1} NaCl. Consequently, the red cultivar showed better water relation adaptability to the stressful environment and higher capacity for osmotic adjustment under salt stress. In Experiment 2, all 100 mmol·L^{-1} NaCl treated plants showed equivalent reductions in both Ψ_w and Ψ_π, suggesting that these parameters may not be reliable for discriminating salt tolerance in radish. Overnight measurements of plant water loss during Experiment 1 (Fig. 2, H) were scarcely correlated with either punctual leaf gas exchange measurements or plant growth parameters, suggesting that these measurements would not allow reliable prediction of plant final yield in response to salinity.

4.3. Salinity tolerance in radish and plant solute accumulation

A 2-fold increase in total proteins content in cultivar SAXA2 was consistent with the higher levels of soluble proteins that are generally encountered in salt tolerant genotypes, as previously demonstrated in barley, sunflower, finger millet and rice (Ashraf, 2009). The absence of a consistent trend of protein content in TP limits the adoption of soluble proteins as a discriminant for salinity tolerance. This result was confirmed also by correlation matrix data that presented a slight positive correlation between salt stress and protein level.

Increased proline content was used as a marker for assisted selection to improve salinity and drought tolerance (Wu et al., 2013). However, the contribution of proline varies among species and cultivars under stress conditions (Ashraf, 2009). In this investigation, SAXA2 and TP cultivars presented comparatively higher amount of proline accumulation at 200 mmol·L^{-1} NaCl. Correspondingly, the correlation matrix showed a high positive correlation between salt stress and proline accumulation. Therefore, we concluded that salt tolerance in SAXA2 cultivar may be attributed, at least partially, to the osmoprotectant effect of proline accumulation.

4.4. MDA and H_2O_2 content and enzyme activities

MDA and H_2O_2 are reliable indicators of free radical formation and peroxidative damage to cell membranes (Abd Elgawad et al., 2016; Ashraf, 2009). In the present study, there was no adverse effect of salt either on MDA or on H_2O_2 levels suggesting that these parameters may not be considered as markers or useful screening tools for salt tolerance in radish.

To endure salt-induced oxidative damages, constitutive and/or induced activities of antioxidative enzymes such as SOD, CAT and GR are crucial (Foyer and Noctor, 2005). Generally, salt tolerant cultivars showed higher activities of these antioxidant enzymes as compared to salt-sensitive ones (Sairam et al., 2005). Many reports showed increased activity of APX upon salt treatment (Oidaire et al., 2000). Higher APX activity was linked to higher level of H_2O_2 production in cell walls and/or cytosol (Mittova et al., 2004). In this investigation, salt stress did not affect APX activity with no genotypic variation among all plants. The stability of APX enzyme under salt stress may be related to its detoxification (reduction) capacity being below the oxidation capacity of H_2O_2 since the H_2O_2 level was also not affected by salt stress (data not shown). Accordingly, H_2O_2 production was low where APX enzyme is located (Doğan, 2012).

Previous works showed that salt stress has a depressive effect on CAT activity, linked to the disruption of cell metabolism and structure (Sairam et al., 2005; Doğan, 2012). In this work, CAT enzyme showed low activities upon salt stress in all cultivars, although the depressive effect of salt was less evident in SAXA2 in 200 mmol·L^{-1} NaCl. Taken together, the decrease in CAT and APX activity upon salinization could indicate that H_2O_2 formation is not a key event in salt stress in radish. Our result was consistent with Karim et al. (2012) that salt stress reduced the CAT activity while APX activity did not change in *Crithmum maritimum*.

GR, plays an essential role in plant defence against ROS (Karim et al., 2012). In our experiment, NaCl treatments induced remarkably GR activity in all radish cultivars and particularly in SAXA2 and TP. The oxidative stress in SAXA2 and TP cultivars appeared to be prevented by the ascorbate-glutathione cycle as shown by the increased GR activity under both salt levels. SOD plays a significant role in protecting living cells against the toxicity of ROS. If this radical is not scavenged by SOD, it disturbs plant vital biomolecules (Ozyigit et al., 2016). The variation in the activity of SOD was found in response to salinity appeared at the interspecific or intra-specific level (Ashraf, 2009). As indicated in Fig. 6, C, SOD activity increased in 100 mmol·L^{-1} NaCl. This may suggest a relevant role of SOD enzyme in adaptation to lower NaCl concentration (100 mmol·L^{-1}) rather than high NaCl level (200 mmol·L^{-1}) in radish.

5. Conclusion

This work tried to identify a rapid screening methodology for salt stress tolerance in radish based on some morphological and biochemical responses, by using two different radish cultivars (round red and long white). The study showed that salt resulted in a lower biomass reduction in round red radish based

on yield, RGR, NAR and leaf area. However, both cultivars showed similar reduction in gas exchange parameters after salt application, suggesting that photosynthetic apparatus is not reliable indicator of salinity tolerance. Besides, higher decreases in water and osmotic potential were recorded in white radish, which may have impaired plant growth, while red plant demonstrated better adaptation to overall water relations and showed higher capacity to maintain osmotic adjustment under salt stress. Based on these considerations, red radish appeared to be more salt tolerant than the white one and accordingly it was chosen for successive comparative biochemical analyses. In the second experiment, the variability of different red radish cultivars was assessed in order to identify some candidate resistant cultivars toward salt stress. Upon salinity, a genotypic variation was presented either morphologically or physiologically where SAXA2 and TP cultivars showed lower yield reduction and better adaptation of overall water relations compared to other cultivars. Moreover, the compatible osmolytes accumulation of proline in SAXA2 and TP cultivars confirmed that proline accumulation is an important strategy to cope with salt stress condition. Furthermore, the induction of antioxidant enzymes activity is an important component of the tolerance adaptation mechanism of radish to salinity. However, H_2O_2 and its main scavenging enzymes (CAT and APX) did not appear significantly affected by salt stress and they might not be considered useful screening tools for salt tolerance in radish plant. In contrast, SAXA2 cultivar showed up-regulation of GR activity, suggesting this plant might activate GR enzyme as a first defence line towards salt stress, and consequently this might greatly sustain plant growth ability in presence of salt stress conditions.

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