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Influence of water deficit and rewatering on the components of the ascorbate–glutathione cycle in four interspecific *Prunus* hybrids

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Abstract

The activities of ascorbate peroxidase (APX, EC 1.11.1.11), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2), as well as the levels of ascorbate pool, glutathione pool and H_2O_2 were studied in plants of four interspecific *Prunus* hybrids subjected to water deficit and shade conditions. After 70 days of water shortage, plants were subjected to a rewatering treatment. During water recovery, leaves fully exposed to sunlight and leaves in shade conditions of about 30% of environmental irradiance were sampled. After 70 days without irrigation, mean pre-dawn leaf water potential of all the hybrids fell from −0.34 to −3.30 MPa and marked decreases in net photosynthesis and transpiration occurred. The activities of APX, MDHAR, DHAR and GR increased in relation to the severity of drought stress in all the clones studied. Generally, APX, MDHAR, DHAR and GR were down-regulated during the rewatering phase and their activities decreased faster in shaded leaves than in non-shaded leaves. The levels of total ascorbate, total glutathione and H_2O_2 were directly related to the increase of drought stress and subsequently decreased during rewatering. This response could limit cellular damage caused by active oxygen species during periods of water deficit. The ability of *Prunus* hybrids to regulate the enzymatic antioxidant system during different water and irradiance conditions might be an important attribute linked to drought tolerance.

Keywords: Antioxidant enzymes; Ascorbate peroxidase; Dehydroascorbate reductase; Drought stress; Glutathione reductase; Oxidative stress

1. Introduction

Acclimation of plants to drought is often associated with increased levels of activated oxygen species (AOS), such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$) and singlet oxygen ($^1O_2$), which are toxic for the cells [1,2]. AOS are by-products of aerobic metabolism and their production is enhanced during drought conditions through the disruption of electron transport system and oxidizing metabolic activities occurring in chloroplasts, mitochondria and microbodies [3,4]. Excessive levels of AOS damage cellular structures and macromolecules, causing photoinhibition of photosynthetic apparatus [1] but the production and accumulation of AOS activate multiple defence responses, thus having also a positive role [4,5]. In particular, the presence of H_2O_2 in the apoplast is toxic for pathogens, is involved in gene transcription and systemic acquired resistance, and slows down the spread of invading organisms by cell death round the infection and a rapid local cross-linking of the cell wall [6,7].

Under non-stressful conditions, AOS are efficiently eliminated by non-enzymatic and enzymatic antioxidants, whereas during drought conditions the production of AOS exceeds the capacity of the antioxidative systems to remove them, causing oxidative stress [1,8]. The antioxidant non-

Abbreviations: AOS, activated oxygen species; APX, ascorbate peroxidase; AsA, ascorbate; CP, control plants; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; LWP, leaf water potential; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; NSL, non-shaded leaves; PAR, photosynthetic active radiation; SL, shaded leaves; SP, stressed plants

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enzymatic system includes ascorbate and glutathione, two constituents of the antioxidative ascorbate–glutathione cycle which detoxify H_2O_2 in the chloroplasts [3] and are located both within the cell and in the apoplast [6,9]. Ascorbate (AsA) is a major primary antioxidant synthesized on the inner membrane of the mitochondria which reacts chemically with $^1O_2$, $O_2^{\bullet -}$, $HO^{\bullet}$ and thiyl radical [3,8], and acts as the natural substrate of many plant peroxidases [10]. Moreover, AsA is involved in other functions such as plant growth, gene regulation, modulation of some enzymes and redox regulation of membrane-bound antioxidant compounds [6–8]. Glutathione (GSH) is a tripeptide synthesized in the cytosol and the choloroplast which scavenges $^1O_2$ and H_2O_2 and is oxidized to glutathione disulfide (GSSG) when acts as an antioxidant and redox regulator [1,8,11]. GSH is the substrate of glutathione *S*-transferases (GSTs), which have a protective role in the detoxification of xenobiotics and dehydroascorbate reductase (DHAR) [9]. Finally, GSH is a precursor of phytochelatins, which regulate cellular heavy metals levels, and is involved in gene expression [8].

The antioxidant enzymatic system includes the enzymes of the ascorbate–glutathione cycle, that operates both in the chloroplasts and in the cytosol: ascorbate peroxidase (APX, EC 1.11.1.11), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2).

The genus *Prunus* comprises more than 400 species adapted to temperate areas and cultivated in Europe [12]. In particular, stone fruit crops, such as peach (*Prunus persica* L.), plum (*Prunus cerasifera* L. and *Prunus domestica* L.), almond (*Prunus dulcis* L.), apricot (*Prunus armeniaca* L.) and cherry tree (*Prunus avium* L.), are typical and economically important cultures mainly localized in Mediterranean regions, where the spring–summer period is often characterized by high temperatures, high irradiance levels and lack of precipitation. Productive stone fruit trees are usually grafted plants with a lower part, the rootstock and an upper grafted part, which is the genotype of the commercial variety. Rootstocks have different genetic background compared to the commercial varieties and can be used to confer various traits, such as drought stress resistance. Many plum genotypes are used as rootstock for almost all other *Prunus* species and, among them, Myrobalan plum (*P. cerasifera* L.) clones show positive agronomic features and are resistant to root-knot nematodes [12]. The response to water deficit of these species is a well documented process [13–17] but only few studies highlighted the importance of antioxidant enzymes in genus *Prunus* and in other fruit trees [18,19], and very little is known about the linkages between drought and the components of the ascorbate–glutathione cycle in these species.

The aim of this work was to study the changes of antioxidant enzyme activities (APX, MDHAR, DHAR and GR) and the level of some compounds (ascorbate and glutathione pools and H_2O_2) involved in the ascorbate–glutathione cycle in plants of four *Prunus* interspecific hybrids grown under water shortage followed by a rewatering phase, and to determine the differences of antioxidant and physiological responses among hybrids during stress conditions. Finally, on the basis of previous findings [19], we also hypothesize different patterns of enzyme activities in leaves under different levels of irradiance during the rewatering phase.

2. Materials and methods

2.1. Study site, plant material and experimental design

The study site was located at the 'Università degli Studi della Basilicata' in Potenza (Southern Italy – Basilicata Region – 40°39′N, 15°47′E). The experimental period started on July 10 and ended on October 24, 2002.

Trials were conducted on virus free plant material obtained from the breeding programs of INRA Bordeaux and SIA Zaragoza (EU funded project FAIR-6-CT-98-4139). The material, presenting different levels of resistance against nematodes of *Meloidogyne* spp., included four interspecific hybrids named 'P3605' (*Prunus amygdalus* L. 'Garfi' × *P. persica* L. 'Nemared'), '8–9' (*P. cerasifera* L. 'P2980' × 'P3605'), '7–7' (*P. cerasifera* L. 'P2175' × *Prunus davidiana* L.) and '6–5' (*P. cerasifera* L. 'P2175' × *P. amygdalus* L. 'Garfi').

The experimental scheme was carried out using 1-year-old rootstocks planted in spring 2001, spaced at 1 m in the row with 1 m between rows and grew uniformly outdoors in 5.0 m^3 containers filled with a silty-clay loam. Trees were irrigated with drip emitters per plant discharging 3 L h^{-1}. Soil water content was maintained at a constant value of around 85% of the field water capacity by integrating the amount of water lost through transpiration during the day. Plants were fertilized at 25-day intervals throughout the period of vegetative growth with 3.5 g of slow release nitrogen complex fertilizer Nitrophoska Gold—BASf-15N-9P-15 K + 2 Ca + 17.5 Mg (Compo Agricoltura, Cesano Maderno, MI, Italy).

Plants were divided in two groups: drought-stressed plants (SP) and control plants (CP). CP were maintained in an optimal soil water conditions (85% of the field water capacity) during the whole experimental period, whereas SP subjected to a water shortage period starting from July 10 to September 18. Containers of SP were covered with plastic film in order to avoid rainfall infiltrations and evaporation from the soil surface. After this 70-day period of drought, stressed plants were subjected to a rewatering treatment of 36 days (from September 19 to October 24).

2.2. Environmental parameters, gas exchange and water status

For each day of the experimental period, measurements of air temperature and relative humidity (RH) were taken by a data logger Tinytag Ultra 2 K (Maeco, Cranleigh, Surrey,

UK) located inside the experimental plot. Vapour pressure deficit (VPD) was calculated from the values of air temperature and RH at 11:00 h, according to Goudriaan and van Laar [20]. The values of soil humidity at 0, 2, 9, 13, 23, 70 and 84 days from the beginning of the experimental period, were determined from the weight differences of soil samples before and after drying at 105 °C for 18 h and expressed as percentages of water on dry matter. Soil samples were taken in different points of the containers at three levels of depth (0–20, 20–40 and 40–60 cm).

Three CP and three SP from each clone were chosen to measure physiological parameters at 0, 2, 9, 13, 23, 70 and 84 days from the beginning of the experimental period on four fully expanded leaves selected from each plant along the median segment of new-growth shoots and marked at the beginning of the experiment. The measurements of transpiration rate, photosynthetic rate, stomatal conductance were carried out using the portable photosynthesis system LCA-4 (Analytical Development Company, Hoddesdon, UK) operated at 200 μmol m^{-2} s^{-1} flow rate, under field conditions at 09:30 h.

The measurements of leaf water potential (LWP) were carried out pre-dawn (at 04:00 h) at 0, 2, 9, 13, 23, 70 and 84 days from the beginning of the experimental period on three fully expanded leaves selected from each plant along the median segment of new-growth shoots using a Sholander pressure chamber (PMS Instrument Co., Corvallis, OR, USA), according to Turner [21].

2.3. Leaf sampling

Three SP plants having similar LWP and three CP from each clone were selected in each date for tissue sampling. Leaves were collected at 0, 2, 9, 13, 23, 63 and 70 days from the beginning of the drought stress period. Each sample contained three fully expanded leaves taken along the median segment of new-growth shoots and fully exposed to sunlight (photosynthetic active radiation, PAR > 1000 μmol m^{-2} s^{-1}). At the end of the rewatering phase, both in rewatered and control plants, two types of leaf samples were collected: the former with leaves fully exposed to sunlight (non-shaded leaves (NSL)) and the latter with leaves picked in the inner part of the canopy in shade conditions of about 30% of environmental PAR (shaded leaves (SL)). Leaf samples were washed with distilled water, dried with filter paper, temporarily covered with an aluminium foil and put in a plastic envelope, and then immediately used for extraction of the different enzymes and for the determination of the levels of ascorbate, glutathione and H_2O_2.

2.4. Enzyme activities

All procedures for enzyme extraction and determination of enzyme activities were carried out at 0 °C on ice bath unless otherwise stated. A 1.0 g aliquot of leaves was homogenized in 10 mL of 50 mM potassium phosphate buffer, pH 7.6, containing 1% (w/v) polyvinylpolypyrrolidone (PVPP) and 1 mM EDTA. For estimations of the activities of APX and DHAR, 1 mM ascorbate and 2 mM 2-mercaptoethanol were added to the homogenizing buffer to prevent inactivation of the respective enzyme. The homogenate was filtered through three layers of cheesecloth (Miracloth) and then centrifuged at 15,000 × *g* for 30 min and 4 °C. The obtained supernatant was recovered, desalted on a Sephadex™ G-25M column and used for the enzyme activity assays.

2.4.1. APX

APX activity was measured spectrophotometrically by recording the decrease in ascorbate content at 290 nm, according to Ushimaru et al. [22] with some modifications. A 1.0 mL aliquot of enzyme extract was treated with 100 μL of 10 mM H_2O_2 and 100 μL of 80 mM hydroxylamine, a selective inhibitor of APX, for 5 min. The same procedure, but with 200 μL of distilled water and without hydroxylamine, was used to determine total (APX + guaiacol peroxidase—GPX, EC 1.11.1.7) activity in a second 1.0 aliquot of the same extract. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 500 μM ascorbate, 100 μM EDTA, 100 μM H_2O_2 and 100 μL of enzyme extract, in a final volume of 3.0 mL. The reaction started with the addition of H_2O_2 and an absorption coefficient of 2.8 mM^{-1} cm^{-1} was used for calculations. One unit of APX activity was defined as the amount of enzyme that oxidizes 1 μmol of ascorbate per min at 20 °C.

2.4.2. MDHAR

MDHAR activity was tested after the method of Hossain et al. [23] with some modifications by following the decrease in absorbance at 340 nm due to NADH oxidation. A 1.0 mL aliquot of enzyme extract was added to a reaction mixture containing 50 mM potassium phosphate, pH 7.6, 0.3 mM NADH and 2.5 mM AsA. The reaction was started by adding AsA oxidase (EC 1.10.3.3) (Sigma–Aldrich A0157) to produce monodehydroascorbate and an absorption coefficient of 6.2 mM^{-1} cm^{-1} was used for calculations. One unit of MDHAR activity was defined as the amount of enzyme that oxidizes 1 nmol of NADH per min at 25 °C.

2.4.3. DHAR

DHAR activity was determined by monitoring the increase in absorbance at 265 nm due to AsA production, according to Hossain and Asada [24] with some modifications. A 1.0 mL aliquot of enzyme extract was added to a reaction mixture containing 50 mM potassium phosphate buffer, pH 6.5, 0.1 mM EDTA, 0.5 mM DHA and 2.5 mM GSH. The non-enzymatic reduction of DHA by GSH was subtracted. An absorption coefficient of 14.6 mM^{-1} cm^{-1} was used for calculations. One unit of DHAR activity was defined as the amount of enzyme that produces 1 nmol of AsA per min at 25 °C.

2.4.4. GR

GR activity was measured by following the decrease in absorbance at 340 nm due to NADPH oxidation after the method of Carlberg and Mannervik [25]. A 200 μL aliquot of enzyme extract was added to a reaction mixture containing 1.5 mL of 0.1 M potassium phosphate buffer, pH 7, 150 μL of 20 mM GSSG, 1 mL of distilled water and 150 μL of 2 mM NADPH (dissolved in Tris–HCl buffer, pH 7), in a final volume of 3.0 mL. An absorption coefficient of 6.2 mM^{-1} cm^{-1} was used for calculations. One unit of GR activity was defined as the amount of enzyme that oxidizes 1 nmol of NADPH per min at 25 °C.

2.5. Ascorbate and glutathione levels

A 0.5 g aliquot of leaves was homogenized in 1.0 mL of ice-cold 2.5 N $HClO_4$. The homogenate was filtered through three layers of cheesecloth (Miracloth) and then centrifuged at 15,000 × *g* for 5 min. The supernatant was neutralized with 5 M K_2CO_3 to pH 4.5 for ascorbate determination and to pH 6.5 for glutathione determination.

Ascorbic acid was measured spectrophotometrically by reading absorbance at 265 nm due to ascorbate oxidation by ascorbate oxidase, according to Foyer et al. [26]. For measurements of total ascorbate (AsA + DHA), 300 μL of neutralized extract were added to 1.2 mL of a reaction mixture containing 20 mM dithiothreitol (DTT) in 50 mM Hepes-KOH, pH 7.0. After incubation for 10 min at 25 °C in a water bath, 100 μL of 0.5 M *N*-ethylmaleimide were added to remove DTT. The reaction was started with the addition of five units of ascorbate oxidase. For reduced ascorbate (AsA) determination, 300 μL of neutralized extract were added to 1.2 mL of a reaction mixture containing 150 mM K_3PO_4, pH 7.4, 5 mM EDTA and five units of ascorbate oxidase. The concentration of dehydroascorbate (DHA) was calculated as the difference between total ascorbate and AsA.

The levels of glutathione were measured spectrophotometrically by monitoring the reduction of 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 412 nm, after the method of Griffith [27]. For measurements of total glutathione (GSH + GSSG), 150 μL of neutralized extract were added to 1.2 mL of 0.3 mM NADPH, 150 μL of 6 mM DTNB and 1 unit of glutathione reductase (GR). For oxidized glutathione (GSSG) determination, 150 μL of neutralized extract were incubated with 2 μL of 2-vinylpyridine for 1 h at 25 °C, and then added to 1.2 mL of 0.3 mM NADPH, 100 μL of 6 mM DTNB, and 1 unit of GR. The concentration of reduced glutathione (GSH) was calculated as the difference between total glutathione and GSSG.

2.6. H_2O_2 content

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 20,000 × *g* for 15 min at 4 °C. The supernatant was recovered and used for H_2O_2 determination, according to Lee and Lee [28]. A 0.5 mL aliquot of supernatant was mixed with 2.5 mL of peroxide reagent, containing of 83 mM sodium phosphate, pH 7.0, 0.005% (w/v) *o*-dianisidine, and 40 μg mL^{-1} 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 N $HClO_4$. After centrifugation at 10,000 × *g* for 5 min, the absorbance at 436 nm of the supernatant was compared to the extinction of a H_2O_2 standard.

2.7. Determination of total soluble protein

The total soluble proteins were determined according to Smith et al. [29], using bovine serum albumine as calibration standard.

2.8. Statistical analysis

The values of gas exchange parameters were represented as means of 12 measurements (±S.E.) from three selected plants (four measurements per plant and three replications of each measurement), whereas the values of LWP were represented as means of nine measurements (±S.E.) from three selected plants (three measurements per plant and three replications of each measurement). Enzyme activities were expressed as means of three measurements (±S.E.) from three plants having a similar value of LWP (one measurement per plant and three replications of each measurement).

Statistical analysis was performed using analysis of variance (ANOVA). Significant differences were determined at $P \leq 0.05$, according to Duncan's multiple range test.

3. Results

3.1. Environmental conditions and physiological parameters

The highest value of air temperature was 29.2 °C after 13 days from the beginning of the drought period. The mean values of all the daily values of air temperature and RH were 21.6 °C and 68.2%, respectively. VPD range was between 2.2 and 0.3 KPa, with a mean value of 0.9 KPa (Fig. 1). The

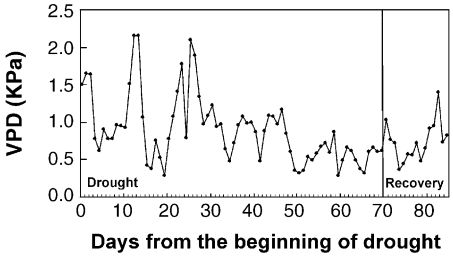

Fig. 1. Vapour pressure deficit at the field site during the experimental period. Measurements were taken during the drought period (values before vertical line) and the following rewatering phase (values after vertical line).

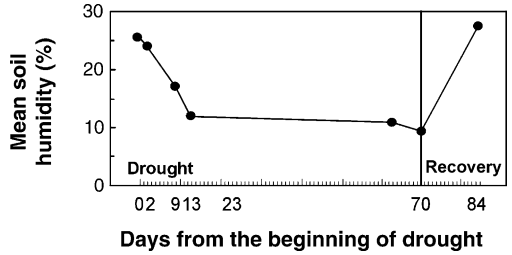

Fig. 2. Mean soil humidity measured at 0, 2, 9, 13, 23, 70 and 84 days from the beginning of the experimental period. Measurements were taken during the drought period (values before vertical line) and the following rewatering phase (values after vertical line).

mean values of soil humidity measured at different levels of depth decreased progressively during the drought phase and then increased during rewatering, showing a range between 9.3 and 27.4% (Fig. 2).

The water shortage during the drought period was paralleled by a substantial decrease in leaf LWP in all the hybrids studied starting from 23 days after the beginning of the drought phase (Fig. 3A). In particular, 6–5 reached lowest values of LWP (mean minimum value of −4.00 MPa after 70 days) if compared to P3605, 8–9 and 7–7 (mean minimum values of −3.15, −2.93 and −3.12 MPa, respectively, after 70 days). In SP, the values of transpiration rate and net photosynthetic rate decreased during the drought period in all the hybrids studied, reaching the lowest values at 23 and 63 days from the beginning of the drought period (Fig. 3B and C). During the rewatering period, at 84 days from the beginning of the experimental period, LWP, net photosynthetic rate and transpiration rate values recovered in all the hybrids tested (Fig. 3).

3.2. Drought phase

APX activity showed an increase starting from 23 days after the beginning of drought in all the hybrids tested, decreasing slightly only at the end of the drought phase (Fig. 4A). In particular, during the last dates of the drought phase, 7–7 and 6–5, presented high values of APX activity if compared with those of P3605 and 8–9. The values of

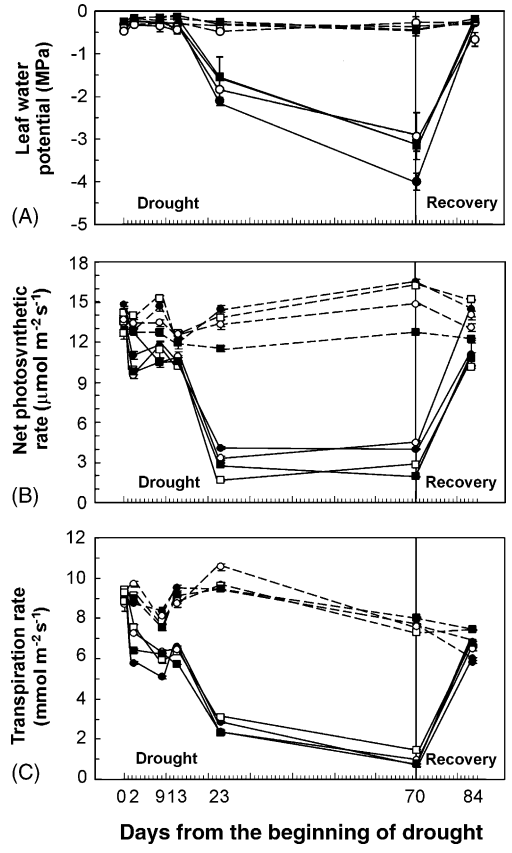

Fig. 3. Trends of leaf water potential (A), net photosynthetic rate (B) and transpiration rate (C) in drought-stressed plants (—) and control plants (- - -) at 0, 2, 9, 13, 23, 70 and 84 days from the beginning of the experimental period. Each value represents the mean of 12 measurements (±S.E.) from three plants. Hybrids: P3605 (■), 6–5 (●), 7–7 (□), 8–9 (○). Measurements in were taken during the drought period (values before vertical line) and the following rewatering phase (values after vertical line).

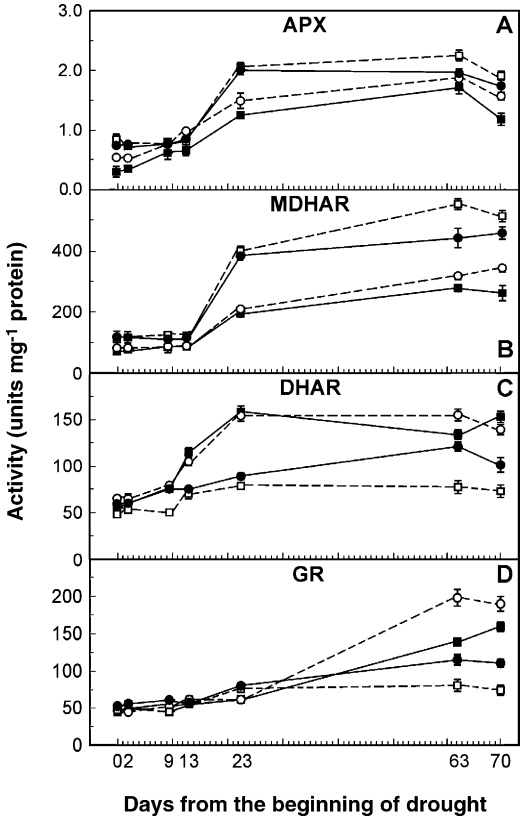

Fig. 4. Activities of ascorbate peroxidase (APX), monodehydroascorbate redictase (MDHAR), dehydroascroabate reductase (DHAR) and glutathione reductase (GR) in drought-stressed plants at 0, 2, 9, 13, 23, 63 and 70 days from the beginning of the drought period. Each value represents the mean of three measurements (±S.E.) from three plants having a similar level of drought stress. Hybrids: P3605 (■ , —), 6–5 (●, —), 7–7 (□, - - -), 8–9 (○, - - -).

MDHAR activity remained stable in all the hybrids during the first 13 days of the experimental period and then increase (Fig. 4B). 7–7 and 6–5 presented the highest values of MDHAR activity among all the hybrids studied (Fig. 4B).

DHAR activity was directly related to drought stress levels in all the hybrids studied, showing a gradual increase starting at 9 days from the beginning of the water deficit (Fig. 4C). In particular, P3605, 8–9 and, in a lesser extent, 6–5 showed high values of DHAR activity if compared with those of 7–7. GR activity showed a sharp increase at 63 days from the beginning of the drought period (Fig. 4D), with the exception of 7–7. The patterns of DHAR and GR activities were similar in all the hybrids examined.

Generally, APX, MDHAR, DHAR and GR were up-regulated during water deficit (Fig. 4). AsA and DHA content increased in relation to the duration of drought stress in all the hybrids examined, with a slight decrease at the highest level of water deficit for DHA (Fig. 5A and B).

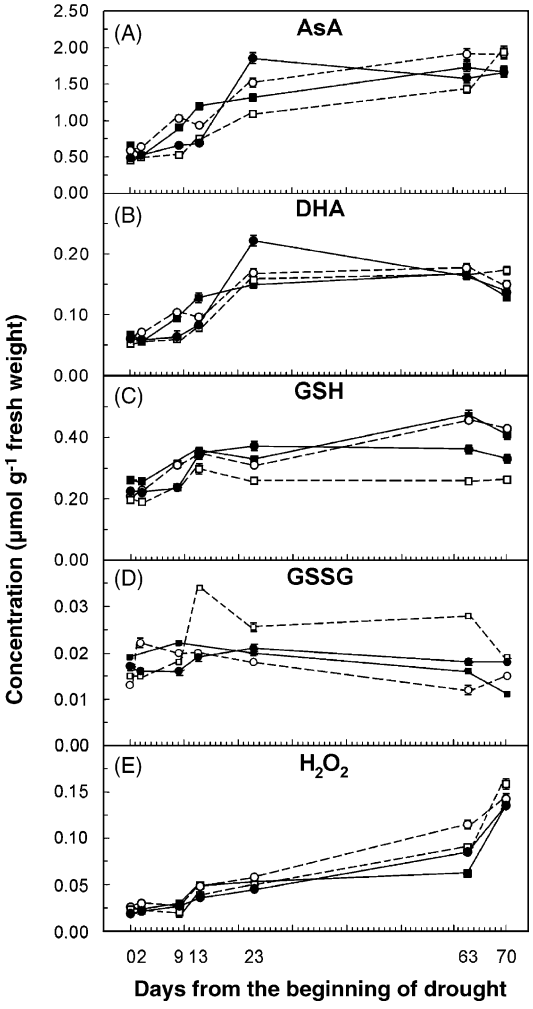

Fig. 5. Levels of ascorbate (AsA), dehydroascorbate (DHA), reduced glutathione (GSH), oxidized glutathione (GSSG) and hydrogen peroxide (H_2O_2) in drought-stressed plants at 0, 2, 9, 13, 23, 63 and 70 days from the beginning of the drought period. Each value represents the mean of three measurements (±S.E.) from three plants having a similar level of drought stress. Hybrids: P3605 (■, —), 6–5 (●, —), 7–7 (□, - - -), 8–9 (○, - - -).

In the first 13 days of the drought phase, the ratios of AsA to DHA were similar to those of CP, whereas, at high level of water deficit, ASA to DHA ratio decreased and subsequently showed an increase in the last days of the drought period (Table 1). Drought stress caused marked increases in GSH content in P3605 and 8–9 and, in a lesser extent, 7–7 and 6–5 (Fig. 5C). GSSG levels in P3605, 8–9 and 6–5 showed a slight increase during the progressive water shortage followed by a decrease in the last dates of the drought period, while in 7–7 the rise was more intense (Fig. 5D). In 7–7, the ratios of GSH to GSSG remained relatively stable during the drought period, whereas in P3605, 8–9 and, in a lesser extent, in 6–5, higher values were found during high levels of water deficit if compared with CP (Table 1). In all the hybrids studies, the levels of H_2O_2 were directly related to the level of drought stress, presenting a continuous increase during all the drought phase (Fig. 5E).

Table 1
Ratios AsA/DHA and GSH/GSSG in leaves of drought-stressed (SP) and irrigated (CP) plants

Days	Hybrids	AsA/DHA		GSH/GSSG	
		SP	CP	SP	CP
0	P3605	9.76	9.88	13.74	14.00
	8–9	9.67	9.58	16.00	10.16
	7–7	8.65	9.35	13.07	12.00
	6–5	8.13	9.30	13.24	13.24
2	P3605	9.39	9.25	16.06	15.56
	8–9	9.01	9.63	10.36	13.36
	7–7	8.72	9.67	12.67	9.44
	6–5	8.98	9.74	13.75	12.24
9	P3605	9.63	9.57	14.23	13.75
	8–9	9.90	9.52	15.50	12.29
	7–7	8.80[a]	10.27	13.22	10.40
	6–5	10.28	9.65	14.81[a]	8.12
13	P3605	9.34	9.32	17.65[a]	13.24
	8–9	9.74	10.04	17.20[a]	10.33
	7–7	9.60	9.78	8.74	11.26
	6–5	8.31[a]	10.85	18.00[a]	10.00
23	P3605	8.77[a]	10.63	16.35[a]	10.13
	8–9	8.99[a]	10.81	17.22[a]	8.91
	7–7	6.94[a]	8.73	10.12[a]	7.97
	6–5	8.36[a]	9.93	17.71[a]	9.04
63	P3605	9.89[a]	8.80	29.31[a]	11.24
	8–9	10.80[a]	9.33	37.92[a]	13.70
	7–7	8.68[a]	10.12	9.18	10.85
	6–5	9.57	10.00	20.00[a]	11.85
70	P3605	13.03[a]	9.12	37.27[a]	8.88
	8–9	12.80[a]	9.57	28.53[a]	12.54
	7–7	11.24[a]	8.88	13.79	10.37
	6–5	12.12[a]	8.75	18.33[a]	10.14

Samples were collected at 0, 2, 9, 13, 23, 63 and 70 days from the beginning of the drought stress period. Each value represents the mean of three measurements from three plants having a similar level of drought stress.

[a] Significant difference at $P \leq 0.05$ from the relevant control value, according to Duncan's Multiple Range Test.

In CP, the activities of the enzymes above mentioned and the levels of ascorbate, glutathione and H_2O_2 remained relatively stable during the experimental period.

3.3. Rewatering phase

Generally, the activities of APX, MDHAR, DHAR and GR were down-regulated during the rewatering phase and their values were lower than those found in the drought-stressed plants (Fig. 6). APX activity decreased both in SL and NSL during rewatering, reaching values similar to those found in the corresponding CP (Fig. 6A). The activities of MDHAR and DHAR declined both in SL and NSL during the rewatering period and maintained higher values with respect to those found in the relevant CP (Fig. 6B and C). The decrements of MDHAR and DHAR activities were more marked in SL than in NSL (Fig. 6B and C). Both in SL and NSL, the values of GR activity decreased during rewatering in all the hybrids studied (Fig. 6D).

In SL and NSL of all the hybrids studied, AsA and DHA content and AsA to DHA ratios decreased during rewatering if compared to the values found during the last days of the drought phase (Fig. 7A and B; Table 2). The rewatering treatment caused decreases in GSH content both in SL and NSL of P3605, 8–9 and 6–5, while the values in 7–7 remained rather constant (Fig. 7C). GSSG levels of rewatered plants were similar to those of CP, with the exception of 7–7, whereas GSH to GSSG ratios decreased both in SL and NSL, if compared to the values observed during the drought period (Fig. 7D). The levels of H_2O_2 showed a strong decrease during the rewatering phase more marked in SL than in NSL (Fig. 7E).

In CP, the activities of the enzymes above studied and the levels of metabolites remained relatively stable during the rewatering period if compared with the

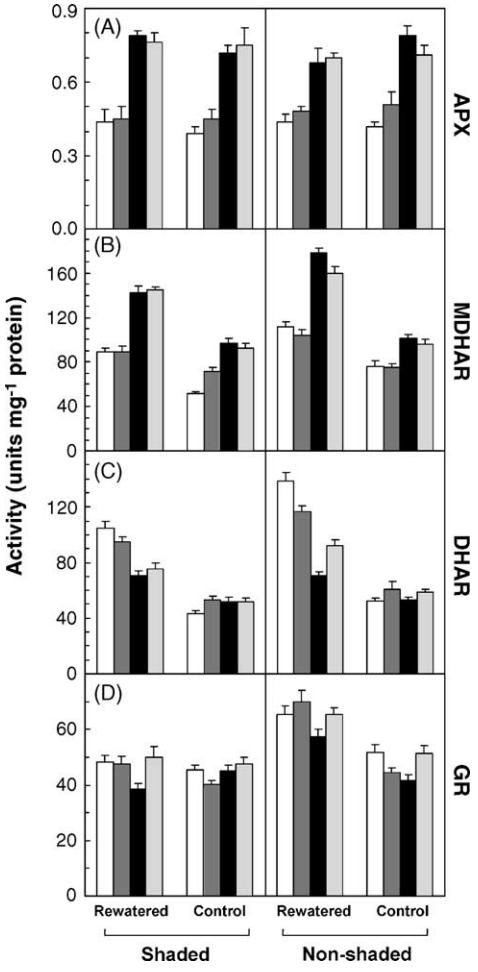

Fig. 6. Activities of ascorbate peroxidase (APX), monodehydroascorbate redictase (MDHAR), dehydroascroabate reductase (DHAR) and glutathione reductase (GR) in shaded and non-shaded leaves of rewatered plants and in the relevant control plants. Samples were collected after 36 days from the beginning of the rewatering period. Each value represents the mean of three measurements (±S.E.) from three plants. Hybrids: P3605 (white columns), 8–9 (deep grey), 6–5 (black), 6–5 (light grey).

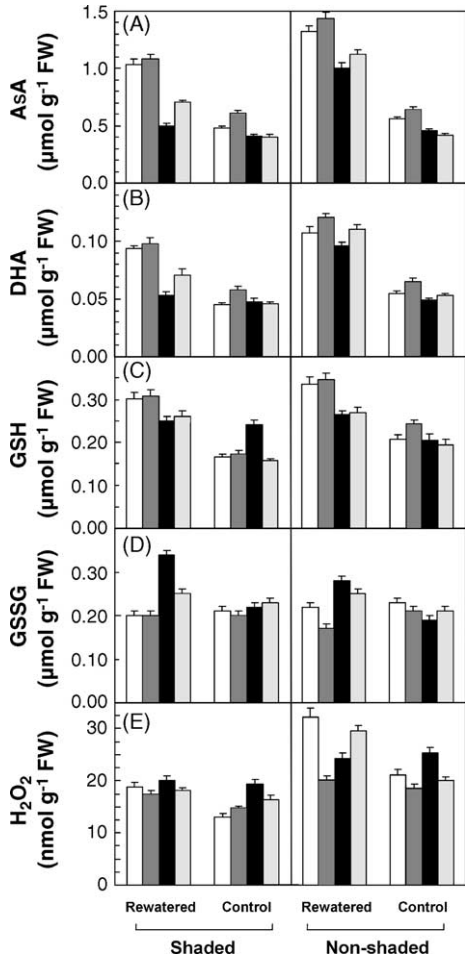

Fig. 7. Ascorbate (AsA and DHA), glutathione (GSH and GSSG) and hydrogen peroxide levels in shaded and non-shaded leaves of rewatered plants (RP) and in the relevant leaves of control plants (CP). Samples were collected after 36 days from the beginning of the rewatering period. Each value represents the mean of three measurements (±S.E.) from three plants. Hybrids: P3605 (white columns), 8–9 (deep grey), 6–5 (black), 6–5 (light grey).

Table 2
Ratios AsA/DHA and GSH/GSSG in shaded and non-shaded leaves of rewatered plants (RP) and in the relevant leaves of control plants (CP)

Leaves	Hybrids	AsA/DHA		GSH/GSSG	
		RP	CP	RP	CP
Shaded	P3605	11.04	10.69	15.05[a]	7.86
	8–9	11.08	10.52	15.40[a,b]	8.55[b]
	7–7	9.43	8.50	7.32[b]	10.95
	6–5	9.90	8.80	10.40[a]	6.83[b]
Non-shaded	P3605	12.36	10.18	15.27[a]	9.00
	8–9	11.88	9.85	20.35[a]	11.62
	7–7	10.46	9.33	9.43	10.79
	6–5	10.23	7.89	10.76	9.24

Samples were collected after 36 days from the beginning of the rewatering period. Each value represents the mean of three measurements (±S.E.) from three plants.

[a] Significant difference at $P \leq 0.05$ from the relevant control value, according to Duncan's Multiple Range Test.

[b] Significant difference at $P \leq 0.05$ from the relevant value in non-shaded leaves, according to Duncan's Multiple Range Test.

values measured during the drought phase (Figs. 6 and 7).

4. Discussion

During periods of water deficit, species of the genus *Prunus*, such as almond, peach and apricot tree, show significant decrease in gas exchange [14,16,30]. Our results confirm that the decrease of soil humidity (Fig. 2), together with high values of VPD (Fig. 1), caused a reduction of LWP and gas exchange in *Prunus* plants (Fig. 3). Recent studies demonstrated that the decrease in net CO_2 assimilation in response to environmental stresses reduces the capacity of the photosynthetic electron transport system, which in turn causes increased levels of AOS [2,3,5]. In this context, some AOS, such as $O_2^{\bullet -}$ and H_2O_2, act as signal molecules during drought stress responses and trigger defence responses [4,5]. The regulation of the activities of some antioxidant enzymes is an immediate and efficacious response to scavenge the excess of AOS and it was observed in some fruit tree species such as olive [19] and apricot tree [18]. We suppose that an excess of reducing power, with the consequent increase in H_2O_2 and other AOS concentration, likely caused the up-regulation of some antioxidant enzymes during the drought period. In fact, our results show that the activities of antioxidant enzymes and the levels of the molecules involved in the ascorbate–glutathione cycle increased in all the hybrids examined in parallel to the severity of drought stress, and in particular from 23 days from the beginning of drought, when soil humidity and LWP reached low values (Figs. 2, 3A and 4).

Among the enzymes examined, it seems evident a linkage between APX and DHAR, which showed an increase of activity directly related to the degree of drought stress in all the tested tissues (Fig. 4). APX isozymes, able to scavenge the H_2O_2 produced by superoxide dismutases using ascorbate as the electron donor, are generally located in chloroplasts, but microsomal, peroxisomal and membrane-bound forms, as well as soluble cytosolic and apoplastic isozymes, also exist [8]. Monodehydroascorbate (MDHA), a free radical intermediate produced by APX catalysis, can disproportionate spontaneously to AsA and dehydroascorbate or be enzymatically reduced to AsA by MDHAR, a FAD enzyme with an high specificity to MDHA which uses NAD(P)H as a reductant [3]. During the whole period of drought stress, the hybrids 6–5 and 7–7 showed high activities of APX and MDHA and low AsA level if compared to 8–9 and 3605 (Figs. 4A and B, and 5A). This suggests that antioxidant protection in these hybrids could be attributed mainly to APX and DHAR, with a partial contribute of the other components of ascorbate–glutathione cycle (Figs. 4 and 5). In 6–5 and 7–7, the high APX activity likely determined low level of AsA, which is the main substrate of APX (Fig. 5A) and is then regenerated by MDHAR, whereas the fast turnover of AsA and MDHA, due to high activities of APX and DHAR caused low levels of MDHA and, indirectly, of DHA (Fig. 5B). We hypothesize that the similar behaviour of the clones 6–5 and 7–7 could be due to the sharing of one parent (*P. cerasifera* L. 'P2175'), common to both the genotypes.

The increase of DHAR and GR activities during the progression of water deficit suggests a strict relationship of these enzymes with drought stress conditions in all the hybrids studied (Fig. 4C and D). DHAR is a monomeric thiol enzyme that reduces DHA to AsA at expense of GSH as an electron donor, with the consequent production of GSSG [31,32]. The isoforms of GR are flavoenzymes with a redox cystine residue in their active sites which maintain the intracellular glutathione pool in the reduced status catalysing the NADPH-dependent reduction of GSSH to GSH [3,33]. DHAR and GR activities, were higher in the hybrids 3605 and 8–9 than those found in 6–5 and 7–7 (Fig. 4C and D). The high levels of GHS in these clones, in particular at high degrees of drought stress (Fig. 5C) was directly related to the observed increase of GR in the last days of the drought phase (Fig. 4D). This highlights the regulative action of GR in the homeostasis of glutathione pool in *Prunus* plants subjected to water deficit conditions. The results of 3605 and 8–9, together with the relative low activities of APX and MDHAR with respect to those found in 6–5 and 7–7, show that antioxidant protection in these clones is mainly due to the enzymes and molecules involved in glutathione metabolism. Therefore, the production of DHA deriving from MDHA disproportionation and it consequent reduction by DAHR plays a key role in these hybrids. Both the patterns and levels of H_2O_2 were similar in all the hybrids studied (Fig. 5E). This confirm that the drought-related reduction of CO_2 assimilation (Fig. 3B) causes an excess of H_2O_2 deriving from the photoreduction of O_2 to $O_2^{\bullet -}$ in PSI and the following disproportionation of $O_2^{\bullet -}$ catalyzed by superoxide dismutases [1,3].

The observed changes in the ratios AsA to DHA starting from 23 days from the beginning of drought reflect the changes in H_2O_2 production and conversion (Table 1; Fig. 5E). In fact, at 23 days from the beginning of drought, the values of AsA to DHA ratios in SP were significantly lower than those found in CP and this was likely due to the high APX activity and H_2O_2 concentration in SP, with the consequent increase in DHA (Fig. 5B). Subsequently, after 63 days from the beginning of drought, APX activity started to decrease slightly (Fig. 4A), while AsA levels and DHAR and MDHAR activities continued to raise (Figs. 4B and C, and 5A), determining an increase in AsA to DHA ratio with respect to CP in the last dates (Table 1).

Our results show that the progressive loss of water from leaf tissues (Fig. 3A) caused an increase of H_2O_2-related oxidative stress (Fig. 5E) which in turn enhanced ascorbate accumulation and AsA-dependent detoxification processes (Fig. 4A and B), in accordance with Smirnoff [1] and Horemans et al. [6]. The differences of GSH to GSSG ratios and GSH level between SP and CP (Table 1; Fig. 5C), indicate that high levels of drought stress induced a synthesis ex novo of GSH. The high activity of GR, especially at 63 and 70 days from the beginning of drought, maintained glutathione pool in its reduced status. This is confirmed by the different behaviour of the hybrid 7–7, in which a relative low GR activity, also at an high degree of drought, was associated to low GSH and high GSSG levels (Figs. 4D and, 5C and D), without showing significant differences in GSH to GSSH ratios if compared to CP (Table 1). The increase of glutathione pool during water shortage confirms the important role of GSH in plant protection against oxidative stress and could be necessary to regulate the levels of AsA and DHA [8,9].

The down-regulation of ascorbate–glutathione system observed in rewatered plants (Figs. 6 and 7), likely due to a reduced need for AOS removal, was accompanied to the recovery of water and gas exchange of the plants (Fig. 3). During the rewatering phase, the lower expression of the enzymatic antioxidant system and the lower levels of total ascorbate and glutathione found in SL if compared to NSL (Figs. 6 and 7) indicate that the synergic action of water deficit and high light intensity determines a higher degree of oxidative stress and antioxidant protection in NSL (Figs. 6 and 7). This was also confirmed by the higher levels of H_2O_2 in NSL in comparison to SL (Fig. 7E). In particular, after 36 days of rewatering, H_2O_2 levels in rewatered SL were lower than those found in control NSL, confirming the low rate of oxidative stress in leaves exposed to low irradiance level. In fruit tree species, the role of light during drought stress is of great importance. In fact, in *Prunus cerasus* L., a continuous illumination determines the photoinhibition of leaves and the irreversible damage of PSII [34]. Moreover, in other fruit species, such as olive tree, water deficit induces the 'down regulation' of PS II electron transport [35] and high levels of irradiance cause higher activities of some antioxidant enzymes [19].

We have found evidence for an up-regulation of AOS-scavenging enzymes and changes in ascorbate and glutathione pools as plants enter water deficit conditions. We can also confirm that different irradiance levels in different parts of the canopy play a key role in the regulation of ascorbate–glutathione cycle, causing different antioxidant responses. Our results highlight the capacity of *Prunus* hybrids to withstand drought conditions by regulating the ascorbate–glutathione cycle. The results obtained in this investigation underline the important role of some antioxidant enzymes and compounds in protecting cellular apparatus during water deficit conditions and may be important for the selection for drought resistance in *Prunus* rootstocks material. This could lead to the characterization of different genotypes with this important characteristic.

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