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ORIGINAL PAPER

Salt stress induction of some key antioxidant enzymes and metabolites in eight Iranian wild almond species

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Abstract The present work describes the changes in the activities of key antioxidant enzymes and the levels of some metabolites in relation to salt tolerance in eight wild almond species. All the species were exposed to four levels of NaCl (control, 40, 80 and 120 mM). Plant fresh biomass, α-, γ- and δ-tocopherol, total soluble proteins, malondialdehyde (MDA_{eq}), H_2O_2, total phenolics, and the activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were analyzed in leaves of salt-stressed and non-stressed plants of the eight almond species. In all the species, salt stress significantly enhanced the activities of SOD and POD, levels of total phenolics and γ- and δ-tocopherols. High levels of salt stress significantly depressed the levels of total soluble proteins, MDA and CAT activity, while salt stress did not significantly affect leaf H_2O_2 contents. Regression analysis showed that the relationship between salt levels and total soluble proteins, CAT, γ-tocopherol, MDA_{eq}, SOD and POD were statistically significant. Principal component analysis discriminated the almond species based on their degree of tolerance/sensitivity to saline conditions: *Prunus reuteri* and *P. glauca* were ranked as salt tolerant, *P. lycioides* and *P. scoparia* as moderately tolerant, and *P. communis*, *P. eleagnifolia*, *P. arabica* and *P. orientalis* as salt sensitive. The results could be used for selecting salt tolerant genotypes to be used as rootstocks for almond cultivation.

Keywords Antioxidants enzymes · Non-enzymatic antioxidants · *Prunus* spp. · Phenolics · Salt stress · Tocopherols

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Introduction

Production of almond [*Prunus dulcis* (Mill.) D.A. Webb syn. *P. amygdalus* (L.) Batsch] in Iran is based on locally adapted clones, with minimum to no mineral inputs and customary management (Moradi 2006). The limited gene pool of cultivated almond restricts its cultivation to specific areas, hit by a variety of abiotic stresses, including salinity (Kester et al. 1991). In Iran, but also in other places worldwide, almond cultivation is confined to agricultural lands characterized by continue drought, high temperatures, and low precipitation, where irrigation induces accumulation of salt in soils following a combination of sustained evaporative and transpirational water losses (Sharma and Rao 1998; Rains and Goyal 2003). Related species of almond demonstrate a greater resistance to abiotic stresses as well as positive tree and nut traits, so represent valuable germplasm sources for breeding (Sorkheh et al. 2009). For this reason, interspecific hybrids between these almond related species have been diffusely used for almond cultivation under saline and drought conditions as rootstocks, including *Prunus spartioides* (Spach) C.K. Schneid. in Iran (Sorkheh et al. 2009), *P. bucharica* (Korsh.) Hand.-Mazz. and *P. fenzliana* Fritsch in Russia (Denisov 1988), and *P. fenzliana*, *P. bucharica*, *P. kuramica* (Korsh.) Kitam., *P. argentea* (Lam.) Rehder, *P. dehiscens* (Batalin) Koehne

and *P. kotschyi* (Boiss. & Hohen. ex Spach) Meikle in France (Kester et al. 1991).

Temperate fruit trees are generally categorized as sensitive to high levels of soluble salts, particularly to chloride, and their degree of salt tolerance also depends on the stage of growth and development (Najafian 2008). For example, woody plants have been generally reported as relatively salt tolerant during seed germination and reproductive stage, but as sensitive during the emergence and young seedling stages (Najafian 2008). Stone fruit trees, including apricot (*Prunus armeniaca* L.), plum (*P. domestica* L.) and peach (*P. persica* L.), are particularly sensitive to salinity (Gucci and Tattini 1997; Rains and Goyal 2003). As the use of interspecific hybrids as a rootstock for a number of stone fruits is strongly recommended, the selection of salt tolerant genotypes for hybridization can be extremely important. A multitude of physiological and biochemical characteristics have been recommended as potential indicators for the identification of salt tolerant genotypes in many herbaceous and tree species (Rains and Goyal 2003; Noreen and Ashraf 2009). Among these physiological selection criteria, the study of the antioxidant system has been successfully used for discriminating genotypes of different *Prunus* crops for their potential to tolerate drought (Sofo et al. 2005; Sorkheh et al. 2011). The oxidative defense system includes different types of enzymatic (e.g., superoxide dismutase SOD, peroxidases POD, catalase CAT, ascorbate peroxidase APX) and non-enzymatic antioxidants (e.g. ascorbic acid, total phenolics, flavonoids, tocopherols), able to eliminate or reduce reactive oxygen species (ROS), generated in plant cells during saline conditions that are toxic for cell metabolism (Sofo et al. 2010).

It is generally proved that salt-tolerant genotypes of most plant species have higher activities/levels of antioxidant enzymes than those of salt sensitive ones (Logan 2005), but in some cases the reverse is true (Munns and Tester 2008). For instance, in the model plant *Arabidopsis thaliana*, mutants lacking one or both cytosolic and chloroplastic APX, involved mainly in H_2O_2 removal, were found to be more tolerant to salt stress (Mittler et al. 2004). Among woody plants, species able to cope with arid and semi-arid environments, such as olive and some *Prunus* species, showed higher activities of antioxidant enzymes and are able to up-regulate antioxidant systems under adverse conditions (Scebba et al. 2001; Gucci and Tattini 1997; Guerfel et al. 2009; Sofo et al. 2010; Sorkheh et al. 2011).

Although a wide range of biochemical and physiological adaptations, including oxidative defense system s to face saline conditions, have been observed in a number of crop species, little information is available in the literature on the underlying mechanisms of salt-induced oxidative stress in cultivated and economically important fruit trees (Gucci and Tattini 1997). Thus, the major objective of the present study was to measure various antioxidants and metabolites in eight genetically different wild almond species, and to understand whether the changes of these biochemical parameters could be related to their degree of salt tolerance.

Materials and methods

Wild almond species and their sources of origin

The wild almond species used in the present investigation, belonging to the genus *Prunus*, subgenus *Amygdalus*, were: *Prunus communis* (L.) Archang, *P. elaeagnifolia* (Spach) Fritsch, *P. orientalis* Mill. (syn. *Prunus argentea* Lam.) in the section 'Euamygdalus' Spach; *P. lycioides* Spach, *P. reuteri* Boiss. et Bushe in the section 'Lycioides' Spach; and *P. arabica* (Olivier) Neikle, *P. glauca* (Browicz) A.E. Murray, and *P. scoparia* Spach in the section 'Spartioides' Spach. The number of plants sampled per site ranged from one to five, depending on habitat diversity and availability at collection time.

Field expeditions were carried out in 2008 and 2009 based on previous published information (Gorttapeh et al. 2006; Moradi 2006; Sorkheh et al. 2007), indigenous information, or prominent presence. Seeds came from both wild and cultivated habitats concentrated in two different regions of Iran. The former, Azerbaijan and Kurdestan (36°00′–38°28′N, 44°51′ to 45°46′E; mean elevation 1,473 m a.s.l.), is characterized by relatively lush environment, mean annual rainfall of 507 mm, high biological diversity, and relatively low agricultural development. The second region, Shahrekord and Shiraz (27°32′–32°26′N, 49°50′–56°50′E; mean elevation 2,030 m a.s.l.), has a mean annual rainfall of 436 mm and is a more xerophytic area with widespread agriculture.

Growth and salt-induced conditions

The study was conducted in a greenhouse under natural sunlight in Shahrekord, Iran (32°17′N, 50°51′E). The average day and night temperatures were 30.1 ± 6.7 and 16.1 ± 9.2°C, respectively and the average light intensity was 1,060 μmol m^{-2} s^{-1}. The relative humidity ranged from 33.3 to 69.5%. 30 seeds of each species were sown per plastic pot (23.5 cm diameter and 29 cm deep), each containing 10 kg of dry sand. Sand was previously washed with distilled water. Successively, 2 L of full strength Hoagland's nutrient solution were applied on alternate days to each pot, in order to remove all the salts previously present in the sand. After 14 days of growth, plants were

thinned to ten plants per pot. Four NaCl treatments (0, 40, 80 and 120 mM) in Hoagland's nutrient solutions were applied to 3-week-old plants. The NaCl concentration was increased step-wise in aliquots of 40 mM every day until the appropriate concentration was attained. Five plants from each pot were uprooted carefully after 40 days from the beginning of the salt treatment and data for shoot fresh biomass were recorded. After the same period, expanded leaves from the remaining five plants per pot were sampled and immediately used for the following determinations. Five pots per treatment ($n = 25$) were used for all the determinations.

Tocopherols and total soluble proteins

For the determination α-, γ- and δ-tocopherol, fresh samples of leaves (0.5 g) were used for the extraction. 4 mL of ethanol were added to the homogenate, and the mixture was centrifuged at 10,000×*g* for 5 min at 4°C. The supernatant was recovered and filtered through a Whatman no.1 paper, and 5 mL of *n*-hexane were added to the filtrate. Tocopherols were extracted twice in the hexane phase, and the collected extract dried under a stream of liquid nitrogen. The dried extract was solubilized in 1 mL of HPLC-quality methanol. The quantification was carried out according to Miller et al. (1984) utilizing absorption spectra at 296 nm. A Hypersil ODS reverse phase (C_{18}) column (5-mm particle size, 250 mm × 4.6 ID Themohypersil GmbH, Germany) fitted with a C_{18} guard column and methanol:acetonitrile:chloroform (47:42:11 v/v) mobile phase at 1 mL min^{-1} flow rate was used.

For total protein determination, fresh leaves (0.5 g) were ground with 10 mL of 50 mM cooled K-phosphate buffer (pH 7.8) placed in an ice bath. The homogenate was centrifuged at 6,000×*g* for 20 min at 4°C and the soluble proteins content of the extract was determined by the Coomassie blue dye binding method, using bovine serum albumin as a standard.

Lipid peroxidation, H_2O_2 and total phenolics

The level of lipid peroxidation was determined in terms of thiobarbituric acid-reactive substances (TBARS) concentration as described by Hodges et al. (1999) with some modifications. Leaf samples of 0.5 g were homogenized in 4 mL of 1% (w/v) trichloroacetic acid (TCA), and then centrifuged at 10,000×*g* for 10 min. To a 1.5 ml aliquot of the supernatant, 1.5 mL of 20% (w/v) TCA containing 0.5% (w/v) TBA was added. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000×*g* for 5 min, the values of absorbance measured at 532, 600 and 440 nm of the supernatant were recorded. The value for aspecific absorption at 600 nm was subtracted and a standard curve of sucrose (from 2.5 to 10 μmol mL^{-1}) was used to correct the results from the interference of soluble sugars in samples. The concentration of TBARS was calculated using an absorption coefficient of 156 $mmol^{-1}$ cm^{-1}, and the results expressed as MDA equivalents (MDA_{eq}).

H_2O_2 determination in leaf samples (0.5 g) was carried out according to the method of Lee and Lee (2000) using peroxidase enzyme and following spectrophotometrically the absorbance at 436 nm.

Total phenolics were extracted from fresh samples of leaves (1 g) placed in a 100 mL methanol-HCl 0.75% (w/w) solution at 20°C in the dark for 24 h. The resulting extracts were filtered through 0.20 μm Minisart SFCA sterile filters (Sartorius Stedim Biotech GmbG, Goettingen, Germany). Total phenolics were determined spectrophotometrically at 750 nm using the Folin–Ciocalteau reagent, according to the method of Safahlan et al. (2009), and the results were expressed as gallic acid equivalents per gram of fresh weight.

Enzyme extraction and antioxidant activity assay

Tissue extraction of the samples were prepared for the analyses by homogenizing 1 g of fresh leaf material in 4 mL of ice cold 50 mM K-phosphate buffer (pH 7.0) containing 2 mM Na-EDTA and 1% (w/v) polyvinyl–polypirrolidone (PVPP). The homogenate was centrifuged at 10,000×*g* at 4°C for 10 min. The supernatant was used for determining the activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT).

For the measurement of SOD activity, the reaction mixture (3 mL) contained 50 mM K-phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 4 mM riboflavin, 0.1 mM EDTA, and 0.25 mL enzyme extract. The test tubes were shaken then placed in a light box consisting of six fluorescent lamps (15 W preheat, daylight 6500 K; light intensity = 900 μmol m^{-2} s^{-1}). The reaction was based on the reduction of nitroblue tetrazolium (NBT) in the presence of riboflavin in light, and it was stopped by switching off the light and placing the test tubes into dark. One unit of enzyme activity was determined as the amount of the enzyme to reach an inhibition of 50% NBT reduction measured at 560 nm.

Activities of CAT and POD were measured according to Osswald et al. (1992), with some modifications. For CAT, the decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The reaction was initiated by adding enzyme extract to 3 mL of reaction mixture containing 50 mM K-phosphate buffer (pH 7.0), 10 mM H_2O_2 and 50 μL of enzyme extract. CAT activity was determined as the consumption of H_2O_2 at 240 nm over a 2-min interval. For POD, the activity was determined by measuring the

oxidation of guaiacol in the presence of H_2O_2, and following an increase in absorbance at 470 nm over a 2-min interval. The assay mixture (3 mL) contained 0.33 mM guaiacol, 10 mM K-phosphate buffer (pH 7.0), and 50 μL of enzyme extract. The reaction was initiated by adding 40 mM H_2O_2. The activity of each antioxidant enzyme was expressed on protein basis.

Statistical analysis

The values of plant fresh biomass, total phenolics, total soluble proteins, H_2O_2, α-, γ- and δ-tocopherol, MDA_{eq}, and the activities SOD, POD and CAT in plants at the highest level of salt stress (120 mM NaCl) were treated by analysis of variance (ANOVA) and regression analysis using the SAS software (SAS Institute, NC, USA), in order to detect significant differences in salt tolerance/sensitivity (PROC GLM).

Principal component analysis (PCA) was applied (PROC FACTOR) on the same parameters listed above (120 mM NaCl) via the correlation matrix in order to characterize the degree of tolerance/sensitivity of almond species to salt stress by classifying treatments according to the changes in metabolites levels and enzyme activities in salt-stressed plants if compared to controls. Each principal component was a linear combination of the original variables with coefficients equal to the eigenvectors of the correlation matrix. According to Kaiser's criterion, only components with eigenvalue >1 were retained. A graphic interpretation was obtained by biplot on the two dimensions of the main principal components. To crosscheck the spurious entry of the variables in to the regression equation and to see the possible increase in the value of R^2 (multiple correlation), the data was also analyzed using the backward selection procedures (Draper and Smith 1981). Confidence ellipses (90%) enclosing the almond species analyzed were plotted.

Results

Effect of NaCl on plant growth

Our data shows that salt stress had a pronounced inhibitory effect on the shoot fresh weight of all the almond species, which differed significantly at each salt level (Table 1). If compared to control plants, the differences in percent growth inhibition for *P. communis, P. eleagnifolia, P. orientalis* and *P. arabica* were prominent (more than 20% inhibition) at the highest salt level (120 mM NaCl) (Table 1). In the remaining species, with the exception of *P. glauca* that presented a statistically higher value of percent growth inhibition at 80 mM NaCl, the values of shoot fresh biomass measured at 80 and 120 mM NaCl were not statistically different (Table 1).

α-, γ- and δ-tocopherols, and total soluble proteins

At the highest NaCl concentration, the maximum level of α-tocopherol was recorded in *P. communis* and the minimum in *P. lycioides* and *P. scoparia* (Fig. 1a). Foliar γ-tocopherol in all *Prunus* spp. increased significantly as salt concentration rose, and at 120 mM NaCl the highest levels were found in *P. communis* and *P. scoparia* (Fig. 1b). The levels of δ-tocopherol in the leaves of all the almond species, in particular in *P. communis* and in *P. reuteri*, significantly increased, showing significant changes at the maximum salt level if compared to the controls (Fig. 1c).

Table 1 Shoot fresh biomass and percent inhibition in shoot fresh weight in the eight wild almond species subjected to different levels of salt concentration. Percent inhibition is calculated on control plants (0 mM NaCl)

Section	Species	Salt stress levels						
		0 mM (control)	40 mM		80 mM		120 mM	
		Shoot fresh biomass (g)	Shoot fresh biomass (g)	% Inhibition	Shoot fresh biomass (g)	% Inhibition	Shoot fresh biomass (g)	% Inhibition
Euamygdalus	*P. commnunis*	16.4 ± 0.01 a	15.3 ± 0.4 a	6.71	14.2 ± 0.2 b	13.41	12.3 ± 0.4 c	25.00
	P. eleagnifolia	15.3 ± 0.02 a	13.5 ± 0.4 b	11.76	13.1 ± 0.6 b	14.38	12.1 ± 0.7 c	20.91
	P. orientalis	17.3 ± 0.2 a	16.1 ± 0.2 a	6.93	15.2 ± 0.3 b	12.14	13.2 ± 0.6 c	23.70
Lycioides	*P. lycioides*	18.2 ± 0.4 a	17.3 ± 0.2 a	4.94	16.2 ± 0.4 b	10.98	16.3 ± 0.3 b	10.43
	P. reuteri	14.2 ± 0.7 a	13.1 ± 0.6 b	7.75	12.1 ± 0.3 b	14.79	13.5 ± 0.2 b	4.93
Spartioides	*P. arabica*	16.2 ± 0.2 a	15.0 ± 0.1 b	7.41	14.0 ± 0.3 c	13.58	12.3 ± 0.1 d	24.07
	P. glauca	15.3 ± 0.1 a	15.1 ± 0.4 a	1.31	12.3 ± 0.6 c	19.61	14.2 ± 0.2 b	7.18
	P. scoparia	17.9 ± 0.7 a	16.8 ± 0.2 a	6.14	15.1 ± 0.4 b	15.64	16.1 ± 0.5 b	10.05

The values represent the averages (±SE) of five independent replicates ($n = 25$). Values followed by different letters among columns are significantly different at $P \leq 0.01$, according to Duncan's multiple range test

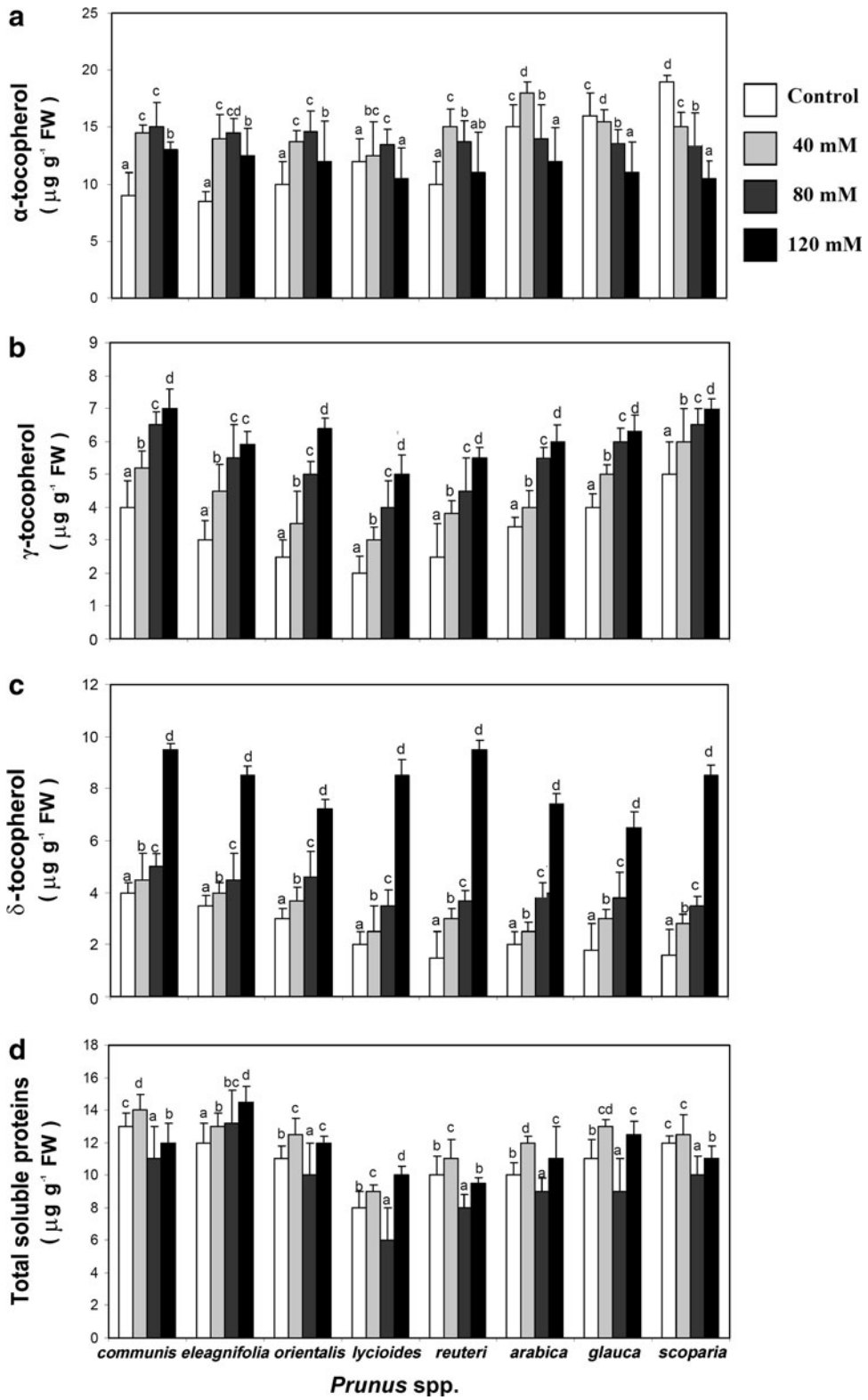

Fig. 1 Levels of **a** α-tocopherol, **b** *β*-tocopherol, **c** *δ*-tocopherol, and **d** total soluble proteins in the eight wild almond species subjected to different levels of salt stress (0, 40, 80 and 120 mM NaCl). The values represent the averages (±SE) of five independent replicates ($n = 25$). Values with different letters are significantly different at $P \leq 0.01$, according to Duncan's multiple range test

Interestingly, the α-tocopherol concentration appeared to be greatest at 80 mM NaCl than at 120 mM NaCl (Fig. 1a), whereas this trend was not observed for *γ*- and *δ*-tocopherol (Fig. 1b, c).

Total soluble proteins in the leaves of *P. eleagnifolia* significantly increased when increasing NaCl while in the other almond species increased at 40 mM NaCl, and then they decreased at 80 mM NaCl (Fig. 1d). At all the salt

levels, total soluble proteins appeared to be higher in *P. communis*, *P. eleagnifolia*, *P. orientalis*, *P. glauca, P. arabica* and *P. scoparia* than in *P. lycioides* and *P. reuteri* (Fig. 1d).

Lipid peroxidation, hydrogen peroxide and total phenolics

Increasing supply of NaCl caused a significant increasing effect on the levels of MDA_{eq} in leaves of *P. lycioides*, *P. reuteri*, *P. arabica* and *P. glauca*, and a decreasing effect in the remaining species (Fig. 2a). The observed increases in MDA_{eq} at 40 mM NaCl were followed by a decrease starting from 80 mM NaCl in *P. reuteri* and *P. glauca*, and strarting at 120 mM NaCl in *P. arabica* (Fig. 2a). At all salt levels, the maximum amount of MDA_{eq} was recorded in *P. glauca* and the minimum in *P. scoparia* (Fig. 2a).

Salt stress did not clearly affect leaf H_2O_2, and all the almond species differed significantly in H_2O_2 content (Fig. 2b). *P. reuteri* and *P. arabica* presented higher leaf H_2O_2 content, particularly at 80 mM NaCl (Fig. 2b). In all almond species, the levels of H_2O_2 at 120 mM NaCl were lower than the corresponding levels at 80 mM NaCl (Fig. 2b).

Addition of varying amounts of NaCl to the growth medium caused a strong and significant increase in the

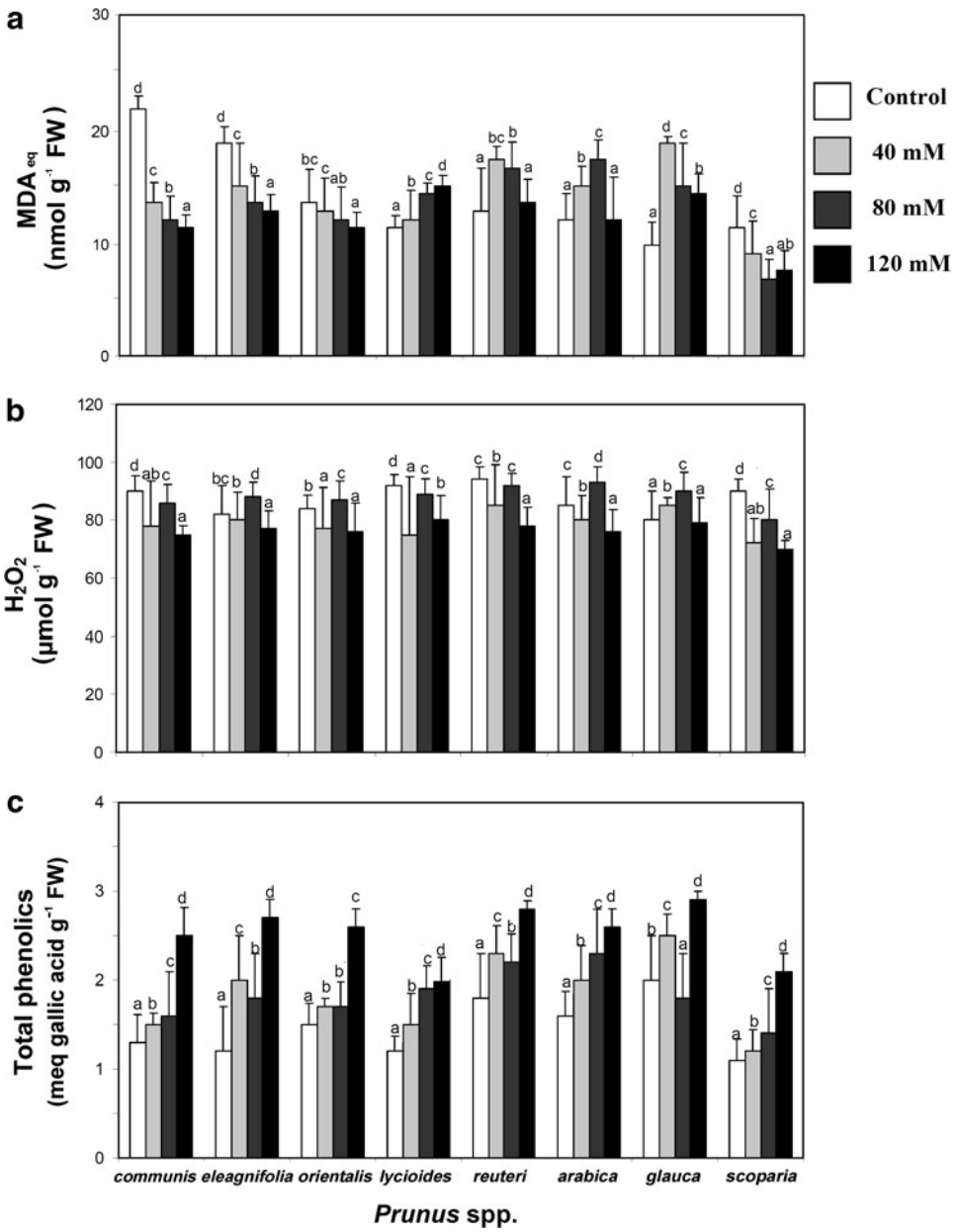

Fig. 2 Levels of **a** malondialdehyde equivalents, **b** hydrogen peroxide, and **c** total phenolics in the eight wild almond species subjected to different levels of salt stress (0, 40, 80 and 120 mM NaCl). Statistics as in Fig. 1

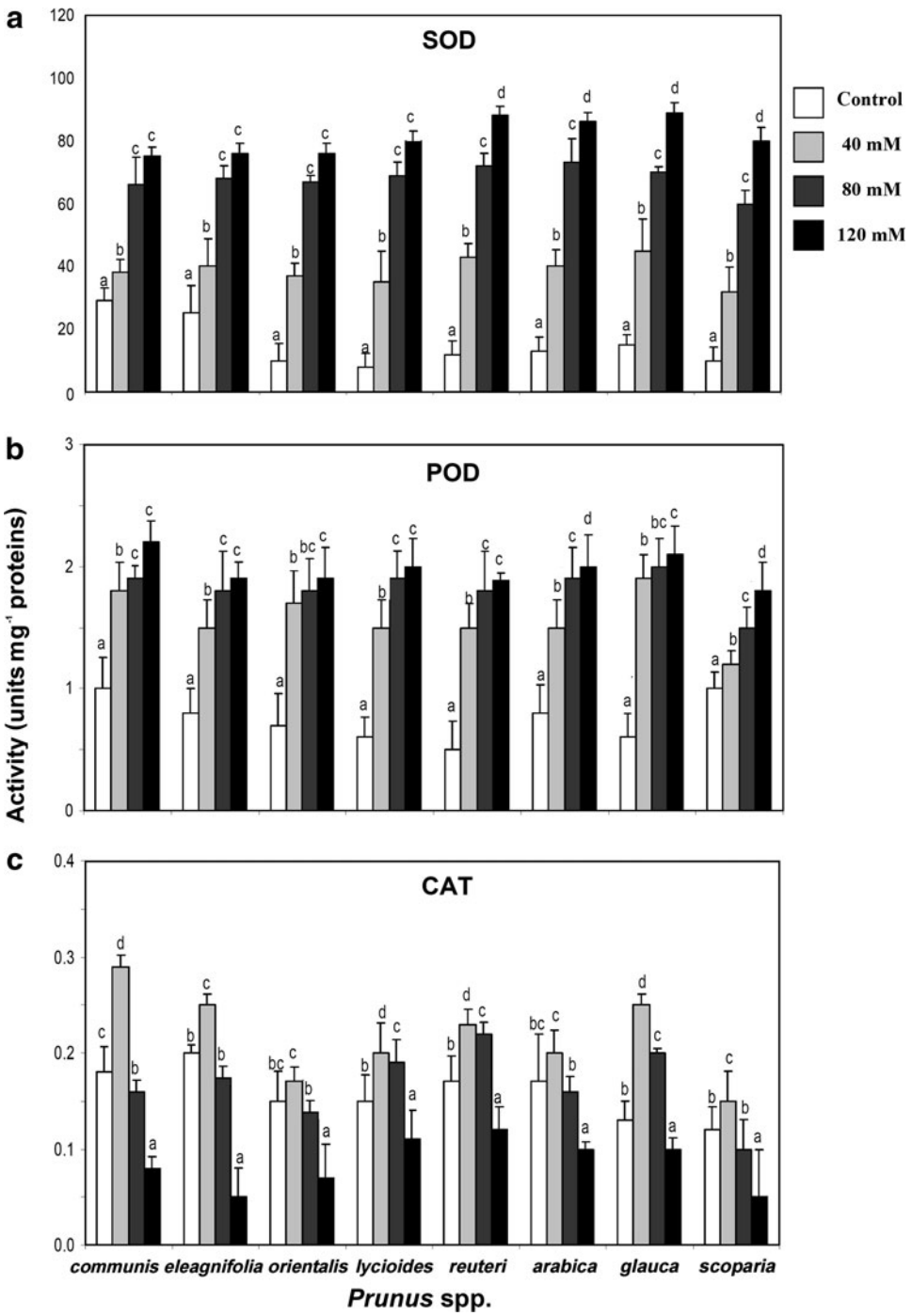

Fig. 3 Activities of **a** superoxide dismutase (SOD), **b** peroxidase (POD), and **c** catalase (CAT) in the eight wild almond species subjected to different levels of salt stress (0, 40, 80 and 120 mM NaCl). Statistics as in Fig. 1

accumulation of leaf total phenolics in all the almond species (Fig. 2c). In particular, *P. eleagnifolia*, *P. reuteri* and *P. glauca* had high levels of total phenolics at the highest salt regime (Fig. 2c).

Enzyme activities

The activities of both SOD and POD increased significantly in all *Prunus* spp. when increasing NaCl concentration (Fig. 3a, b). At the highest salt level, *P. reuteri*, *P. arabica*, *P. glauca* and *P. scoparia* presented the highest SOD activities, and they were significantly higher than those measured at 80 mM NaCl (Fig. 3a). Regarding POD, *P. communis* and *P. glauca*, followed by *P. lycioides* and *P. arabica* presented the highest activities, particularly at the highest salt level (Fig. 3b). *P. arabica* and *P. scoparia* showed significant increases in POD activity passing from 80 to 120 mM NaCl, whereas in the other species this did not occur (Fig. 3b).

Addition of 40 mM NaCl to the growth medium caused a significant increase in CAT activity for all *Prunus* spp. (Fig. 3c). However, higher levels of salt (80 and 120 mM NaCl) caused a significant decline in CAT activity (Fig. 3c). In particular, *P. scoparia* and *P. eleagnifolia*, followed by *P. orientalis* and *P. communis*, showed lower values of CAT activity when compared to the other species (Fig. 3c).

Data analysis

The *F* values obtained from statistical analysis showed that total soluble protein, γ-tocopherol, MDA_{eq}, and the activities of SOD, POD and CAT were significantly affected ($P < 0.01$) by salt treatment, whereas the other parameters showed no significant differences (Table 2).

The principal component analysis identified three components with eigenvalue >1 (PC 1, PC 2 and PC 3 were 13.98, 10.12, 8.89, respectively). Biplot in Fig. 4 showed the first two components to account for most variance (76%). The eight almond species were quite clearly separated in three groups from each other. In particular, as we discuss later, PC 1 (accounting for 44% of total variance) separated salt tolerant from moderately tolerant almond species while PC 2 (32% of the total variance) clearly separated salt sensitive species from the other two groups (Fig. 4).

Table 2 Regression analysis of metabolite levels and enzyme activities in the eight species of almond at the highest level of salt stress (120 mM NaCl)

Variable	Coefficient	Standard error	R^2 partial	R^2 model	*F* test
Intercept	0.430	0.70			
Total soluble protein	0.530	0.04	0.6330	0.6330	7.176**
Catalase	0.060	0.02	0.0990	0.7320	6.135**
γ-tocopherol	0.230	0.35	0.0730	0.8050	5.509**
Malondialdehyde eq.	–0.050	0.13	0.0120	0.8316	5.357**
Superoxide dismutase	0.250	0.25	0.0073	0.8482	3.229**
Peroxidase	3.800	1.46	0.0061	0.8543	3.148**
Total phenolics	–0.030	0.05	0.0146	0.8196	ns
Hydrogen peroxide	0.002	0.01	0.0093	0.8409	ns
α-Tocopherol	0.048	0.05	0.0058	0.8601	ns
δ-Tocopherol	0.030	0.02	0.0022	0.8623	ns

NS non-significant

** Significant at $P \leq 0.01$

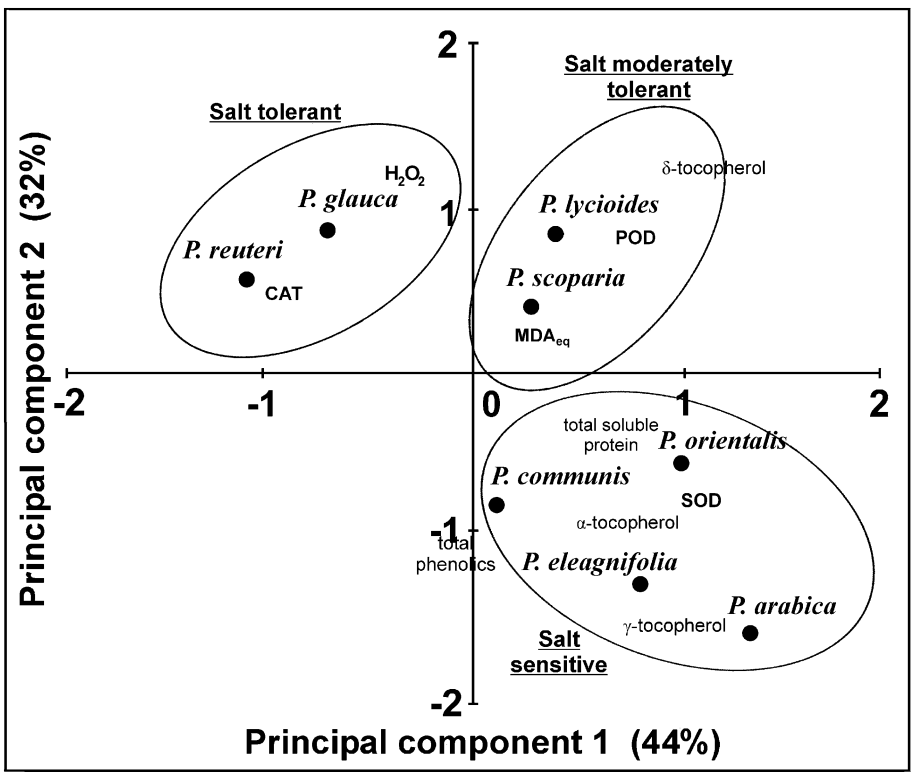

Fig. 4 Score plot obtained from principal component analysis (PCA) of metabolite levels and enzyme activities in the eight wild almond species subjected to the maximum level of salt stress (120 mM NaCl). The values represent the average of five independent replicates ($n = 25$). The variables considered (the same of Table 2) were reported. Confidence ellipses (90%) enclosing the almond species analyzed are shown

Discussion

Regression analysis highlighted that highest level of salt stress (120 mM NaCl) in the different almond species significantly affected total soluble protein, γ-tocopherol, MDA_{eq}, and the activities of SOD, POD and CAT (Table 2). Multivariate statistical analysis has been necessary to take into account the complexity of the data sets. The visualization of the scores and loadings by PCA biplot allowed to highlight significant differences in the degree of tolerance/sensitivity to saline conditions of the almond species studied (Fig. 4). The discrimination carried out by PCA reflected the differences in shoot fresh biomass observed after NaCl application. Indeed, at 120 mM NaCl *P. reuteri* and *P. glauca* (tolerant) showed less than 8% inhibition, *P. communis*, *P. eleagnifolia*, *P. orientalis* and *P. arabica* (sensitive) more than 20% inhibition, and *P. lycioides* and *P. scoparia* (moderately tolerant) approximately 10% inhibition (Table 1). Based on PCA analysis (Fig. 4) and growth results (Table 1), *Prunus* spp. were ranked for salt tolerance. Therefore, *P. reuteri* and *P. glauca* were classified as salt tolerant, *P. lycioides* and *P. scoparia* as salt moderately tolerant, and *P. communis*, *P. eleagnifolia*, *P. orientalis* and *P. arabica* as salt sensitive.

The growth of all the wild almond species examined in the current study was limited by increasing levels of NaCl (Table 1). The observed reduced growth under salt conditions was probably caused by the perturbation of various physiological and biochemical processes at the cellular, tissue or whole-plant level, such as photosynthesis, nutrient uptake accumulation of compatible solutes, and enzymatic activities (Sharma and Rao 1998; Rains and Goyal 2003; Munns and Tester 2008; Sofo et al. 2010). The negative effects of salt stress on shoot growth were particularly marked when NaCl levels were above 40–80 mM NaCl for salt sensitive almond species, and above 80–120 mM for moderately tolerant and tolerant species (Table 1).

In all the almond species, the levels of α-tocopherol were less affected by salt stress (Fig. 1a) if compared to those of γ- and δ-tocopherol, that increased significantly under high NaCl (Fig. 1b, c). Tocopherols are potential scavengers of ROS and lipid radicals (Hollander-Czytko et al. 2005; Munné-Bosch 2005). This is an important finding, since γ- and δ-tocopherol are directly able to scavenge oxidizing radicals, preventing the chain propagation step during lipid auto-oxidation (Foyer and Noctor 2005).

MDA, a product of lipid peroxidation in plants exposed to adverse environmental conditions, is a reliable indicator of free radical formation and peroxidative damage to cell membranes (Logan 2005). Furthermore, MDA has been found to be significantly associated to drought-related oxidative stress in woody plants adapted to arid conditions (Bacelar et al. 2007; Sofo et al. 2010). The amount of MDA_{eq} in the leaves was strongly variable in the different almond species studied here (Fig. 2a). For example, in the salt sensitive species *P. arabica*, MDA_{eq} increased, while in the other salt sensitive *P. communis*, *P. eleagnifolia* and *P. orientalis* it decreased when increasing salt concentration, suggesting that membrane peroxidation is not in this case the main detrimental effect caused by salt stress (Fig. 2a).

Generally, it is known that salt stress enhances the production of ROS in plants (Amor et al. 2005). In particular, it is known that H_2O_2 is a strong inhibitor of the Calvin cycle, and for this reason, it must be eliminated by conversion to H_2O in reactions involving APX, POD, and CAT. The almond species here studied did not differ in leaf H_2O_2 contents (Fig. 2b). Mittova et al. (2004) found that the mitochondria and peroxisomes of salt-treated roots present increased levels of lipid peroxidation and H_2O_2, but in our experiment, we did not observe significant changes in H_2O_2 content after the application of salt stress in all the species studied (Fig. 2b). Interestingly, the decreases in CAT activity, the main H_2O_2-scavenging enzyme, at 120 mM NaCl (Fig. 3c) corresponded to significant decreases in H_2O_2 levels (Fig. 2b), so indicating a decrease in H_2O_2 metabolism at high NaCl levels. Moreover, CAT is an enzyme particularly susceptible to photoinactivation and degradation under severe abiotic stresses (Sofo et al. 2010).

Phenolics are the main class of secondary metabolites with important roles in abiotic stress tolerance due to their antioxidant properties (Ruiz et al. 2001). Although almond species differed in growth performance at varying salt concentrations (Table 1), salt stress caused significant increases of total phenolics in all the almond species (Fig. 2c). It is noteworthy that almond species tolerant to salinity (*P. reuteri* and *P. glauca*) presented higher increase in total phenolics if compared to the remaining species (Fig. 2c). These results are similar to those of Wahid and Ghazanfar (2006), who observed that enhanced synthesis of soluble phenolics is directly correlated with salt and heat tolerance in sugarcane.

To cope with oxidative damage under extremely adverse conditions, plants finely regulate the enzymatic antioxidant defense system, that includes SOD, POD, and CAT (Foyer and Noctor 2005). Mittler et al. (2004) reported that all these interactive processes are regulated by complex biochemical pathways controlled by more than 150 genes in Arabidopsis. However, how these pathways are regulated, it is still not clearly known. Furthermore, the levels of antioxidant enzymes are usually higher in tolerant than in sensitive woody species under various environmental stresses (Sofo et al. 2010). In the present study, the activities of SOD and POD (Fig. 3a, b) rose in all the almond species studied, and this increase was generally higher for SOD in the species tolerant and moderately tolerant to salt

stress. A fine regulation of the antioxidant system and related metabolites has already been demonstrated to occur in wild, cultivated and hybrid *Prunus* spp. subjected to drought stress (Sofo et al. 2005, 2010; Sorkheh et al. 2011).

In conclusion, our data suggest that induction of antioxidant defenses is an important component of the tolerance mechanism of almond to salinity. Among the various enzymes and metabolites involved in oxidative stress response, the activities of SOD, POD and CAT as well as the amounts of γ-tocopherols and MDA_{eq} were found to be related to salt stress in all the almond species (Table 2). Our results demonstrated that antioxidant enzyme levels could be used as specific markers also for studying salt tolerance and variance in salinity tolerance among almond species. The high levels of salt tolerance showed by some of the species studied (in particular *P. reuteri* and *P. glauca*) can provide useful information for the selection of rootstocks for almond cultivation in sodic soils typical of arid environments. These soils generally have percent exchangeable $Na^+ > 15$, and NaCl concentration in the soil water >3 g L^{-1} (the level of 120 mM NaCl used here corresponds to a solution of 7 g L^{-1} NaCl) (Sharma and Rao 1998; Rains and Goyal 2003). Our results could also be a baseline necessary to conduct further studies related to the genetic basis of salt tolerance in almond.

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