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RESEARCH PAPERS

Exogenous Proline Alleviates the Effects of H_2O_2-Induced Oxidative Stress in Wild Almond Species[1]

K. Sorkheh[a], B. Shiran[a], M. Khodambashi[a], V. Rouhi[a], S. Mosavei[a], and A. Sofo[b]

[a] *Department of Agronomy and Plant Breeding, Faculty of Agriculture, Shahrekord University, P.O.Box 115, Shahrekord, Iran*

[b] *Dipartimento di Scienze dei Sistemi Colturali, Forestali e dell' Ambiente, Universita` degli Studi della Basilicata, Via dell' Ateneo Lucano 10, 85100 Potenza, Italy;*

fax: +39 0971 20-5378; e-mail: adriano.sofo@unibas.it

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Abstract—The effect of proline on the antioxidant system in the leaves of eight species of wild almond (*Prunus* spp.) exposed to H_2O_2-mediated oxidative stress was studied. The levels of endogenous proline (Pro) and hydrogen peroxide, and the activities of total superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and guaiacol peroxidase (POD) were measured. The degradation of chlorophyll but not carotenoids occurred in leaves in the solution of 5 mM H_2O_2 . An increase in membrane lipid peroxidation was observed in H_2O_2 treatment, as assessed by MDA level and percentage of membrane electrolyte leakage (EL). Significant increases in total SOD and CAT activities, as well as decreases in APX and POD activities, were detected in H_2O_2-treated leaves. The three SOD isoforms showed different behavior, as Mn-SOD activity was enhanced by H_2O_2, whereas Fe-SOD and Cu/Zn-SOD activities were inhibited. In addition, Pro accumulation up to 0.1 μmol/g fr wt, accompanied by significant decreases in ascorbate and glutathione levels, was observed in H_2O_2-treated leaves. After two different treatments with 10 mM Pro + 5 mM H_2O_2, total SOD and CAT activities were similar to the levels in control plants, while POD and APX activities were higher if compared to the leaves exposed only to H_2O_2. Pro + H_2O_2 treatments also caused a strong reduction in the cellular H_2O_2 and MDA contents and EL. The results showed that Pro could have a key role in protecting against oxidative stress injury of wild almond species by decreasing membrane oxidative damage.

Keywords: Prunus, antioxidant enzymes, electrolyte leakage, hydrogen peroxide, lipid peroxidation, oxidative damage, proline, SOD isoforms

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INTRODUCTION

The almond (*Prunus dulcis* Miller (D.A Webb) syn. *P. amygdalus* Batsch) is one of the most important nut crops and today represents the largest production of any commercial tree nut product. Almond species grow under subtropical Mediterranean climate, with mild wet winters and warm and dry summer [1]. They originated from central Asia and represent divergent evolution under xerophytic environments [2]. Related *Prunus* species are found growing wild from eastern China to mountainous areas and deserts of Western China, Kurdistan, Turkistan, Afghanistan, and Iran [1].

Plants functioning in an aerobic environment are often subjected to continuous threats due to ROS, such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$) and singlet oxygen ($^1O_2$), which can damage cellular structures and macromolecules, causing photoinhibition of photosynthetic apparatus and other metabolic disorders [3, 4]. ROS are by-products of aerobic metabolism, and their production is enhanced when plants are subjected to abiotic and biotic stresses through the disruption of electron transport system and oxidizing metabolic activities occurring in chloroplasts, mitochondria, and microbodies [5]. The most stable form of the ROS, H_2O_2, is a product of oxidative reactions occurring in mitochondria, peroxisomes, and chloroplasts, and it is one of the main causes of oxidative damage to cells, resulting in disruption of metabolic function and the loss of cellular integrity [6]. Furthermore, H_2O_2 is able to initiate an antioxidant response by acting as a signal of oxidative stress; it is involved in the regulation of the transcription of many genes, and its presence in the

[1] This text was submitted by the authors in English.

Abbreviations: APX—ascorbate peroxidase; ASC—total ascorbate; CAT—catalase; Cu/Zn-SOD—copper/zinc superoxide dismutase; EL—electrolyte leakage; Fe-SOD—iron superoxide dismutase; FW—fresh weight; GLU—total glutathione; GR—glutathione reductase; Mn-SOD—manganese superoxide dismutase; POD—guaiacol peroxidase; SOD—superoxide dismutase; TCA—trichloroacetic acid.

apoplast is toxic for pathogens [3, 4, 7]. The balance between ROS production and removal is tightly controlled by the antioxidant system [6]. It consists of three general classes of compounds: (a) water-soluble reductants, such as ascorbate (ASC) and glutathione (GLU), (b) lipid soluble, membrane-associated antioxidants, and (c) enzymatic antioxidants, such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), guaiacol peroxidase (POD; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.11.1.11), and glutathione reductase (GR; EC 1.6.4.2). In particular, different SOD isoforms convert $O_2^{\bullet -}$ radicals into H_2O_2 and O_2, whereas CAT, APX, and PODs scavenge the accumulated H_2O_2, transforming it into water [3]. Furthermore, glutathione reductase (GR, EC 1.6.4.2) is a flavoenzymes with a redox cystine residue in their active sites, which maintains the intracellular glutathione pool in the reduced status [8].

Another important response occurring in many plant species when exposed to different abiotic stresses is the accumulation of compatible organic solutes, such as proline (Pro) [9]. Pro accumulation has been shown in different plant species grown under oxidative stress [7]. It has been suggested that Pro protects plants by functioning as a cellular osmotic regulator between the cytoplasm and vacuole and by detoxifying ROS, thus protecting membrane integrity and stabilizing antioxidant enzymes [10]. Although much effort has been devoted to genetically engineered plants for overproduction of various osmoprotectants, including Pro, there has been little success in achieving the desired protective levels of these osmolytes in plants [11]. The actual role of Pro accumulation, as well as its physiological importance, still remains unclear and hot debates continue to find the answer whether its accumulation is a response to abiotic stresses or a plant response associated with stress tolerance.

The aim of this study was to investigate the protective effect of exogenous Pro against H_2O_2-related oxidative stress in leaves of eight wild species of almond. We hypothesize that the tolerance level of almond plants grown under oxidative stress conditions could be increased by exogenous Pro application. The almond species were chosen on the basis of their agronomic traits, breeding potential, and degree of tolerance against abiotic stresses [12–14]. Related almond species, interspecific crosses, and spontaneous interspecific hybrids have a greater resistance to abiotic and biotic stresses, such as water deficit, and so represent valuable germplasm sources for rootstock breeding [2, 12]. Thus, our approach might be of great importance for the breeding for drought resistance in almond rootstock material and for the increase in the quality of cultivated almond production under stress conditions determining oxidative stress.

MATERIALS AND METHODS

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

Field expeditions were carried out in 2007 and 2008 on the basis of previous recent literature [13, 14], indigenous information, or conspicuous presence. Collections came from both wild and cultivated habitats, which were concentrated in two different regions in Iran. The first region (Azerbaijan and Kurdistan, 36°00′ to 38.28′N, 44°51′ to 45°46′E, mean elevation of 1473 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′ to 32.26′N, 49°50′ to 56°50′E, mean elevation of 2030 m a.s.l.), with a mean annual rainfall of 436 mm, is in a more xerophytic area with widespread agriculture.

Leaf sampling and stress treatments. Hardwood cuttings of *Prunus* spp. with three nodes were sampled at the dormant bud stage of the trees and cultured in a growth chamber at 24 ± 1°C, 60% humidity, and a 16-h photoperiod. When these plants were more than 5-month-old, nodal explants containing a single auxiliary bud were excised from the main shoots. The nodal explants (0.5–1.0 cm long) were sterilized in 10% (v/v) NaClO solution for 15 min and then rinsed three times with sterile distilled water for 5 min. Thereafter, explants were transferred to Magenta™ GA7 culture vessels (67 × 67 × 97 mm) (Sigma-Aldrich, United States), each containing five explants and 80 mL of shoot induction medium. Root induction medium was composed of MS supplemented with 1 mg/L BA, 40.53 mg/L adenine sulfate, 218.4 mg/L monobasic sodium phosphate, 3% sucrose, and 0.6% agar, at pH 5.8. The cultures were maintained at 24 ± 1°C and a 16 h photoperiod (PAR = 300 μmol/m^2 s). After 60 days of culture, fully expanded uniform leaves of the plants were excised from leaf petiole at 10:00 a.m. and used for the following treatments.

Almond leaf samples were taken and divided into four groups with five replicates, then submerged in a beaker containing 250 mL of a solution with or without 5 mM H_2O_2 and 10 mM Pro for different incubation periods. Incubation was carried out in a growth chamber at 24 ± 1°C, and on an orbital rotating plate under light (150 μmol/(m^2 s).

The four treatments were: (a) incubation in sterile distilled H_2O for 12 h [control], (b) incubation in H_2O_2 solution for 12 h [12 h H_2O_2], (c) incubation in H_2O_2 solution with Pro for 12 h [12 h Pro/H_2O_2], and (d) incubation in Pro solution for 20 h followed by incubation in H_2O_2 solution for 4 h [24 h Pro/H_2O_2]. Subsequently, treated leaves were washed with double distilled H_2O, frozen in liquid N_2, and stored at –80°C until use for the analyses.

Proline and pigments content. For Pro content determination, the modified method of Sofo et al. [15] was used. A 5.0-mL aliquot of 3% (w/v) sulfosalicylic acid was added to 0.5 g of leaves and boiled in a water bath at 100°C for 30 min in glass tubes covered at the top. The mixture was centrifuged at 2000 *g* for 5 min at 25°C. A 300-μL aliquot of the extract was mixed with 300 μL of distilled water and 2.0 mL of the reagent mixture (30 mL of glacial acetic acid, 20 mL of distilled water, and 0.5 g of ninhydrin), and boiled at 100°C for 1 h. After cooling the reaction mixture, 5.0 mL toluene was added. The chromophore-containing toluene was separated, and A_{520} was read, using toluene as a blank. Pro concentration was calculated using L-Pro for the standard curve.

For pigment determination, leaves were homogenized in cold 80% acetone, and successively chlorophyll and carotenoids were extracted and estimated according to Upadhyaya et al. [11].

H_2O_2 levels, lipid peroxidation, and electrolyte leakage. For H_2O_2 determination, a 0.5-g aliquot of leaves was homogenized in 2 mL of 100 mM sodium-phosphate buffer, pH 6.8. The homogenate was filtered through three layers of cheesecloth and then centrifuged at 20000 *g* for 15 min at 4°C. A 0.5-mL aliquot of the supernatant was mixed with 2.5 mL of the peroxide reagent, containing of 83 mM sodium phosphate, pH 7.0, 0.005% (w/v) *o*-dianisidine, and 40 mg/mL peroxidase. The mixture was thermostated at 30°C for 10 min in a water bath, and then the reaction was stopped by adding 0.5 mL of 1 M $HClO_4$. After centrifugation at 10000 *g* for 5 min, the absorbance at 436 nm of the supernatant was compared to the extinction of a H_2O_2 standard, according to Sofo et al. [5].

The level of lipid peroxidation was determined in terms of thiobarbituric acid-reactive substances (TBARS) concentration as described by Hodges et al. [16] 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 10000 *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 10000 *g* for 5 min, the values of absorbance at 532, 600, and 440 nm of the supernatant were recorded. The value for a specific absorption at 600 nm was subtracted and a standard curve of sucrose (from 2.5 to 10 μmol/mL) 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/(mM cm), and the results expressed as MDA equivalents.

Electrolyte leakage (EL) was measured by using a HI 9933 conductimeter (Hanna Instruments, United States). Leaf samples were cut into equal sized pieces (0.3 g per treatment), placed in 25 × 150 mm culture vessels containing 15 mL of distilled water, and shaken on an orbital shaker (100 rpm) for 24 h at room temperature. The initial conductance of the bathing solution was measured using a conductivity meter. The tubes were then autoclaved at 115°C for 10 min, and final readings were taken following autoclaving and additional 24-h incubation at room temperature.

Total ascorbate and glutathione. For the extraction and estimation of total ascorbate (ASC), a 5-g aliquot of leaves was homogenized in 5.0 mL of ice-cold 2.5 N $HClO_4$, and the homogenate was filtered through three layers of cheesecloth (Miracloth) and then centrifuged at 15000 *g* for 5 min [5]. The supernatant was neutralized with 5 M K_2CO_3 to pH 4.5. The estimation of ASC was done by using a reaction mixture consisted of 2 mL of 2% sodium molybdate, 2 mL of 0.15 N H_2SO_4, 1 mL of 1.5 mM Na_2HPO_4, and 1 mL of the tissue extract. The mixture was incubated at 60°C in a water bath for 40 min. After centrifugation at 3000 *g* for 10 min, the absorbance was read at 660 nm over 2-min intervals.

For the extraction and estimation of total glutathione (GLU), leaves were homogenized in 5% (w/v) sulfosalicylic acid and centrifuged at 10000 *g* for 10 min. The supernatant (1 mL) was neutralized with 0.5 mL of 0.5 M potassium phosphate buffer (pH 7.5). Total GLU was measured by adding 1 mL of neutralized supernatant to a standard solution mixture consisting of 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.5) containing EDTA, 0.2 mL of 6 mM 5,5-dithiobis(2-nitrobenzoic acid), 0.1 mL of 2 mM NADPH, and 1 mL of 1 U glutathione reductase (GR) from *Saccharomyces cerevisiae* (Sigma-Aldrich; catalog no. G3664) [5]. The changes in the absorbance at 412 nm were followed at 25 ± 2°C until the absorbance reached 5 U, according to Griffith et al. [17].

Enzyme extraction and activities. Tissue samples were prepared for analyses by homogenizing 1 g of 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) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 10000 *g* and 4°C for 10 min.

For the measurement of total SOD activity, the reaction mixture (3 mL) contained 50 mM K-phosphate buffer (pH 7.8), 13 mM methionine, 75 mM nitroblue tetrazolium (NBT), 4 mM riboflavin, 0.1 mM EDTA, and 0.25 mL of the enzyme extract. One unit of enzyme activity was determined as the amount of the enzyme to reach an inhibition of 50% NBT reduction rate by monitoring absorbance at 560 nm [15]. The test tubes were shaken and then

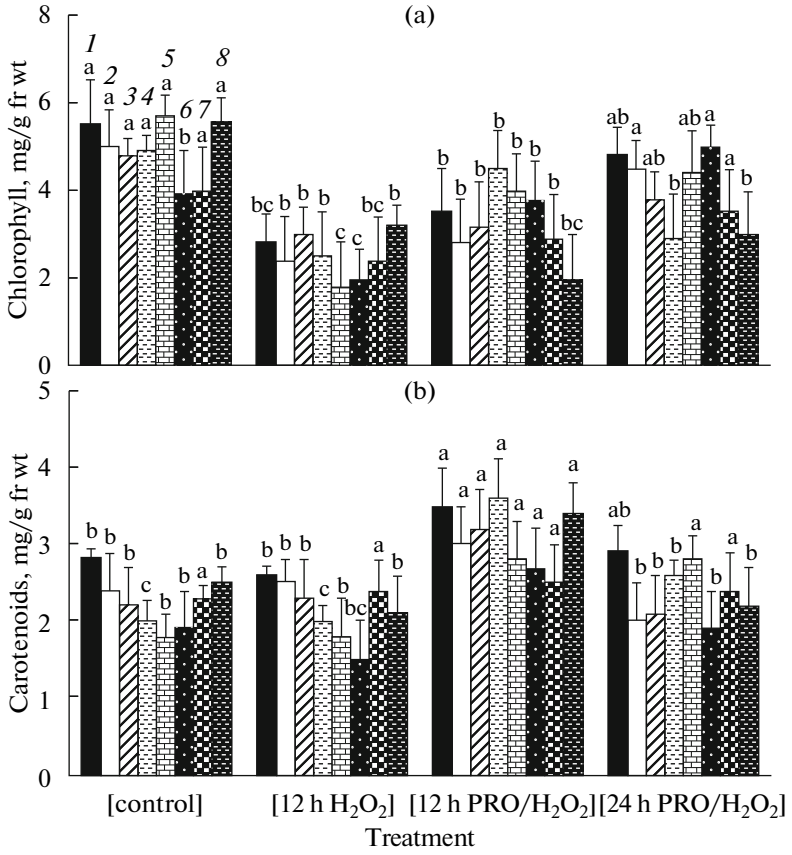

Fig. 1. Levels of chlorophyll (a) and carotenoids (b) in leaves of eight wild almond species incubated in H_2O for 12 h [control], in H_2O_2 solution for 12 h [12 h H_2O_2], in H_2O_2 solution + Pro for 12 h [12 h Pro/H_2O_2], in proline solution for 20 h and then in H_2O_2 solution for 4 h [24 h Pro/H_2O_2].

Data represent means of five replicates ± SE. Values followed by different letters above columns are significantly different at $P \leq 0.05$, according to Fisher's LSD Test.

(*1*) *P. communis*; (*2*) *P. eleagnifolia*; (*3*) *P. orientalis*; (*4*) *P. lycioides*; (*5*) *P. reuteri;* (*6*) *P. arabica*; (*7*) *P. glauca*; (*8*) *P. scoparia.*

placed in a light box consisting of six fluorescent lamps (15 W preheat, daylight 6500 K; light intensity = 900 μmol/(m^2 s) for 10 min. The reaction was based on the reduction of NBT in the presence of riboflavin in light, and it was stopped by switching off the light and placing the test tubes into darkness.

In order to measure spectrophotometrically the activities of different SOD isoforms, either 10 μL of 100 mM KCN (inhibitor of Cu/Zn-SOD) or 25 μL of 80 mM H_2O_2 (inhibitor of both Cu/Zn-SOD and Fe-SOD) were added to the reaction mixture [18]. The activity of Mn-SOD was determined in the presence of both KCN and H_2O_2 and calculated according to Yu and Rengel [18]. Detection of different SOD isoforms was performed electrophoretically in 13% nondenaturing PAAG with a 5% stacking gel, using 40 μg of the original extract per well. The gels were pre-equilibrated in a solution of 0.05 M K-phosphate buffer (pH 7.9) and 1 mM EDTA for 30 min and then immersed in 0.24 mM NBT, 33.2 μM riboflavin, and 0.2% N,N,N',N'-tetramethylethylenediamine for 30 min in darkness [19]. The same inhibitors used for spectrophotometric analysis (2 mM KCN and 5 mM H_2O_2, alone or in combination) were used in PAAG pre-equilibration buffer, in order to detect different SOD isoforms, and final gels were prepared without inhibitors.

APX activity was determined using a reaction mixture with 50 mM K-phosphate buffer (pH 7.0), 1 mM Na-EDTA, 0.5 mM ascorbic acid, 0.1 mM H_2O_2, and 50 mL of the crude enzyme extract [15]. APX activity was calculated by measuring the change in absorbance at 290 nm. One unit of APX activity was defined as the amount of enzyme that oxidized 1 μmol of ascorbate per min at 20°C. Activities of CAT and POD were measured by the methods of Chance and Maehly [20] and Osswald et al. [21], respectively, with some modifications. For CAT activity determination, the decomposition of H_2O_2 was followed by a decline in absorbance at 240 nm. The reaction was initiated by adding

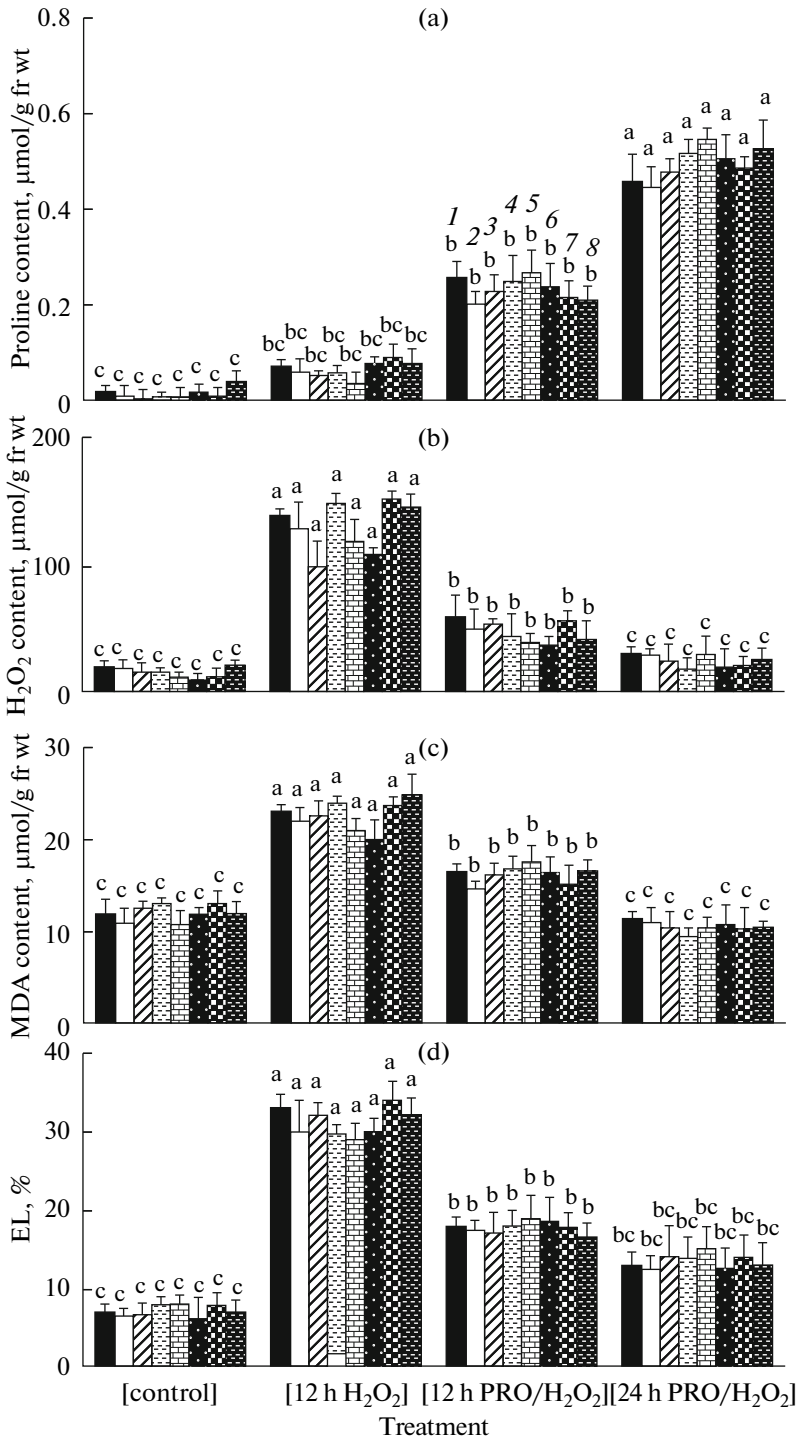

Fig. 2. Levels of endogenous proline (a), hydrogen peroxide (b) MDA (c), and percentage of electrolyte leakage (EL) (d) in leaves of eight wild almond species incubated in H_2O for 12 h [control], in H_2O_2 solution for 12 h [12 h H_2O_2], in H_2O_2 solution + Pro for 12 h [12 h Pro/H_2O_2], in proline solution for 20 h and then in H_2O_2 solution for 4 h [24 h Pro/H_2O_2].

Statistics as in Fig. 1.(*1*) *P. communis*; (*2*) *P. eleagnifolia*; (*3*) *P. orientalis*; (*4*) *P. lycioides*; (*5*) *P. reuteri*; (*6*) *P. arabica;* (*7*) *P. glauca*; (*8*) *P. scoparia*.

the enzyme extract to 3 mL of the reaction mixture containing 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2, and 50 μL of the enzyme extract. CAT activity was determined by following the consumption of H_2O_2 at 240 nm over 2-min intervals. One unit of CAT activity was defined as the amount of enzyme that decom-

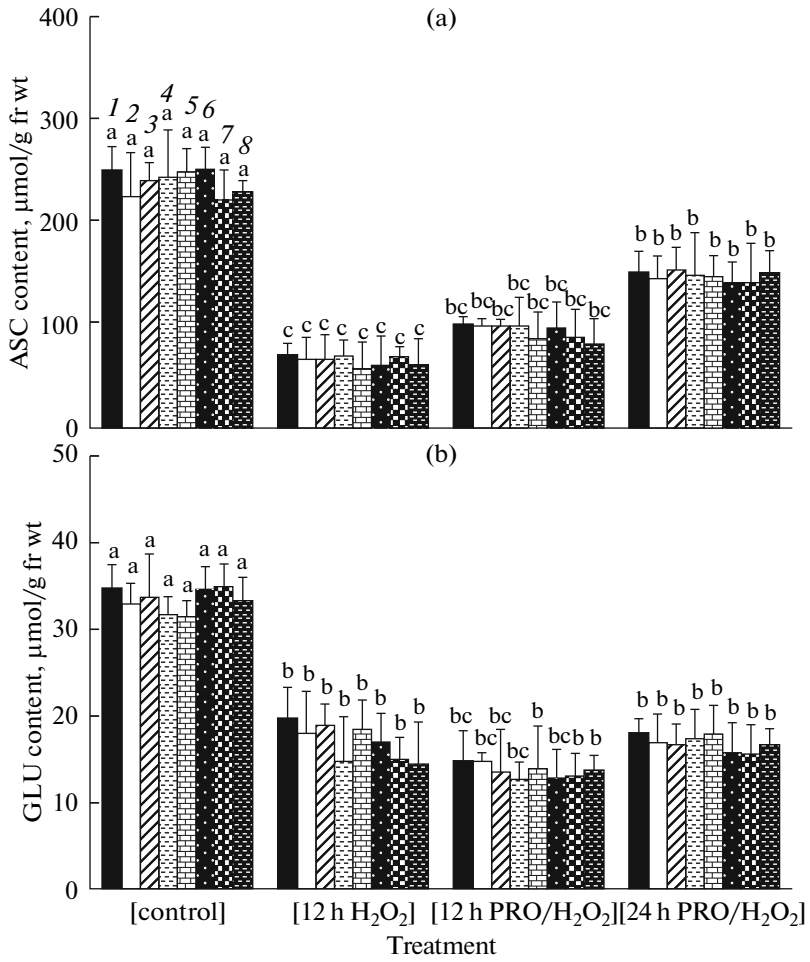

Fig. 3. Levels of total ascorbate (ASC) (a) and total glutathione (GLU) (b) in leaves of eight wild almond species incubated in H_2O for 12 h [control], in H_2O_2 solution for 12 h [12 h H_2O_2], in H_2O_2 solution + proline for 12 h [12 h Pro/H_2O_2], in proline solution for 20 h and then in H_2O_2 solution for 4 h [24 h Pro/H_2O_2].

Statistics as in Fig. 1.(*1*) *P. communis*; (*2*) *P. eleagnifolia*; (*3*) *P. orientalis*; (*4*) *P. lycioides*; (*5*) *P. reuteri*; (*6*) *P. arabica*; (*7*) *P. glauca*; (*8*) *P. scoparia*.

posed 1 μmol of H_2O_2 per minute. For POD, the activity was determined by measuring the oxidation of guaiacol in the presence of H_2O_2, and following the increase in absorbance at 470 nm over 2-min intervals. The assay mixture contained 0.05 mL of 20 mM guaiacol, 2.9 mL of 10 mM, K-phosphate buffer (pH 7.0), and 50 μL of the enzyme extract. The reaction was initiated by adding 20 μL of 40 mM H_2O_2. One unit of POD activity was defined as the amount of enzyme that decomposed 1 μmol of guaiacol per minute.

The soluble protein content was determined by the Coomassie blue dye binding method, using BSA for plotting the standard curve.

Statistical analysis. Data were represented as mean values ± SE for five replicates and treated by analysis of variance (ANOVA) using the SAS software (SAS Institute, United States) in order to detect significant differences among the different treatments (PROC GLM) at $P \leq 0.05$, according to Fisher's LSD Test.

RESULTS

Levels of Pigments, Proline, and H_2O_2

In comparison with control plants, a significant decrease in chlorophyll content was observed in leaves under treatment with H_2O_2 for 12 h for all the cultivars examined, but the application of Pro reversed this trend (Fig. 1a). In particular, *P. eleagnifolia, P. arabica*, and *P. glauca* reached the levels of control plants after [24 h Pro/H_2O_2] application (Fig. 1a). The positive effect of Pro was confirmed for carotenoid content, which statistically increased in [12 h Pro/H_2O_2], with exception of *P. glauca*, and remained generally unchanged in [24 h Pro/H_2O_2] treatment as compared to the control plants (Fig. 1b).

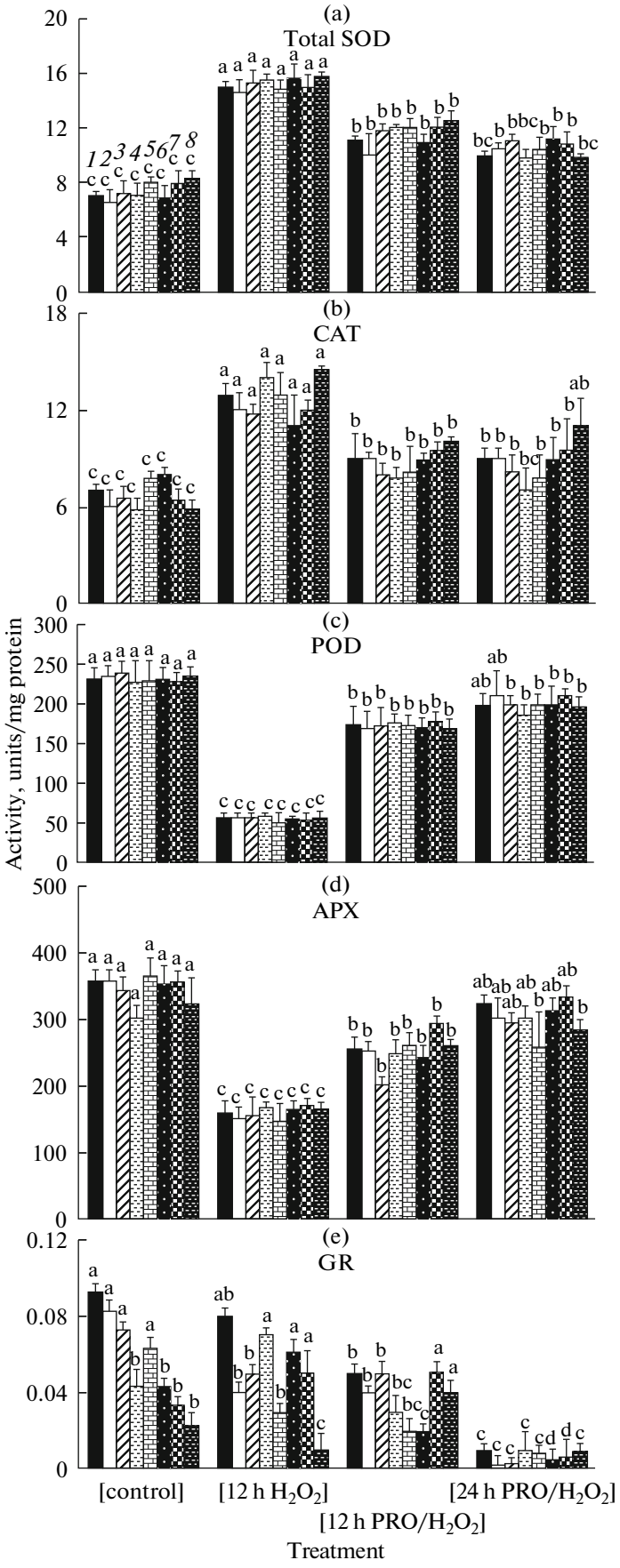
(a)
Total SOD
(b)
CAT
(c)
POD
(d)
APX
(e)
GR
Activity, units/mg protein
1 2 3 4 5 6 7 8
[control]
[12 h H_2O_2]
[24 h PRO/H_2O_2]
[12 h PRO/H_2O_2]
Treatment

Fig. 4. Activities of total superoxide dismutase (SOD) (a), catalase (CAT) (b), guaiacol peroxidase (POD) (c), ascorbate peroxidase (APX) (d), and glutathione reductase (GR) (e) in leaves of eight wild almond species incubated in H_2O for 12 h [control], in H_2O_2 solution for 12 h [12 h H_2O_2], in H_2O_2 solution + proline for 12 h [12 h Pro/H_2O_2], in proline solution for 20 h and then in H_2O_2 solution for 4 h [24 h Pro/H_2O_2].

Statistics as in Fig. 1. (*1*) *P. communis*; (*2*) *P. eleagnifolia*; (*3*) *P. orientalis*; (*4*) *P. lycioides*; (*5*) *P. reuteri*; (*6*) *P. arabica*; (*7*) *P. glauca*; (*8*) *P. scoparia*.

Pro concentration ranged from 0.009 to 0.038 μmol/g fr wt in control leaves, and it increased up to 0.105 μmol/g fr wt under [12 h H_2O_2] treatment (Fig. 2a). Endogenous Pro concentration of samples increased to 0.27 μmol/g fr wt, after [12 h Pro/H_2O_2] treatment, and the samples subjected to [24 h Pro/H_2O_2] treatment showed a drastic increase (up to 0.53 μmol/g fr wt) (Fig. 2a). The endogenous H_2O_2 content of sample was 10 to 22 μmol/g fr wt under no stress condition (Fig. 2b), but it sharply increased to about 150 μmol/g fr wt after [12 h H_2O_2] treatment. Interestingly, the addition of Pro during the oxidative stress caused a significant decrease in the endogenous H_2O_2 concentration to a mean value of 38 μmol/g fr wt in leaves under [12 h H_2O_2] treatment (Fig. 2b). Almost similar endogenous Pro concentration (30 μmol/g fr wt) was observed in leaves subjected to the treatments [12 h Pro/H_2O_2] and [24 h Pro/H_2O_2] (Fig. 2b).

Membrane Oxidative Damage

The range of endogenous MDA content was 10.8 to 13.0 μmol/g fr wt in control leaves, and MDA level increased in leaves subjected to [12 h H_2O_2] treatment (Fig. 2c). However, a significant decrease in MDA content was observed when the samples were subjected to the treatments [12 h Pro/H_2O_2] and [24 h Pro/H_2O_2] (Fig. 2c).

Electrolyte leakage (EL), increased drastically under the [12 h H_2O_2] treatment as compared to control plants (Fig. 2d). The mean values of EL among all the almond species under the [12 h Pro/H_2O_2] and [24 h Pro/H_2O_2] treatments were 19 and 14%, respectively, so indicating a significant lower membrane damage if compared to that of leaves subjected only to H_2O_2 (mean EL = 34%).

Levels of Total Ascorbate and Glutathione, and Antioxidant Enzyme Activities

The content of ASC and GLU significantly decreased in relation to the duration of oxidative stress in all the species examined (Fig. 3). Pro application in the treatments [12 h Pro/H_2O_2] and [24 h Pro/H_2O_2] partially mitigated ASC decreases, while this did not happen for GLU (Fig. 3).

Responses of SOD and CAT activities to the different oxidative stress treatments were the opposite to the responses of POD and APX under the assay conditions (Fig. 4). Treatment of leaf samples with H_2O_2 for 12 h drastically increased total SOD activity, whereas the two treatments with Pro caused a reduced increase as compared to the treatment [12 h H_2O_2] (Fig. 4a). Responses of CAT enzyme to different oxidative stress treatments were similar to those of total SOD activity, as it was directly related to the level of oxidative stress in all the species studied (Fig. 4b). In particular, *P. glauca* and *P. scoparia* under oxidative stress manifested the higher CAT activity if compared with the other species (Fig. 4b).

The two almond species that showed the lower and the higher total SOD activity in the control treatment (*P. eleagnifolia* and *P. scoparia*, respectively) were chosen to measure the activities of the different SOD isoforms both spectrophotometrically and electrophoretically (table, Fig. 5). Both Fe-SOD and Cu/Zn-SOD of the two species were clearly inhibited after [12 h H_2O_2] treatment, but not statistically affected by [24 h Pro/H_2O_2] (table, Fig. 5). The activities of these two SOD isoforms were inhibited under [12 h Pro/H_2O_2] in *P. eleagnifolia* but not in *P. scoparia* (table, Fig. 5). In both species, the behavior of Mn-SOD was opposite, as its activity was significantly enhanced in plants

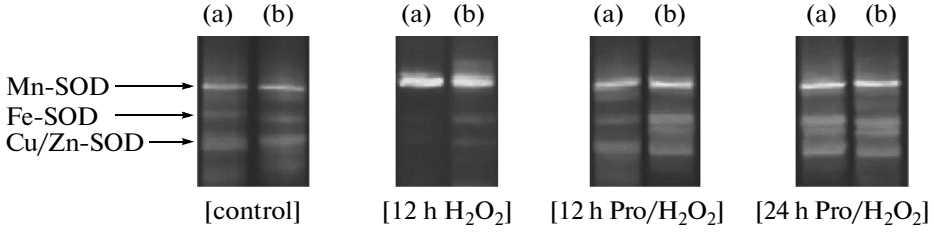

Fig. 5. Separation of SOD isoforms by nondenaturing protein PAGE in leaves of two wild almond species ((a) *Prunus eleagnifolia* and (b) *P. scoparia*) incubated in H_2O for 12 h [control], in H_2O_2 solution for 12 h [12 h H_2O_2], in H_2O_2 solution + proline for 12 h [12 h Pro/H_2O_2], in proline solution for 20 h and then in H_2O_2 solution for 4 h [24 h Pro/H_2O_2].

The different SOD isoforms are indicated in the left margin.

Activities of the different superoxide dismutase (SOD) isoforms in leaves of two wild almond species (*Prunus eleagnifolia* and *P. scoparia*)

Treatment	SOD isoform	SOD activity, units/mg protein	
		P. elaeagnifolia	*P. scoparia*
[control]	Mn-SOD	3.6 ± 0.5^{c}	4.3 ± 0.7^{c}
	Fe-SOD	2.0 ± 0.2^{a}	2.2 ± 0.2^{a}
	Cu/Zn-SOD	1.2 ± 0.4^{a}	1.8 ± 0.5^{a}
[12 h H_2O_2]	Mn-SOD	13.2 ± 2.9^{a}	14.2 ± 1.4^{a}
	Fe-SOD	0.7 ± 0.1^{c}	1.0 ± 0.2^{b}
	Cu/Zn-SOD	0.3 ± 0.1^{b}	0.5 ± 0.1^{b}
[12 h Pro/H_2O_2]	Mn-SOD	7.5 ± 1.4^{b}	8.9 ± 1.8^{b}
	Fe-SOD	1.4 ± 0.2^{b}	1.7 ± 0.4^{a}
	Cu/Zn-SOD	0.8 ± 0.1^{ab}	1.2 ± 0.6^{a}
[24 h Pro/H_2O_2]	Mn-SOD	7.2 ± 1.6^{b}	6.4 ± 1.2^{b}
	Fe-SOD	1.8 ± 0.3^{a}	1.9 ± 0.4^{a}
	Cu/Zn-SOD	1.2 ± 0.4^{a}	1.3 ± 0.2^{a}

Notes: Leaves were incubated in H_2O for 12 h [control], in H_2O_2 solution for 12 h [12 h H_2O_2], in H_2O_2 solution + Pro for 12 h [12 h Pro/H_2O_2], in proline solution for 20 h and then in H_2O_2 solution for 4 h [24 h Pro/H_2O_2]. Data represent means of five replicates ± SE. Values in two columns followed by different letters are significantly different at $P \leq 0.05$, according to Fisher's LSD Test.

subjected to [12 h H_2O_2], [12 h Pro/H_2O_2], and [24 h Pro/H_2O_2] treatments (table, Fig. 5).

The application of [12 h H_2O_2] treatment caused a drastic decrease in POD activity (about 25% of the control), whereas leaves subjected to [12 h Pro/H_2O_2] and [24 h Pro/H_2O_2] treatments showed significantly higher activities as compared to [12 h H_2O_2] (Fig. 4c). Moreover, the levels of POD activity were highly uniform among the almond species. The patterns of APX activity in all the oxidative stress treatments were similar to POD activity but the differences among the species were more marked as compared to POD, and the activities under [24 h Pro/H_2O_2] treatment more similar to those of control plants (Fig. 4d). In particular, after [24 h Pro/H_2O_2] treatment, *P. communis* and *P. glauca* presented high values of APX activity as compared with the other species (Fig. 4d), while *P. eleagnifolia* and *P. glauca* presented the highest values of POD activity after the same treatment (Fig. 4c). With some exceptions, the effects of [24 h Pro/H_2O_2] treatment on total SOD, CAT, POD, and APX activities were found to be significantly different from those determined after [12 h Pro/H_2O_2] treatment (Figs. 4a–4d).

In *P. lycioides, P. arabica*, and *P. glauca* leaves, GR activity in [12 h H_2O_2] was the higher than in control plants, whereas in the remaining almond species a significant decrease occurred (Fig. 4e). This trend was not reversed after [12 h Pro/H_2O_2] treatment, with exception of *P. scoparia*, and GR activities decline even more in plants subjected to [24 h Pro/H_2O_2] (Fig. 4e). *P. communis* presented the highest values of GR activity under all the four treatments (Fig. 4e).

DISCUSSION

Plant pigments are of key importance for photosynthetic processes and for preventing photo-oxidation and photo-inhibition [4, 6], and their degradation in senescing leaves is due to the cytotoxic effect of H_2O_2 [22]. The leaves of the almond species here studied showed a partial degradation of chlorophylls when subjected to [12 h H_2O_2] treatment that was mitigated with the simultaneous presence of Pro (Fig. 1a). A similar protective role of Pro was observed for carotenoids, whose levels increased after Pro treatment (Fig. 1b). The values observed for both chlorophylls and carotenoids in [12 h H_2O_2] treatment indicated that the H_2O_2 concentration used (5 mM) was sufficient for inducing a certain degree of oxidative stress without irreversibly oxidizing pigments or damaging chloroplast thylakoids, where they are located. Upadhyayal et al. [11] demonstrated that the negative effects of H_2O_2 on pigments of rice leaves were mitigated by the addition of radical scavengers, such as mannitol and sodium benzoate. Our results confirmed that the presence of Pro during the oxidative stress protected chlorophylls and carotenoids from degradation (Figs. 1a, 1b).

Cellular content of MDA, H_2O_2, and level of EL reflect cellular damage resulting from oxidative stress [6]. The results show that there was a positive direct correlation between oxidative stress induced by H_2O_2 and increases in MDA and EL (Figs. 2b-2d), as reported for many plant species [8]. Ozden et al. [23] recently found that a treatment with H_2O_2 increased lipid peroxidation and endogenous accumulation of H_2O_2, and decreased membrane stability in grapevine. It is known that drought, salt, metal exposure, and other abiotic stresses induce endogenous H_2O_2 accumulation in plants [4, 8]. Our results showed that when Pro was exogenously supplied together with H_2O_2 or prior to H_2O_2 treatment, the production of H_2O_2 into the cells was significantly reduced (Fig. 2b). In our experiment, the Pro content in the leaves increased in relation to the length of the incubation time of leaves with Pro (Fig. 2a). The observed increase in the Pro concentration in leaf samples after the treatment with H_2O_2 alone for 12 h (Fig. 2a) can be attributed to the *ex novo* synthesis of Pro, that can act as a ROS scavenger [11, 24]. Generally, there is a strong positive correlation between oxidative stress tolerance and Pro accumulation in higher plants [7]. A longer incubation time with Pro ([24 h Pro/H_2O_2] treatment) significantly reduced the concentrations of H_2O_2 and MDA, and the values of EL as compared to the leaves subjected to [12 h Pro/H_2O_2] treatment (Figs. 2c, 2d). From all these data, Pro appeared to have a protective

action which prevents membrane damage and protein denaturation in oxidative conditions [25, 26].

Under normal growth conditions, the antioxidant system is usually sufficient to prevent oxidative damage but environmental stresses are known to increase H_2O_2 and other toxic oxygen species production in cellular compartments, so enhancing oxidative stress and its deleterious effects [4, 5]. The decrease in the total ASC and GLU contents in H_2O_2-stressed almond leaves (Fig. 3), only partially mitigated by Pro in the case of ASC (Fig. 3a), demonstrated a low non-enzymatic antioxidant protection [5, 8]. On the contrary, the enzymatic antioxidant defense of almond leaves has been partly up-regulated under oxidative stress conditions (Figs. 4a, 4b). Indeed, the increase in the total SOD and CAT activities (Figs. 4a, 4b) was strongly induced by the oxidative stress at [12 h H_2O_2] treatment (Figs. 4a, 4b). Their decrease due to Pro application (Figs. 4a, 4b), as compared to [12 h H_2O_2] treatment, could be attributed to the decreased H_2O_2 levels (Fig. 2b), not sufficient to up-regulate the activities of these enzymes and/or the transcription of the corresponding genes, as described by other authors [4, 5, 8]. Interestingly, the activities of both Fe-SOD and Cu/Zn-SOD isoforms of both *P. eleagnifolia* and *P. scoparia* plants (table) were negatively affected by the high levels of H_2O_2 present in [12 h H_2O_2] treatment (Fig. 2b). In contrast, Mn-SOD activity (table) was finely up-regulated on the basis of the levels of H_2O_2 in different treatments (Fig. 2b), and this could be due to the extremely variable level of Mn-SOD mRNA observed by some authors in *Prunus* genus [27]. The observed changes in the activities of SOD isoforms were confirmed by PAGE that highlighted how the enzymes responded to H_2O_2 action (Fig. 5). Thus, the application of Pro seems to act indirectly on SOD activity regulating the H_2O_2 level. Recently, similar differences between SOD isoforms in relation to proline were found in other species, such as *Salvia officinalis* [28]. Moreover, as Mn-SOD is mainly mitochondrial, Fe-SOD plastidic, and Cu/Zn-SOD cytosolic, Pro regulation is likely based on various extents of oxidative stress in different cell compartments [28].

The marked decreases in POD and APX activities after [12 h H_2O_2] treatment (Figs. 4c, 4d) suggest a signaling role of H_2O_2 in the down-regulation of these enzymes, as observed in other agronomically important crop species subjected to oxidative stress [3]. This depression was likely due to the generation of very high levels of oxidative stress (up to 157 μmol H_2O_2/g fr wt in *P. glauca*; Fig. 2b) and to the rapid inhibition of APX and POD (Figs. 4c, 4d). Furthermore, the values of APX activity (Fig. 4d) strongly reflected the changes of ASC levels in leaves observed in the four treatments (Fig. 3a), and they were inversely related to H_2O_2 levels (Fig. 2b), that so acts as a negative feedback regulator. Our results indicate that two different treatments of almond leaves with Pro did not eliminate the inhibitory effect of H_2O_2 on POD and APX but partially restored their activities as compared to [12 h H_2O_2] treatment (Figs. 4c, 4d), so confirming the important role of Pro in the regulation of these antioxidant enzymes during oxidative events. Finally, as observed for GLU (Fig. 3b), the decrease in GR activities decreased during oxidative stress for some almond species and was not reversed by Pro treatment (Fig. 4e). We suggest that this is due to the fact that GR isoforms are mainly cytosolic [3, 8] and so less affected by the protective action of Pro against membrane damage (Figs. 2c, 2d). As in the case of APX, the values of GR activity (Fig. 4e) strongly reflected those of its substrate (GLU; Fig. 3b), but they were not related to the levels of H_2O_2 (Fig. 2b), probably because H_2O_2 is not able to regulate the activity of this enzyme [5, 8].

Different ROS compounds show different reactivity with biomolecules and play different roles in the complex signaling system [4, 6]. The paradox consists in the fact that plants developed several systems to control the ROS level, but on the other hand, plants produce and need ROS for their development, according to the rule "no stress no development" [6]. The concerted action of the antioxidant enzymes here studied (Fig. 4) are finely regulated by the H_2O_2 level (Fig. 2b) and affected by Pro application; they could improve the tolerance of wild almond species against oxidative stress.

In conclusion, our results highlight that the damage to cell membranes is a direct consequence of oxidative stress induced by H_2O_2 and that the application of Pro can alleviate these detrimental effects (Figs. 2c, 2d). The external application of Pro allowed the almond leaves to better face oxidative stress by acting as an efficacious H_2O_2 scavenger (Fig. 2b). Excluding GLU levels and GR activity (Figs. 3b, 4e), all the activities of the antioxidant enzymes studied (Fig. 4) and ASC content (Fig. 3) were found to be finely up- or down-regulated in response to oxidative stress, in accordance to other studied carried out on the same wild almond species under drought stress [12]. It is still debated if the observed effects of Pro application could be due to a direct effect or an indirect effect mediated by H_2O_2 detoxification, but, on the basis of the results discussed here, it is possible to recommend exogenous Pro treatment of wild almond in order to increase its antioxidant defense against the adverse environmental conditions typical of the semi-arid regions, where this species grows.

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