Physiological and biochemical response to photooxidative stress of the fundamental citrus species

Jérémie Santini, Jean Giannettini, Stéphane Herbette, Olivier Pailly, Patrick Ollitrault, Luro François, Liliane Berti

Physiological and biochemical response to photooxidative stress of the fundamental citrus species

Jérémie Santini[a,b], Jean Giannettini[b,*], Stéphane Herbette[c,d], Olivier Pailly[a], Patrick Ollitrault[e], François Luro[a], Liliane Berti[b]

[a] INRA, UR Génétique et Ecophysiologie de la Qualité des Agrumes, F-20230 San Giuliano, France

[b] CNRS, UMR 6134 SPE, Laboratoire Biochimie & Biologie Moléculaire du Végétal, F-20250 Corte, France

[c] INRA, UMR 547 PIAF, F-63100 Clermont-Ferrand, France

[d] Clermont Université, Université Blaise-Pascal, UMR 547 PIAF, BP 10448, F-63000 Clermont-Ferrand, France

[e] CIRAD, UPR 75, Avenue Agropolis, TA A-75/02, F-34398 Montpellier cedex 5, France

*Corresponding author: Jean Giannettini
Université de Corse Pasquale-Paoli
UMR 6134 Sciences pour l'Environnement (SPE)
Laboratoire de Biochimie et Biologie moléculaire
Quartier Grossetti, B.P 52
F-20250 Corte, France.
Tel: +33495450674; fax: +33495450154. *E-mail address*: gianetti@univ-corse.fr

ABSTRACT

Despite the economic importance of citrus, insights on the genetic response to stress are scarce. The aim of the present study was to compare fundamental citrus species for their response to photooxidative stress. The experiment was conducted under orchard conditions on three fundamental citrus species *C. medica* L., *C. reticulata* Blanco and *C. maxima* (Burm.) Merr., and on *Fortunella japonica* (Thunb.) Swing.. We examined their respective net photosynthesis (*P*net), stomatal conductance (*G*s) and chlorophyll fluorescence (*F*v/*F*m) on sun-acclimated leaves and shade–acclimated leaves returned under natural sunlight irradiance. To compare the respective response mechanism, we analyzed changes in oxidative status (hydrogen peroxide (H_2O_2) and malondialdehyde (MDA)), reactive oxygen species (ROS)-scavenging enzymes (superoxide dismutase (SOD), catalase, ascorbate peroxidase), recycling enzymes (monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase) and antioxidant metabolites (ascorbate and glutathione). Kumquat and pummelo exposed lower down-regulation and full recovery of photosynthetic parameters, lower accumulation of oxidized compounds associated with greater production of reduced glutathione (Gsh) and enhanced activity of the three ROS scavenging enzymes, especially SOD. Citron and mandarin showed a marked decrease and incomplete recovery in photosynthetic performance, mainly in *P*net and *F*v/*F*m, larger accumulation of oxidative parameters, slighter induction of antioxidant enzymes and down-regulation of reduced ascorbate (Asa) and Gsh synthesis. These results suggest that kumquat and pummelo have a greater tolerance to photooxidative stress than citron and mandarin.

Keywords: Antioxidant system, *Fortunella japonica*, *Citrus maxima*, *Citrus medica*, *Citrus reticulata*, light stress

1. Introduction

Citrus is the world's most economically important fruit crop. Strictly, true citrus plants comprise six genera: *Clymenia*, *Eremocitrus*, *Microcitrus*, *Poncirus*, *Fortunella* and *Citrus* . Scora (1975) and Barrett and Rhodes (1976) considered *Citrus medica* L. (citron), *C. maxima* (Burm.) Merr. (pummelo) and *C. reticulata* Blanco (mandarin) to be the three fundamental species of *Citrus*, the other species resulting from hybridization of these true species. This view has recently gained support from various biochemical and molecular studies (Federici et al., 1998; Barkley et al., 2006; Fanciullino et al., 2006). Allopatric evolution has resulted in strong genetic and also phenotypic differentiation between these *Citrus* taxa (Garcia-Lor et al., 2012).

Citrus trees are continuously exposed to changes in light and temperature in their natural environment. Global climatic warming may cause these changes to become increasingly pronounced in both frequency and magnitude, particularly in the north Mediterranean area. In this region, the summer season is characterized by high temperatures and dryness, whereas in winter, day temperature is generally moderate and night temperatures often dip below 5 °C. At these two periods, the radiation loads can reach high levels. Sunlight contains high-energy ultraviolet radiation (UV, 280–400 nm) and photosynthesis is one of the processes most sensitive to high irradiance (Demmig-Adams and Adams, 1992). Under such conditions, trees are liable to suffer photoinhibition, defined as the slow, reversible decline in photochemical efficiency that occurs under photooxidative stress (Krause et al., 2001). This process is frequent in trees of warm regions, where the light intensity can reach levels over 1800 $\mu mol.m^{-2}\ s^{-1}$ photosynthetic photon flux density (PPFD) (Favaretto et al., 2011). The ability to cope with photoinhibition ranges greatly among plant species (Kitao et al., 2006). Numerous studies have shown that photosystem II (PSII) is the primary target of photoinhibitory damage

(Aro et al., 1993). Photoinhibition of PSII can be easily detected *in vivo* by a decrease in the dark-adapted ratio of variable to maximum chlorophyll a fluorescence (*F*v/*F*m) (Krause and Weis, 1991). A decrease in this ratio indicates a stressful condition, and a reduction in the maximum quantum efficiency of PSII, which thereby compromises the plant's photosynthetic potential (Maxwell and Johnson, 2000).

A common effect of most environmental factors is an increased production of reactive oxygen species (ROS) in green plant cells, a situation called photooxidative stress, driven by the light energy absorbed in excess of assimilatory requirements (Foyer et al., 1994). These harmful ROS such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) are involved in the mechanism of photoinhibition (Asada, 1999). The production of ROS in plant cells is enhanced by conditions that limit CO_2 fixation, such as drought, salt, heat and cold stresses, and by the combination of these conditions with strong light (Foyer and Noctor, 2003). Because aerobic organisms, such as plants, live in a highly oxidative environment, they have evolved efficient antioxidant systems protecting them from the damaging effects of ROS (Asada, 1999) such as decreased protein synthesis, damage to DNA and membrane lipids (Frohnmeyer and Staiger, 2003; Mackerness et al., 2001). These antioxidant mechanisms employ (i) ROS-scavenging enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and ascorbate peroxidase (APX, 1.11.1.11), (ii) recycling enzymes of the ascorbate-glutathione cycle, such as 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), and (iii) low molecular weight antioxidants, such as reduced ascorbic acid (Asa) and reduced glutathione (Gsh). Some authors have reported that antioxidative systems play a major role in protecting plants from the harmful effects of excess light energy (Foyer et al., 1994; Favaretto et al., 2011).

Thus, antioxidative systems have been found to be of paramount importance in the response and tolerance of trees to environmental stress (Polle and Rennenberg, 1993).

Some authors have shown that citrus physiology is adversely affected by abiotic stresses, such as drought (Avila et al., 2012), waterlogging (Hossain et al., 2009) and salinity (Balal et al., 2012; Brumos et al., 2009). Currently, experiments have been mainly performed on the most common rootstocks under the superimposition of a specific stress with strong light. For instance, it was found that the Cleopatra mandarin was very sensitive to flooding stress and tolerant to salt stress, whereas Carrizo citrange showed the opposite behavior (Arbona et al., 2008; Brumos et al., 2009). Thus, a heterogeneous response to oxidative stress between rootstocks exists under homogeneous cultural conditions. To date, no study has focused on the possible differences of stress response that could exist between citrus species and, especially, for the species at the origin of the broad genetic diversity of cultivated citrus.

The main objective of this work was to compare fundamental citrus species for their response to oxidative stress. Thus, individual trees grown under orchard conditions were submitted to photooxidative stress by controlling light conditions of the leaves. We measured the main photosynthetic traits (net photosynthesis, stomatal conductance and chlorophyll a fluorescence), the oxidative status (H_2O_2 and malondialdehyde (MDA) contents), the activities of the main antioxidant enzymes (SOD, CAT, APX, MDHAR, DHAR, GR) and the level of the main hydrophilic antioxidant molecules (ascorbate and glutathione) of the four fundamental citrus species. These measurements were performed on sun-acclimated leaves and on one-week shade-acclimated leaves returned under natural sunlight irradiance. The results allow discussing the responses of the citrus species to photooxidative stress.

2. Materials and methods

136 *2.1. Plant material and growth conditions*

137

138 Experiments were carried out on leaves from 8-year-old trees with genotypes belonging to
139 the *Citrus* and *Fortunella* genera (Table 1) growing in the experimental orchards of the
140 Station de Recherches Agronomiques INRA-CIRAD of San Giuliano, Corsica, France
141 (42° 18′ 55″ N, 9° 29′ 29″ E; 51m a.s.l., under a Mediterranean climate and on soil derived
142 from alluvial deposits and classified as fersiallitic, pH range 6.0–6.6). The trees were
143 about 2.0 m high, spaced 6 × 4 m, and subjected to homogeneous growing conditions to
144 reduce environmental effects. Water was supplied every day on the basis of 100%
145 replacement of actual evapotranspiration estimated from the equation of Monteith (1965).
146 Fertilizers were supplied, and insects and diseases were controlled according to the
147 recommendations of the local Department of Agriculture.

148 The experiment was conducted from September 23, 2010 to October 10, 2010 on clear
149 days. For each of the basic true species of the *Citrus* genus and of the *Fortunella* genus (Table
150 1), three trees were analyzed. We isolated two independent sections on each of the three trees.
151 The first section did not undergo any special treatment, and served as control. On this section,
152 the leaves were kept uncovered throughout the experimental period to receive 100% sunlight
153 irradiance. The leaves of the second section were shaded using a 90% shade cloth. This shade
154 cloth allowed the actual transmission of 9.2% sunlight irradiance (90% shade cloth). We
155 checked that spectra were not modified by shading, using a Li-Cor Li-1800 spectrometer.
156 After one week of adaptation, the photooxidative stress was applied. The leaves were
157 completely uncovered and received full light. Each treatment was allocated among the three
158 selected trees of each genotype of the trial at three different periods (Fig. 1). At each period,
159 one tree of each genotype was studied. In this way, the variability associated with different
160 days of measurement was included in the intraspecific variability. On each section,

physiological measurements and samplings were performed 0, 3, 6, 24 and 48 hours after the end of the shading period. The same leaves were used for physiological parameter measurements. On each tree, three fully expanded leaves from spring of the current year's growth were selected. Thus nine measurements per genotype were made for each genotype and for each time. For biochemical assays, on each tree, two samples of 15 fully expanded leaves from the current year's growth were collected and immediately frozen in liquid nitrogen and stored at −80 °C. Thus six samples of 15 leaves were separately analyzed for each genotype and for each time point of the kinetics. Before analysis, each leaf sample was ground to a fine powder in liquid nitrogen using a pre-chilled pestle and mortar.

Temperatures and daily total radiation were recorded throughout the experiment (Fig. 1).

2.2. Gas exchange measurements

Measurements of net photosynthetic rate (*P*net) and stomatal conductance (*G*s) were made with a portable open gas exchange system (GFS 3000, WALZ, Effeltrich, Germany). Air flow rate was 750 µmol.s^{-1}. In a gas exchange chamber, photosynthetic photon flux density (PPFD) was controlled using a LED radiation source, and was fixed at a PPFD of 1400 µmol.m^{-2}.s^{-1}. The use of this LED source ensured a constant, uniform light across all measurements. Carbon dioxide concentration was set at 380 µmol.mol^{-1}.

2.3. Chlorophyll a fluorescence measurements

In vivo chlorophyll fluorescence was measured using a portable chlorophyll fluorometer (Hansatech, Norfolk, England) on sunny days on the same leaves as previously used for gas exchange measurements,. Intact leaves were dark-adapted with leaf clips for 20 min to allow

relaxation of fluorescence quenching associated with thylakoid membrane energization (Krause et al., 1983). Minimal fluorescence (*F*o) and maximal fluorescence (*F*m) were obtained by imposing a 1 s saturating flash to reduce all the PSII reaction centers. The maximum potential photochemical efficiency of PSII was expressed as the ratio *F*v/*F*m (= (*F*m − *F*o)/*F*m). The degree of photoinhibition was evaluated by the reduction in the value of *F*v/*F*m.

2.4. Measurement of H_2O_2 and MDA levels

H_2O_2 levels were measured following the protocol described by Zhou et al. (2006). For extraction, 200 mg of frozen leaf powder was homogenized in 3 mL of trichloroacetic acid (TCA) 5% (w:v) containing 60 mg of activated charcoal. The mixture was then centrifuged at 5000 × *g* for 20 min at 4 °C.

The MDA concentration of leaves was determined using a thiobarbituric acid (TBA) reaction described by Hodges et al. (1999). For extraction, 100 mg of frozen leaf powder was homogenized with inert sand in 2.5 mL of 80% ethanol (v/v), followed by centrifugation at 3000 × *g* for 10 min at 4 °C.

All the measurements were performed using a V-630 spectrophotometer (Jasco Inc., Tokyo, Japan).

2.5. Assay of antioxidant metabolites

Total ascorbate (tAsa) and reduced ascorbate (Asa) contents were measured according to the method of Gillespie and Ainsworth (2007). For extraction, 40 mg of frozen leaf powder was homogenized in 2.0 mL of a 6% (w/v) TCA solution and centrifuged at 13,000 × *g* for

5 min at 4 °C. Oxidized ascorbate (DHA) was calculated by subtracting Asa concentration from the tAsa concentration.

Total glutathione (tGsh) and oxidized glutathione (GssG) contents were measured according to the DTNB-GR recycling procedure of Rahman et al. (2006). For extraction, 50 mg of frozen leaf powder was homogenized in 2.0 mL of mixed buffer (100 mM potassium phosphate, pH 7.5, 5 mM EDTA, 0.1% (v:v) Triton X-100 and 23 mM sulfosalicylic acid) and centrifuged at $8000 \times g$ for 10 min at 4 °C. Gsh concentration was calculated by subtracting GssG concentration from the tGsh concentration.

All measurements were performed using a V-630 spectrophotometer (Jasco Inc., Tokyo, Japan).

2.6. Assay of antioxidant enzyme activities

For all enzymatic assays, frozen leaf powder was homogenized in extraction medium (100 mM potassium phosphate buffer, pH 7.5, containing 0.1% (v/v) TritonX-100 and 1% (w/v) polyvinylpyrolidone (PVP)) using 27 mg FW per mL of buffer. The homogenate was then centrifuged at 13,000 $\times g$ for 30 min at 4 °C. The supernatant was used for the protein and enzyme analysis (except for SOD, where the extract was diluted 20-fold). Protein concentration was determined by the method of Bradford (1976). All kinetic measurements were made using a V-630 spectrophotometer (Jasco Inc., Tokyo, Japan).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured using the method of Oberley and Spitz (1984), modified: 100 µL of diluted extract was added to a solution containing 1 mM DETAPAC buffer (pH 7.8), 1.25 units of catalase, 0.07 mM NBT, 0.2 mM xanthine and 0.010 units of xanthine oxidase in a total volume of 1.0 mL. One unit of SOD

was defined as the amount of enzyme causing 50% inhibition in the rate of NBT reduction at 560 nm, at 25 °C.

Catalase (CAT, EC 1.11.1.6) activity was measured according to the method of Aebi (1984). The reaction mixture (1.1 mL) contained 100 µL of crude enzyme extract, 37.8 mM sodium phosphate buffer (pH 7.0) and 4.4 mM H_2O_2. The decrease in absorbance was measured at 240 nm (ε = 39.4 $mM^{-1}.cm^{-1}$). One unit of CAT was expressed as 1 µmol H_2O_2 degraded per min at 25 °C.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to a modified method described by Asada (1984). The standard reaction mixture (1.0 mL) contained 0.17 mM ascorbate, and 33 µL of crude enzyme extract in a 60.3 mM potassium phosphate buffer (pH 7.0). The reaction was triggered when 4.95 mM H_2O_2 was added. The rate of ascorbate oxidation was evaluated at 290 nm for 3 min (ε = 2.8 $mM^{-1}.cm^{-1}$). One unit of APX was expressed as the oxidation of 1 µmol ascorbate per min at 25 °C.

Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity was established by monitoring the MDHA-dependent oxidation of NADH according to the slightly modified method of Drew et al. (2007). 100 µL of crude enzyme extract was added to a solution containing 9.7 mM potassium phosphate, 0.125% Triton X100 (pH 8), 2.5 mM ascorbate, 0.128 units of ascorbate oxidase in a total volume of 1.0 mL. The reaction was started by adding 0.2 mM NADH. The decrease in absorbance was measured at 340 nm (ε = 6.3 $mM^{-1}.cm^{-1}$). One MDHAR unit was defined as the amount of enzyme required to oxidize 1 µmol NADH per min at 340 nm at 25 °C.

Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was assayed by measuring the rate of appearance of ascorbate measured at 265 nm (ε = 14.5 $mM^{-1}.cm^{-1}$) (Asada, 1984). The standard reaction mixture (1.0 mL) contained 41 mM potassium phosphate buffer (pH 6.5), 5 mM Gsh, 0.11 mM EDTA, and 75 µL of crude enzyme extract, with 0.5 mM DHA added to

initiate the reaction. One DHAR unit was defined as the amount of enzyme that allowed the formation of 1 μmol ascorbate per min at 25 °C.

Glutathione reductase (GR, EC 1.6.4.2) activity was measured according to the modified method of Smith et al. (1988). The standard reaction mixture (1.0 mL) contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM GssG, 0.75 mM DTNB and 100 μL of crude enzyme extract. 0.1 mM NADPH was added to initiate the reaction. The increase in absorbance due to the formation of TNB was measured at 412 nm (ε = 14.15 $mM^{-1}.cm^{-1}$). One GR unit was defined as the amount of enzyme that allowed the formation of 1 μmol TNB per min at 25 °C.

2.7. Statistical analyses

The experimental designs were split-plot, with genotype as the main plot and time after exposure of the shaded leaves to light as the subplot. Data were analyzed using two-way ANOVA, and comparisons between means were made with the least significant difference (LSD) test at $P < 0.05$ using R statistical software (http://www.R-project.org). Data were compared between genotypes for each parameter, at each point of the time course. In addition, for each genotype, the data obtained along the time course were compared. The mean values and standard errors of the mean values are shown in the figures.

3. Results

In order to minimize the effects of changes in environmental conditions during the experiment, the results were expressed as relative data. Thus, only the effect of the light treatment was taken into account.

3.1. Effect of light stress on net photosynthesis (Pnet), stomatal conductance (Gs) and maximum photochemical efficiency of PSII (Fv/Fm)

In all the genotypes, *P*net and *G*s values were lower under the shade treatment (ratio values below 1 at 0 h) (Figs. 2a, b). Leaves acclimated to shade conditions showed a reduction in Pnet of ~30%, ~50%, ~50% and ~60% in MK, WLM, CC and PP, respectively, compared to control leaves acclimated to full light conditions.

Likewise, the decrease in *G*s (Fig. 2b) was very marked in CC (~−52%), moderate in WLM and PP (~−40%) and much lower in MK (~−30%). At the end of the time course, *P*net was totally recovered in PP and MK (ratio values close to 1), whereas in CC and WLM, this recovery was incomplete (for *P*net: ~80% of the control value). *G*s was completely recovered in all genotypes after 48 h.

Under shade conditions, all the genotypes displayed *F*v/*F*m values equivalent to the control (ratio values close to 1) (Fig. 2c). For CC and WLM, the *F*v/*F*m value had dropped sharply at 3 h (~69% and ~80% of the initial value, respectively), whereas it remained unchanged for PP and MK. After 48 h of exposure to full light, the *F*v/*F*m recovery was complete for PP and MK (ratio values close to 1) compared with CC and WLM (only ~85% of the control value).

3.2. Effect of light stress on the oxidative status

The shade treatment caused a decrease in the concentration of oxidative compounds in three (CC, MK and PP) of the four genotypes studied (ratio values below 1 at 0 h, Fig. 3). In WLM, only the MDA concentration was lower in the shade-acclimated leaves compared to

light-acclimated leaves. Highly variable accumulations of H_2O_2 and MDA were found between genotypes after exposure to full light (Figs. 3a**,** b). CC and WLM maintained relatively high levels of leaf H_2O_2 and MDA contents. Along the time course, the rate of H_2O_2 and MDA remained high and stable in CC compared with the control (more than 1.5 times higher), whereas in WLM, the high level of H_2O_2 was transitory. In MK, increase in H_2O_2 and MDA levels was also transitory with a maximum value at 6 h (~1.5 times higher than the control). PP displayed a very specific pattern with no changes in H_2O_2 and MDA levels along the time course.

3.3. Effect of photooxidative stress on the antioxidant system

Acclimatization to shade conditions caused a decrease in antioxidant concentration and antioxidant enzyme activities in all the genotypes studied (Fig. 4). In CC and PP, the increase in the total ascorbate (tAsa) concentration was due more to a rise in the concentration of the oxidized form (DHA) than in that of the reduced form (Asa). Conversely, in MK, the reduced form appeared more markedly improved than WLM, in which the variations were equivalent between the different forms. MK and WLM were the only genotypes to increase leaf redox Asa/DHA ratio during the time course compared with control (~1.3 times higher at 24 h). These increases occurred earlier in MK (from 3 h) and later in WLM (from 24 h). By contrast, for CC and PP, these values remained unchanged along the time course, and were approximately equal to control.

For the glutathione concentration, GssG was the most significantly increased in CC (~3.5 times higher at 48 h compared with control) and WLM (~2.5 times higher at 6 h compared with control), whereas in MK and PP little difference was observed (Fig. 5). Conversely, MK and PP showed significantly higher increases in tGsh from 6 h (~2 times higher for PP and

~1.5 times higher for MK compared with control), caused essentially by a higher incremental Gsh concentration (~2 times higher from 6 h compared with control) than in GssG concentration, which remained very low. Throughout the experiment, a significant increase in Gsh/GssG compared with the control was observed in response to photooxidative stress in MK (~2.1-fold increase at 6 h) and PP (~2-fold increase at 24 h). By contrast, CC and WLM displayed a very marked decrease in the ratio values compared with the control (~−84% and ~−77% at 3 h, respectively), and these values continued to decrease in CC to 48 h, but remained essentially unchanged along the time course in WLM.

We analyzed the activities of various enzymes acting as ROS scavengers, i.e. SOD, CAT and APX, or ensuring the supply/regeneration of primary antioxidants, i.e. MDHAR, DHAR and GR (Fig. 6). For all the genotypes, SOD activity increased rapidly after 3 h of exposure to photooxidative stress and more intensively in CC, MK and PP. A decline was observed in CC and WLM at 24 h to reach values equivalent to the control (ratio values close to 1). By contrast, in MK and PP, SOD remained very active at 48 h (~1.5 times and ~1.3 times higher than the control, respectively). CC was the only genotype studied with a specific CAT pattern. From 3 h, CAT activity increased significantly compared with 0 h, but remained depressed relative to control in the light section (ratio values below 1). In MK and PP, a peak of activity was observed at 24 h (~1.6 and ~2.2 times higher than control, respectively), whereas this peak was present at 3 h in WLM, and was followed by a significant loss of activity. Overall, APX activity was rapidly increased, with a peak at 3 h in all the genotypes. At the end of the kinetics, the activity became equivalent to control (ratio values close to 1) in MK and PP whereas in CC and WLM its activity was blocked (ratio values below 1). MDHAR was the antioxidant enzyme whose activity was the most strongly increased. We observed an early peak of activity from 3 h in all the genotypes (~3 times higher than control), followed by a slow decrease up to 48 h except for WLM, where the decrease was very marked from 6 h.

Considering DHAR activity, the increase was very rapid and equivalent in all the genotypes (more than 2-fold increase at 3 h compared with control) and was followed by a decline. However, MK was the only genotype that maintained a very high activity after 3 h (~2.4 times higher than the control at 24 h) compared with other genotypes, in which the decrease was very marked. Like the two previous regenerating enzymes, rapid activation of GR was observed at 3 h, with a peak of activity similar in all the genotypes (~1.6 times higher than control), except for WLM, where it took place later, at 24 h. MK maintained its activity more effectively than the other genotypes at the beginning of the time course.

4. Discussion

Currently, most of the citrus species cultivated for the fresh fruit consumption or juice processing are secondary species. These species are the result of hybridization between fundamental species. The first step in understanding and improving their response to environmental challenges requires better characterization of the physiological and biochemical mechanisms that govern stress tolerance of basic species. Thus, this study compares four fundamental citrus species for their response to photooxidative stress. In the past, similar experiments were used to induce photooxidative stress in many plants including trees (Gonzalez-Rodriguez et al., 2001; Jiao and Li, 2001). Our results clearly show that the photosynthetic response to photooxidative stress differ depending on the species and that it could be related to dissimilarities in the oxidative status.

4.1. Differences in the sensitivity to photooxidative stress between fundamental citrus species

To date no relationship between taxonomic affiliations and ability to tolerate photooxidative stress has ever been evidenced. The biochemical and physiological parameters studied enabled us to highlight the contrasting strategies implemented by ancestral genotypes of citrus to cope with photooxidative stress. When plants are exposed to high irradiation, the stomata normally close to prevent water loss, resulting in a decrease in the intercellular CO_2 concentration and a depression of photosynthesis (Favaretto et al., 2011). Although *P*net followed the same pattern as *G*s in all the genotypes up to 24 h, light stress-induced changes in photosynthesis were primarily caused by non-stomatal factors, as they were accompanied by similar CO_2 intercellular concentrations (data not shown), as previously shown in 'Xuegan' orange (*Citrus sinensis*) or in 'Sour' pummelo (*Citrus grandis*) during boron or magnesium deficiency (Han et al., 2009; Yang et al., 2012). Our results also show that whereas *Gs* recovered 48 hours after exposure to full light in all the genotypes, *P*net did not, in either CC or WLM. This suggests that factors additional to stomatal closure must limit photosynthetic activity in the latter. Arbona et al. (2009) demonstrated that Carrizo citrange, a flooding-tolerant rootstock, had a better *P*net and Gs recovery after subsequent drainage, unlike Cleopatra mandarin, a flooding-sensitive rootstock, in which *G*s recovered, but *P*net did not.

We measured photoinhibition and oxidative damage to investigate potential mechanisms for tolerance to photooxidative stress. PSII maximum quantum efficiency (*F*v/*F*m) was the best indicator for photoinhibition (Maxwell and Johnson, 2000). Photoinhibition can be considered as a photoprotective process only when decreases in *F*v/*F*m are slight (Adams et al., 2006). We found that *F*v/*F*m decreased in all the genotypes during the first hours of photooxidative stress, indicating compromised PSII efficiency in utilizing incident light (Jung et al., 1998). In MK and PP, the less marked decrease in the *F*v/*F*m value suggested better protection of PSII, whereas the greatest and fastest down-regulation of photochemical activities from 3 h observed in CC and WLM could reflect photodamage to PSII (Genty et al.,

1989). In addition, the incomplete recovery after 48 h of treatment in CC and WLM could be attributable to an increased proportion of closed, reversibly inactivated or destroyed PSII reaction centers, probably caused by enhanced ROS accumulation (Foyer and Noctor, 2000). These results were confirmed by the greatest increase of *F*o in CC and WLM compared to MK and PP (data not shown). An increase in *F*o is considered to be the characteristic of inhibition of the acceptor side of PSII (Setlik et al., 1990) and is interpretable in terms of photodamages (Wingler et al., 2004). Previous studies showed that PSII, but not PSI, was a target during high temperature stress in Satsuma mandarin (*Citrus unshiu*) and Navel orange (*Citrus sinensis*) (Guo et al., 2006). Genotype differences in stress sensitivity was further demonstrated by parameters that estimate oxidative stress. Oxidative damage is caused by increased production of ROS. Among the most abundant ROS, H_2O_2 produced in peroxisomes and chloroplasts might diffuse to the cytosol, where it reacts with transition metal ions (Fe^{2+}) during the Fenton reaction, yielding hydroxyl radical ($OH^{\bullet}$), considered as the main cell-damaging product responsible for lipid peroxidation (Foyer et al., 1994). Considering MDA as an important indicator of lipid peroxidation, the concomitant and significant accumulation of H_2O_2 and MDA in CC and WLM indicated that these two genotypes suffered a higher oxidative pressure than MK and PP. These results were consistent with their probably greater sensitivity and vulnerability to the light stress discussed above. The occurrence of an H_2O_2 and MDA burst had previously been observed in flooding-sensitive Cleopatra mandarin, or in a chilling-sensitive rice genotype IR50, whereas smaller amounts of these two compounds had been highlighted in flooding-tolerant Carrizo citrange, or chilling-tolerant rice genotype L2825CA (Arbona et al., 2008; Bonnecarrere et al., 2011). In response to photooxidative stress, CC also exhibited high amounts of DHA and GssG, the oxidized forms of ascorbate and glutathione, implying that the cells had undergone greater oxidative pressure. Whereas PP and WLM displayed different patterns with a large amount of

DHA in the former and GssG in the latter, MK did not accumulate these two compounds, suggesting less susceptibility to oxidative pressure than CC. This agrees with previous reports on waterlogging stress (Arbona et al., 2008), in which the most sensitive genotype, Cleopatra mandarin, showed higher DHA and GssG increments than the most tolerant one, Carrizo citrange. It also confirmed results obtained on magnesium-deficient leaves of 'Xuegan' orange (*Citrus sinensis*), in which the concentration of DHA and GssG were strongly increased (Yang et al., 2012) compared with control.

Based on the whole results, we propose the following classification of the fundamental citrus species according to their degree of tolerance to photooxidative : *Citrus medica* L. (CC) < *Citrus deliciosa* Ten. (WLM) < *Fortunella japonica* (Thunb.) Swingle (MK) < *Citrus maxima* (Burm.) Merr. (PP). MK and PP proved to be more tolerant (i.e. smaller decline and complete recovery of photosynthetic parameters, and lower accumulation of indicators of the cell oxidation state) in comparison to CC and WLM (i.e. greater decrease and incomplete recovery of photosynthetic parameters and higher accumulation of oxidative compounds).

4.2. Could Antioxidant system explain the differences of response to photooxidative stress between citrus species ?

The antioxidant system is fundamentally important in protecting the photosynthetic apparatus, and it was assumed that higher antioxidant protection would be needed to compensate for higher light-mediated oxidative stress (Hansen et al., 2002). Various studies have highlighted the importance of antioxidant in tolerance to stress. Here, the complexity of the antioxidant system regulation is highlighted by the number of antioxidant components and genotypes analysed. Such a complexity has already been observed (Mai et al., 2010).

459 However, general trends and specific behaviors were observed between tolerant and sensitive
460 genotypes.

461 The more tolerant genotypes, PP and MK, limited the oxidative stress by a fast and great
462 increase in the activities of the three main ROS-scavenging enzymes and the antioxidant
463 molecules concentration. SOD directly dismutates $O_2^{\bullet -}$ into H_2O_2. H_2O_2 produced during the
464 SOD reaction can then be metabolized to oxygen and water by CAT in peroxisomes or
465 exclusively by APX in the chloroplasts (Foyer et al., 1994; Foyer and Noctor, 2000). Yabuta
466 et al. (2002) found transgenic plants over-expressing SOD and APX to be more tolerant than
467 wild-type to a combination of temperature and strong light. Consequently, the higher
468 induction of SOD and CAT, and the maintained activity of APX at 48 h, might account for the
469 lower accumulation of H_2O_2 and MDA previously observed. Arbona et al. (2008) have
470 already observed a marked increase in the activity of these three enzymes in the flooding-
471 tolerant genotype Carrizo citrange, supporting a synergistic action in tolerant genotypes. In
472 our results, the high production of Gsh and the improvement of the Gsh/GssG ratios
473 confirmed that glutathione played a crucial role in the protection of tolerant genotype from
474 photooxidative stress as indicated by Arbona et al. (2008). Generally, precise metabolic
475 tuning of GR allows the cell to maintain the favorable Gsh/GssG ratio for cellular redox
476 regulation. The Gsh/GssG ratio can also be improved by an increased synthesis of Gsh
477 (Queval et al., 2007). Equivalent results were found in citrumelo CPB4475 (*Citrus paradisi* L.
478 Macf. × *Poncirus trifoliata* L. Raf.) during waterlogging stress (Hossain et al., 2009).
479 Interestingly, despite the marked increase in MDHAR activity, diminution of DHAR activity
480 along the time course was accompanied by no change in the Asa/DHA ratio in PP. Conversely,
481 the significant rise in MDHAR and DHAR activities along the time course were coupled with
482 a consequent increase in the ratio Asa/DHA in MK. This suggests that a collaborative action
483 between these two enzymes was needed to regulate the redox state of ascorbate. These results

484 agree with previous studies showing that DHAR is especially important during stress response
485 and adaptation to regulate ascorbate levels (Chen et al., 2003; Mai et al., 2010).

486 The two sensitive genotypes CC and WLM showed sharply contrasting behaviors
487 compared with the tolerant ones. The slight increases in SOD and APX activity could
488 probably explain the very high accumulation of H_2O_2 and MDA. These results are consistent
489 with their previously observed greater sensitivity. Some authors had already observed a slight
490 increase in these two enzymes in *Hevea* chilling-sensitive clones (Mai et al., 2010) or even a
491 depression in the flooding-sensitive rootstock, Cleopatra Mandarin (Arbona et al., 2008). CC
492 presented the peculiarity of significantly inactivating CAT compared with WLM. Favaretto et
493 al. (2011) and Yang et al. (2012) also respectively reported a decline in CAT activity in
494 pioneer tree species and in magnesium-deficient leaves of 'Sour' pummelo (*Citrus grandis*),
495 probably because this enzyme is light-sensitive and suffers from photoinactivation caused by
496 oxidative damage initiated *via* direct absorption of light by the heme moieties of the enzyme
497 itself (Shang and Feierabend, 1999). It was also postulated that inactivation of CAT could
498 also be mediated by photo-oxidative events initiated through light absorption by chlorophyll
499 (Feierabend and Engel, 1986). Earlier induction of CAT and APX in WLM could explain why
500 this genotype tended to decrease the accumulation of H_2O_2 from 6 h compared with CC, in
501 which these two enzymes were completely inactivated from 6 h. The significant decrease in
502 Gsh/GssG and Asa/DHA ratios suggested that the increased activity of the recycling enzyme
503 was insufficient to produce enough glutathione/ascorbate to regulate the redox status, and that
504 no new synthesis was occurring as previously reported by Yang et al. (2012) in citrus
505 magnesium-deficient leaves or by Arbona et al. (2008) in the flooding-sensitive rootstock
506 Cleopatra mandarin.

507

508 **5. Conclusions**

Each ancestral species of citrus had a physiological and biochemical response to photooxidative stress that was specific. Based on the whole results, several conclusions may be drawn: (i) There are different levels of sensitivity to photooxidative stress between ancestral citrus species, (ii) *F*v/*F*m appears as a good parameter to screen citrus species for their sensitivity to photooxidative stress, (iii) a coordinated action between the three main ROS-scavenging enzymes seems necessary to limit the harmful effects of photooxidative stress in tolerant genotypes, (iv) glutathione appears as a key compound in stress tolerance. The present work performed on fundamental citrus species may serve as a reference to investigate the genetic response of citrus species to environmental stresses, especially in screening programs aimed to maintain fruit quality and productivity under adverse conditions like chilling stress.

Acknowledgments

We thank the Collectivité Territoriale de Corse (CTC) for the financial support of this study. We are grateful to Anne-Laure Fanciullino and Isabelle Poggi for their assistance in leaf harvesting, and to Jérôme Barbaggio and Isabelle Tur for technical assistance in the grinding of leaves.

References

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121-126.

Adams, W.W., Zarter, C.R., Mueh, K.E., Amiard, V.E., Demmig-Adams, B., 2006. Energy dissipation and photoinhibition: a continuum of photoprotection, In: Demmig-Adams, B., Adams, W.W., Mattoo, A.K. (Eds.), Photoprotection, photoinhibition, gene regulation and environment. Springer Netherlands, pp. 49-64.

Arbona, V., Hossain, Z., Lopez-Climent, M.F., Perez-Clemente, R.M., Gomez-Cadenas, A., 2008. Antioxidant enzymatic activity is linked to waterlogging stress tolerance in citrus. Physiol. Plant. 132, 452-466.

538 Arbona, V., Lopez-Climent, M.F., Perez-Clemente, R.M., Gomez-Cadenas, A., 2009.
539 Maintenance of a high photosynthetic performance is linked to flooding tolerance in
540 citrus. Environ. Exp. Bot. 66, 135-142.
541 Aro, E.M., Virgin, I., Andersson, B., 1993. Photoinhibition of photosystem II. Inactivation,
542 protein damage and turnover. Biochim. Biophys. Acta. 1143, 113-134.
543 Asada, K., 1984. Chloroplasts - Formation of active oxygen species. Methods Enzymol. 105,
544 422-429.
545 Asada, K., 1999. The water-water cycle in chloroplasts: Scavenging of active oxygens and
546 dissipation of excess photons. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 601-639.
547 Avila, C., Guardiola, J.L., Nebauer, S.G., 2012. Response of the photosynthetic apparatus to a
548 flowering-inductive period by water stress in Citrus. Trees 26, 833-840.
549 Balal, R.M., Khan, M.M., Shahid, M.A., Mattson, N.S., Abbas, T., Ashfaq, M., Garcia-
550 Sanchez, F., Ghazanfer, U., Gimeno, V., Iqbal, Z., 2012. Comparative Studies on the
551 Physiobiochemical, Enzymatic, and Ionic Modifications in Salt-tolerant and Salt-
552 sensitive Citrus Rootstocks under NaCl Stress. J. Am. Soc. Hortic. Sci. 137, 86-95.
553 Barkley, N.A., Roose, M.L., Krueger, R.R., Federici, C.T., 2006. Assessing genetic diversity
554 and population structure in a citrus germplasm collection utilizing simple sequence
555 repeat markers (SSRs). Theor. Appl. Genet. 112, 1519-1531.
556 Barrett, H.C., Rhodes, A.M., 1976. A numerical taxonomic study of affinity relationships in
557 cultivated Citrus and its close relatives. Syst. Bot. 1, 105-136.
558 Bonnecarrere, V., Borsani, O., Diaz, P., Capdevielle, F., Blanco, P., Monza, J., 2011.
559 Response to photoxidative stress induced by cold in japonica rice is genotype
560 dependent. Plant Sci. 180, 726-732.
561 Bradford, M.M., 1976. Rapid and sensitive method for quantitation of microgram quantities
562 of protein utilizing principle of protein-dye binding. Anal. Biochem. 72, 248-254.
563 Brumos, J., Colmenero-Flores, J.M., Conesa, A., Izquierdo, P., Sanchez, G., Iglesias, D.J.,
564 Lopez-Climent, M.F., Gomez-Cadenas, A., Talon, M., 2009. Membrane transporters
565 and carbon metabolism implicated in chloride homeostasis differentiate salt stress
566 responses in tolerant and sensitive Citrus rootstocks. Funct. Integr. Genomics 9, 293-
567 309.
568 Chen, Z., Young, T.E., Ling, J., Chang, S.C., Gallie, D.R., 2003. Increasing vitamin C content
569 of plants through enhanced ascorbate recycling. Proc. Natl. Acad. Sci. USA 100,
570 3525-3530.
571 Demmig-Adams, B., Adams, W.W., 1992. Photoprotection and other responses of plants to
572 high light stress. Annu. Rev. Plant. Physiol. Plant Mol. Biol. 43, 599-626.
573 Drew, D.P., Lunde, C., Lahnstein, J., Fincher, G.B., 2007. Heterologous expression of cDNAs
574 encoding monodehydroascorbate reductases from the moss, Physcomitrella patens and
575 characterization of the expressed enzymes. Planta 225, 945-954.
576 Fanciullino, A.L., Dhuique-Mayer, C., Luro, F., Casanova, J., Morillon, R., Ollitrault, P.,
577 2006. Carotenoid diversity in cultivated citrus is highly influenced by genetic factors. J.
578 Agric. Food Chem. 54, 4397-4406.
579 Favaretto, V.F., Martinez, C.A., Soriani, H.H., Furriel, R.P.M., 2011. Differential responses
580 of antioxidant enzymes in pioneer and late-successional tropical tree species grown
581 under sun and shade conditions. Environ. Exp. Bot. 70, 20-28.
582 Federici, C.T., Fang, D.Q., Scora, R.W., Roose, M.L., 1998. Phylogenetic relationships within
583 the genus Citrus (Rutaceae) and related genera as revealed by RFLP and RAPD
584 analysis. Theor. Appl. Genet. 96, 812-822.
585 Feierabend, J., Engel, S., 1986. Photoinactivation of catalase in vitro and in leaves. Arch.
586 Biochem. Biophys. 251, 567-576.

587 Foyer, C.H., Lelandais, M., Kunert, K.J., 1994. Photooxidative stress in plants. Physiol. Plant.
588 92, 696-717.
589 Foyer, C.H., Noctor, G., 2000. Oxygen processing in photosynthesis: regulation and
590 signalling. New Phytol. 146, 359-388.
591 Foyer, C.H., Noctor, G., 2003. Redox sensing and signalling associated with reactive oxygen
592 in chloroplasts, peroxisomes and mitochondria. Physiol. Plant. 119, 355-364.
593 Frohnmeyer, H., Staiger, D., 2003. Ultraviolet-B radiation-mediated responses in plants.
594 Balancing damage and protection. Plant Physiol. 133, 1420-1428.
595 Garcia-Lor, A., Luro, F., Navarro, L., Ollitrault, P., 2012. Comparative use of InDel and SSR
596 markers in deciphering the interspecific structure of cultivated citrus genetic diversity:
597 a perspective for genetic association studies. Mol. Genet. Genomics 287, 77-94.
598 Genty, B., Briantais, J.M., Baker, N.R., 1989. The relationship between the quantum yield of
599 photosynthetic electron-transport and quenching of chlorophyll fluorescence. Biochim.
600 Biophys. Acta 990, 87-92.
601 Gillespie, K.M., Ainsworth, E.A., 2007. Measurement of reduced, oxidized and total
602 ascorbate content in plants. Nat. Prot. 2, 871-874.
603 Gonzalez-Rodriguez, A.M., Tausz, M., Wonisch, A., Jimenez, M.S., Grill, D., Morales, D.,
604 2001. The significance of xanthophylls and tocopherols in photo-oxidative stress and
605 photoprotection of three Canarian laurel forest tree species on a high radiation day. J.
606 Plant Physiol. 158, 1547-1554.
607 Guo, Y.P., Zhou, H.F., Zhang, L.C., 2006. Photosynthetic characteristics and protective
608 mechanisms against photooxidation during high temperature stress in two citrus
609 species. Sci. Hort. 108, 260-267.
610 Han, S., Tang, N., Jiang, H.X., Yang, L.T., Li, Y., Chen, L.S., 2009. CO(2) assimilation,
611 photosystem II photochemistry, carbohydrate metabolism and antioxidant system of
612 citrus leaves in response to boron stress. Plant Sci. 176, 143-153.
613 Hansen, U., Fiedler, B., Rank, B., 2002. Variation of pigment composition and antioxidative
614 systems along the canopy light gradient in a mixed beech/oak forest: a comparative
615 study on deciduous tree species differing in shade tolerance. Trees 16, 354-364.
616 Hodges, D.M., DeLong, J.M., Forney, C.F., Prange, R.K., 1999. Improving the thiobarbituric
617 acid-reactive-substances assay for estimating lipid peroxidation in plant tissues
618 containing anthocyanin and other interfering compounds. Planta 207, 604-611.
619 Hossain, Z., Lopez-Climent, M.F., Arbona, V., Perez-Clemente, R.M., Gomez-Cadenas, A.,
620 2009. Modulation of the antioxidant system in citrus under waterlogging and
621 subsequent drainage. J. Plant Physiol. 166, 1391-1404.
622 Jiao, D.M., Li, X., 2001. Cultivar differences in photosynthetic tolerance to photooxidation
623 and shading in rice (Oryza sativa L.). Photosynthetica 39, 167-175.
624 Jung, S., Steffen, K.L., Lee, H.J., 1998. Comparative photoinhibition of a high and a low
625 altitude ecotype of tomato (Lycopersicon hirsutum) to chilling stress under high and
626 low light conditions. Plant Sci. 134, 69-77.
627 Kitao, M., Yoneda, R., Tobita, H., Matsumoto, Y., Maruyama, Y., Arifin, A., Azani, A.M.,
628 Muhamad, M.N., 2006. Susceptibility to photoinhibition in seedlings of six tropical
629 fruit tree species native to Malaysia following transplantation to a degraded land.
630 Trees 20, 601-610.
631 Krause, G.H., Briantais, J.M., Vernotte, C., 1983. Characterization of chlorophyll
632 fluorescence quenching in chloroplasts by fluorescence spectroscopy at 77-K.1. delta-
633 pH-dependent quenching. Biochim. Biophys. Acta 723, 169-175.
634 Krause, G.H., Koroleva, O.Y., Dalling, J.W., Winter, K., 2001. Acclimation of tropical tree
635 seedlings to excessive light in simulated tree-fall gaps. Plant Cell Environ. 24, 1345-
636 1352.

637 Krause, G.H., Weis, E., 1991. Chlorophyll fluorescence and photosynthesis: the basics. Annu.
638 Rev. Plant Physiol. Plant Mol. Biol. 42, 313-349.
639 Mackerness, S.A.H., John, C.F., Jordan, B., Thomas, B., 2001. Early signaling components in
640 ultraviolet-B responses: distinct roles for different reactive oxygen species and nitric
641 oxide. FEBS Lett. 489, 237-242.
642 Mai, J., Herbette, S., Vandame, M., Cavaloc, E., Julien, J.L., Ameglio, T., Roeckel-Drevet, P.,
643 2010. Contrasting strategies to cope with chilling stress among clones of a tropical tree,
644 Hevea brasiliensis. Tree Physiol. 30, 1391-1402.
645 Maxwell, K., Johnson, G.N., 2000. Chlorophyll fluorescence: a practical guide. J. Exp. Bot.
646 51, 659-668.
647 Monteith, J.L., 1965. Evaporation and environment. Symp. Soc. Exp. Biol. 19, 205-234.
648 Oberley, L.W., Spitz, D.R., 1984. Assay of superoxide dismutase activity in tumor-tissue.
649 Methods Enzymol. 105, 457-464.
650 Polle, A., Rennenberg, H., 1993. Significance of antioxidants in plant adaptation to
651 environmental stress, In: Folden, L., Mansfield, F., Stoddard, J. (Eds.), Plant
652 adaptation to environmental stress. Chapman and Hall, London, pp. 263-273.
653 Queval, G., Issakidis-Bourguet, E., Hoeberichts, F.A., Vandorpe, M., Gakiere, B., Vanacker,
654 H., Miginiac-Maslow, M., Van Breusegem, F., Noctor, G., 2007. Conditional
655 oxidative stress responses in the Arabidopsis photorespiratory mutant cat2
656 demonstrate that redox state is a key modulator of daylength-dependent gene
657 expression, and define photoperiod as a crucial factor in the regulation of H2O2-
658 induced cell death. Plant J. 52, 640-657.
659 Rahman, I., Kode, A., Biswas, S.K., 2006. Assay for quantitative determination of glutathione
660 and glutathione disulfide levels using enzymatic recycling method. Nat. Prot. 1, 3159-
661 3165.
662 Scora, R.W., 1975. Symposium on biochemical systematics, genetics and origin of cultivated
663 plants .9. history and origin of citrus. Bull. Torrey Bot. Club 102, 369-375.
664 Setlik, I., Allakhverdiev, S.I., Nedbal, L., Setlikova, E., Klimov, V.V., 1990. Three types of
665 photosystem II photoinactivation.1. Damaging processes on the acceptor side.
666 Photosynth. Res. 23, 39-48.
667 Shang, W., Feierabend, J., 1999. Dependence of catalase photoinactivation in rye leaves on
668 light intensity and quality and characterization of a chloroplast-mediated inactivation
669 in red light. Photosynth. Res. 59, 201-213.
670 Smith, I.K., Vierheller, T.L., Thorne, C.A., 1988. Assay of glutathione-reductase in crude
671 tissue-homogenates using 5,5'-dithiobis(2-nitrobenzoic acid). Anal. Biochem. 175,
672 408-413.
673 Wingler, A., Mares, M., Pourtau, N., 2004. Spatial patterns and metabolic regulation of
674 photosynthetic parameters during leaf senescence. New Phytol. 161, 781-789.
675 Yabuta, Y., Motoki, T., Yoshimura, K., Takeda, T., Ishikawa, T., Shigeoka, S., 2002.
676 Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative
677 systems under photo-oxidative stress. Plant J. 32, 915-925.
678 Yang, G.H., Yang, L.T., Jiang, H.X., Li, Y., Wang, P., Chen, L.S., 2012. Physiological
679 impacts of magnesium-deficiency in Citrus seedlings: photosynthesis, antioxidant
680 system and carbohydrates. Trees doi:10.1007/s00468-012-0699-2.
681 Zhou, B., Wang, J., Guo, Z., Tan, H., Zhu, X., 2006. A simple colorimetric method for
682 determination of hydrogen peroxide in plant tissues. Plant Growth Regul. 49, 113-118.

683

684

685
686

Table 1

Genotypes used for physiological and biochemical analysis and their corresponding rootstocks

Genotype				Corresponding rootstock		
Abbreviation	Common name	Tanaka system	ICVN[a] No.	Common name	Tanaka system	ICVN[a] No.
CC	Corsican citron	*Citrus medica* L.	0100613	Volkamer lemon	*Citrus limonia* Osbeck	0100729
WLM	Willowleaf mandarin	*Citrus deliciosa* Ten.	0100133	Volkamer lemon	*Citrus limonia* Osbeck	0100729
MK	Marumi kumquat	*Fortunella japonica* (Thunb.) Swingle	0100482	Volkamer lemon	*Citrus limonia* Osbeck	0100729
PP	Pink pummelo	*Citrus maxima* (Burm.) Merr.	0100322	Trifoliate orange	*Poncirus trifoliata (*L.) Raf**.**	0110480

[a]International citrus variety numbering.

Figure captions

Fig. 1. Meteorological data, from September 23 to October 10, 2010 at San Giuliano (Corsica, France). Closed symbols represent the minimum daily temperature (T_{min}), the maximum daily temperature (T_{max}) and mean daily temperature (T_{mean}). Open symbols correspond to the daily total radiation (DTR). On the bottom of the figure, the black bars stand for the shade-acclimatization phase and the white bars represent the light treatment. For each of the three periods, physiological measurements and samplings were performed just before the end of the shading phase (0 h) and 3, 6, 24 and 48 h after the beginning of the light treatment.

Fig. 2. Changes in (**a**) net photosynthesis (*P*net), (**b**) stomatal conductance (*G*s) and (**c**) maximum quantum yield of photosystem II (Fv/Fm) in four citrus genotypes during time course of photooxidative stress. Leaves acclimated under shade conditions for one week were suddenly exposed to full light conditions. Photosynthesis parameters were measured just before exposure to full light (0h), and 3, 6, 24 and 48 h after full light exposure. The results are expressed as ratios relative to the values obtained on control leaves acclimated to full light conditions. All data are presented as mean values (±S.E.) of nine independent measurements ($n = 9$). Data were analyzed using ANOVA and Fisher LSD tests ($P < 0.05$). Different upper case letters indicate significant differences between genotypes at a point of the time course and different lower case letters indicate significant differences along the time course for one genotype. Bold roman corresponds to CC, bold italics to MK, regular italics to WLM and regular roman to PP. See the Table 1 for abbreviations information

Fig. 3. Time course of changes in (**a**) hydrogen peroxide (H_2O_2) and (**b**) malondialdehyde (MDA) concentration in leaves of four citrus genotypes during photooxidative stress. Leaves acclimated under shade conditions for one week were suddenly exposed to full light

conditions. Compounds were assayed just before exposure to full light (0h), and 3, 6, 24 and 48 h after full light exposure. The results are expressed as ratios relative to the values obtained on control leaves acclimated to full light conditions. All data are presented as mean values (±S.E.) of six independent measurements ($n = 6$). Data were analyzed using ANOVA and Fisher LSD tests ($P < 0.05$). Different upper case letters indicate significant differences between genotypes at a point of the time course and different lower case letters indicate significant differences along the time course for one genotype. See the Table 1 for abbreviations information

Fig. 4. Time course of changes in reduced ascorbate concentration (Asa), oxidized ascorbate concentration (DHA), total ascorbate concentration (tAsa) and redox status (Asa/DHA) in leaves of four citrus genotypes during photooxidative stress. Leaves acclimated under shade conditions for one week were suddenly exposed to full light conditions. Compounds were assayed just before exposure to full light (0h), and 3, 6, 24 and 48 h after full light exposure. The results are expressed as ratios relative to the values obtained on control leaves acclimated to full light conditions. All data are presented as mean values (±S.E.) of six independent measurements ($n = 6$). Data were analyzed using ANOVA and Fisher LSD tests ($P < 0.05$). Different upper case letters indicate significant differences between genotypes at a point of the time course and different lower case letters indicate significant differences along the time course for one genotype. See the Table 1 for abbreviations information.

Fig. 5. Time course of changes in reduced glutathione concentration (Gsh), oxidized glutathione concentration (GssG), total glutathione concentration (tGsh) and redox status (Gsh/GssG) in leaves of four citrus genotypes during photooxidative stress. Leaves acclimated under shade conditions for one week were suddenly exposed to full light conditions. Compounds were assayed just before exposure to full light (0h), and 3, 6, 24 and 48 h after full light exposure. The results are expressed as ratios relative to the values

obtained on control leaves acclimated to full light conditions All data are presented as mean values (±S.E.) of six independent measurements (n = 6). Data were analyzed using ANOVA and Fisher LSD tests ($P < 0.05$). Different upper case letters indicate significant differences between genotypes at a point of the time course and different lower case letters indicate significant differences along the time course for one genotype. See the Table 1 for abbreviations information.

Fig. 6. Time course of changes in antioxidant enzyme specific activities (SOD, CAT, APX, MDHAR, DHAR, GR) in leaves of four citrus genotypes during photooxidative stress. The results are expressed as ratios relative to control values. Leaves acclimated under shade conditions for one week were suddenly exposed to full light conditions. Activities were assayed just before exposure to full light (0h), and 3, 6, 24 and 48 h after full light exposure. The results are expressed as ratios relative to the values obtained on control leaves acclimated to full light conditions. All data are presented as mean values (±S.E.) of six independent measurements (n = 6). Data were analyzed using ANOVA and Fisher LSD tests ($P < 0.05$). Different upper case letters indicate significant differences between genotypes at a point of the time course and different lower case letters indicate significant differences along the time course for one genotype. See the Table 1 for abbreviations information.

Figure 1

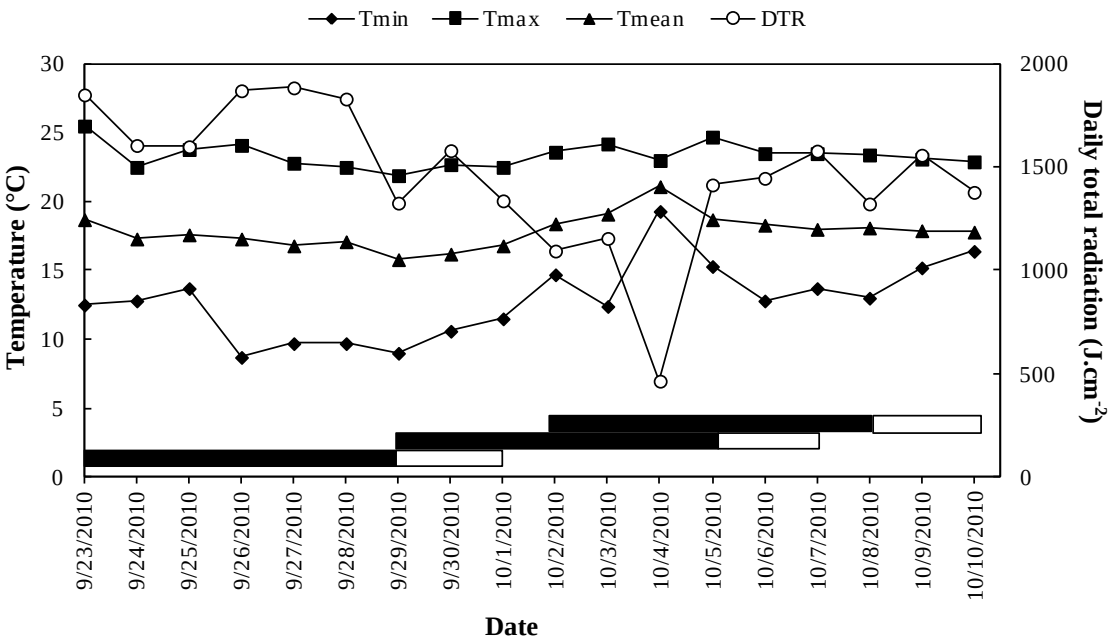

Tmin
Tmax
Tmean
DTR
Temperature (°C)
Daily total radiation (J.cm-2)
Date
0
5
10
15
20
25
30
0
500
1000
1500
2000
9/23/2010
9/24/2010
9/25/2010
9/26/2010
9/27/2010
9/28/2010
9/29/2010
9/30/2010
10/1/2010
10/2/2010
10/3/2010
10/4/2010
10/5/2010
10/6/2010
10/7/2010
10/8/2010
10/9/2010
10/10/2010

Figure 2

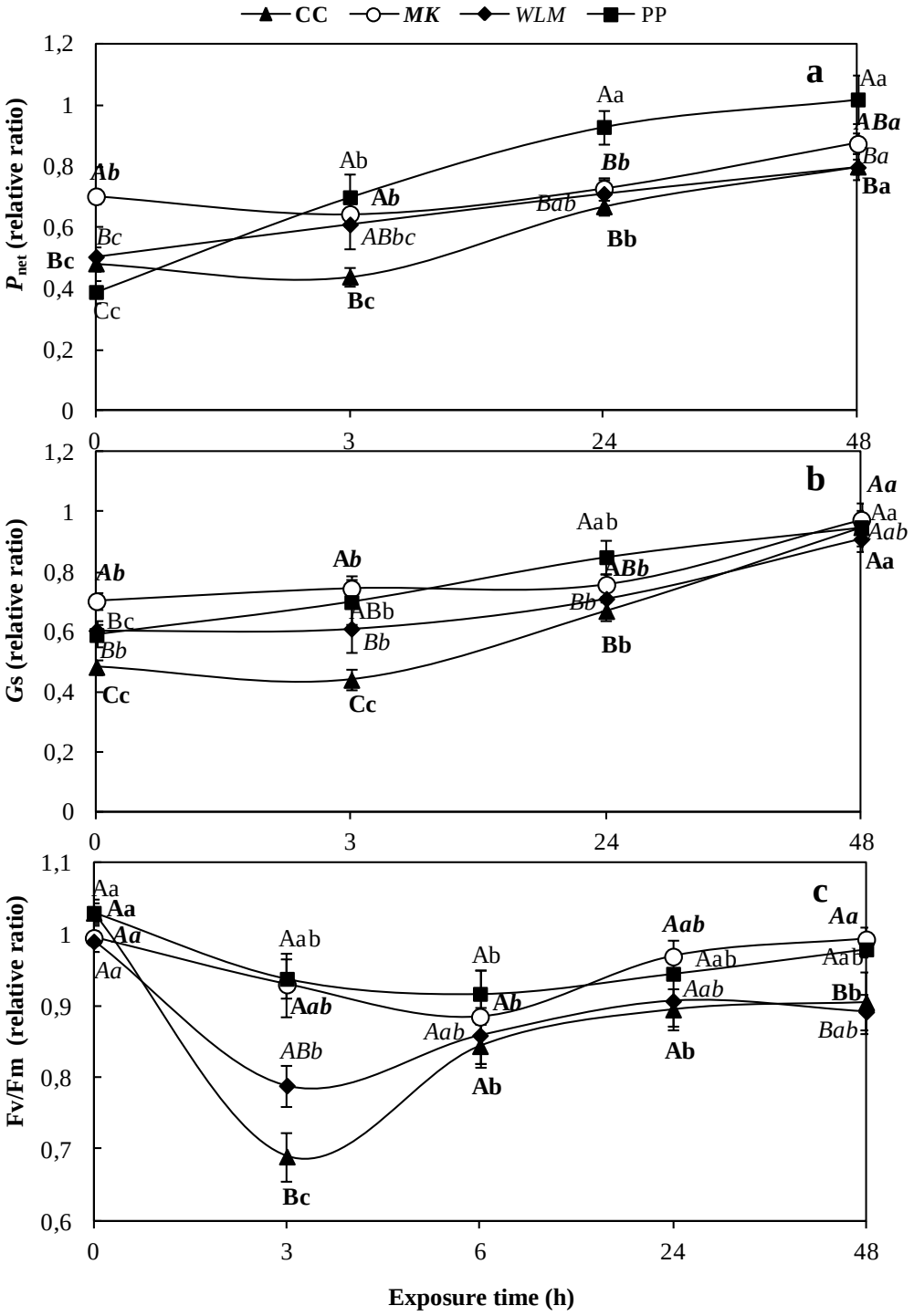

CC
MK
WLM
PP
a
P_{net} (relative ratio)
b
Gs (relative ratio)
c
Fv/Fm (relative ratio)
Exposure time (h)

Figure 3

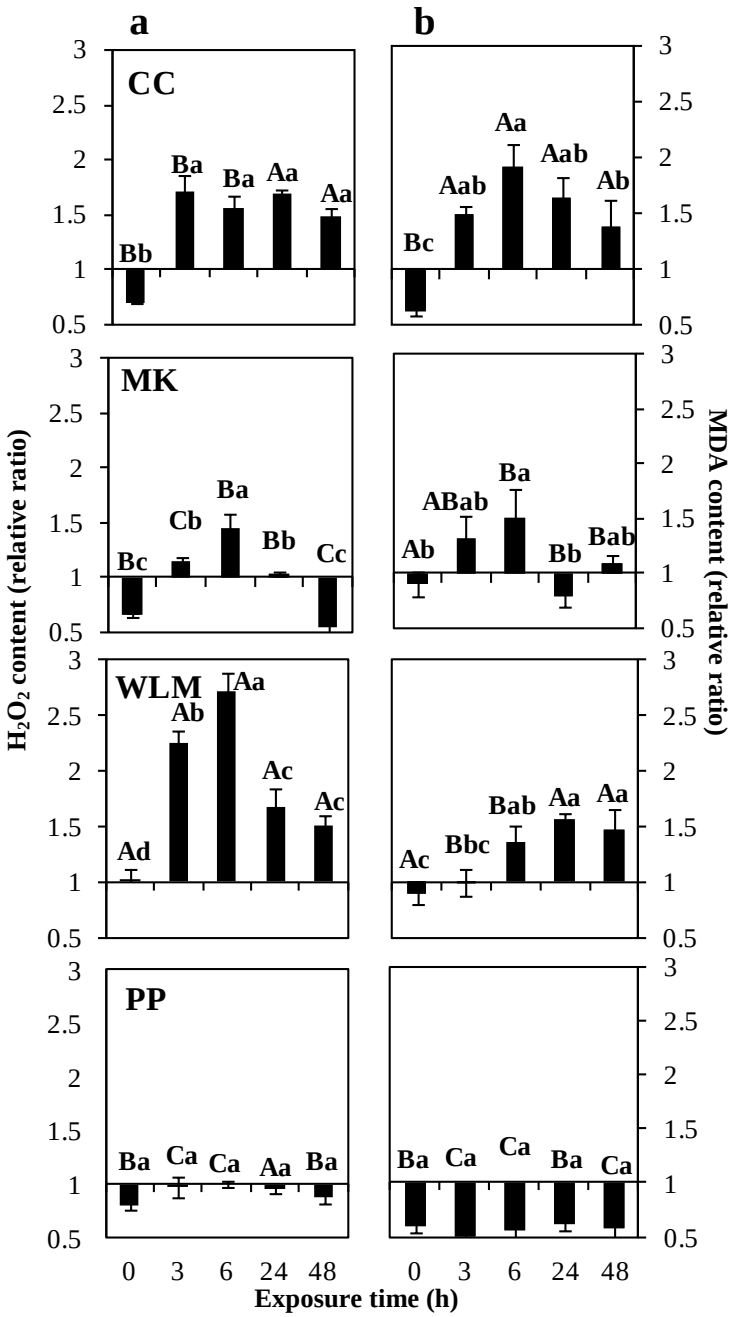

a
b
CC
MK
WLM
PP
Bb Ba Ba Aa Aa
Bc Aab Aa Aab Ab
Bc Cb Ba Bb Cc
Ab ABab Ba Bb Bab
Ad Ab Aa Ac Ac
Ac Bbc Bab Aa Aa
Ba Ca Ca Aa Ba
Ba Ca Ca Ba Ca
0 3 6 24 48
Exposure time (h)
H2O2 content (relative ratio)
MDA content (relative ratio)

Figure 4

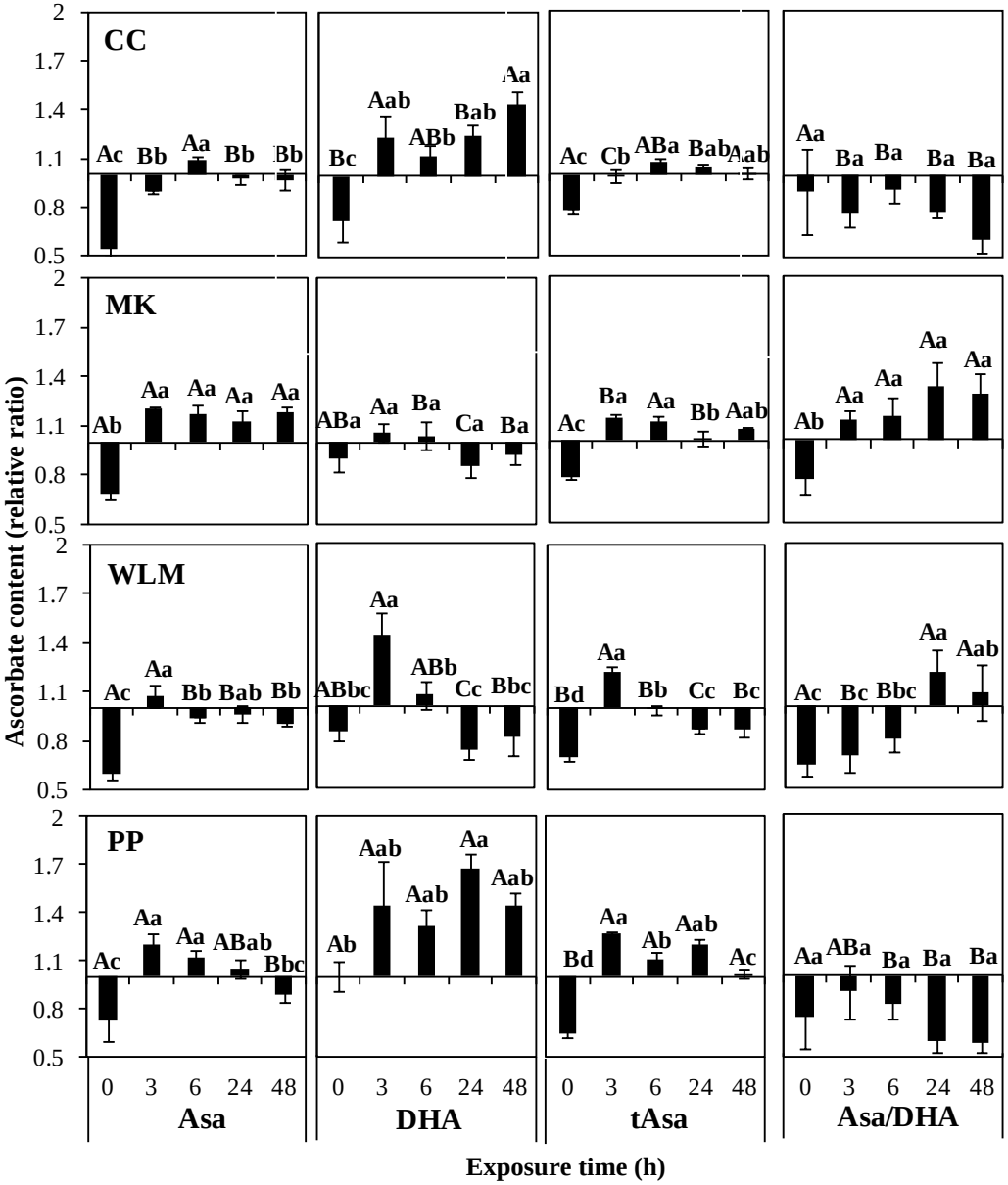

CC
MK
WLM
PP
Ascorbate content (relative ratio)
Asa
DHA
tAsa
Asa/DHA
Exposure time (h)
0 3 6 24 48

Figure 5

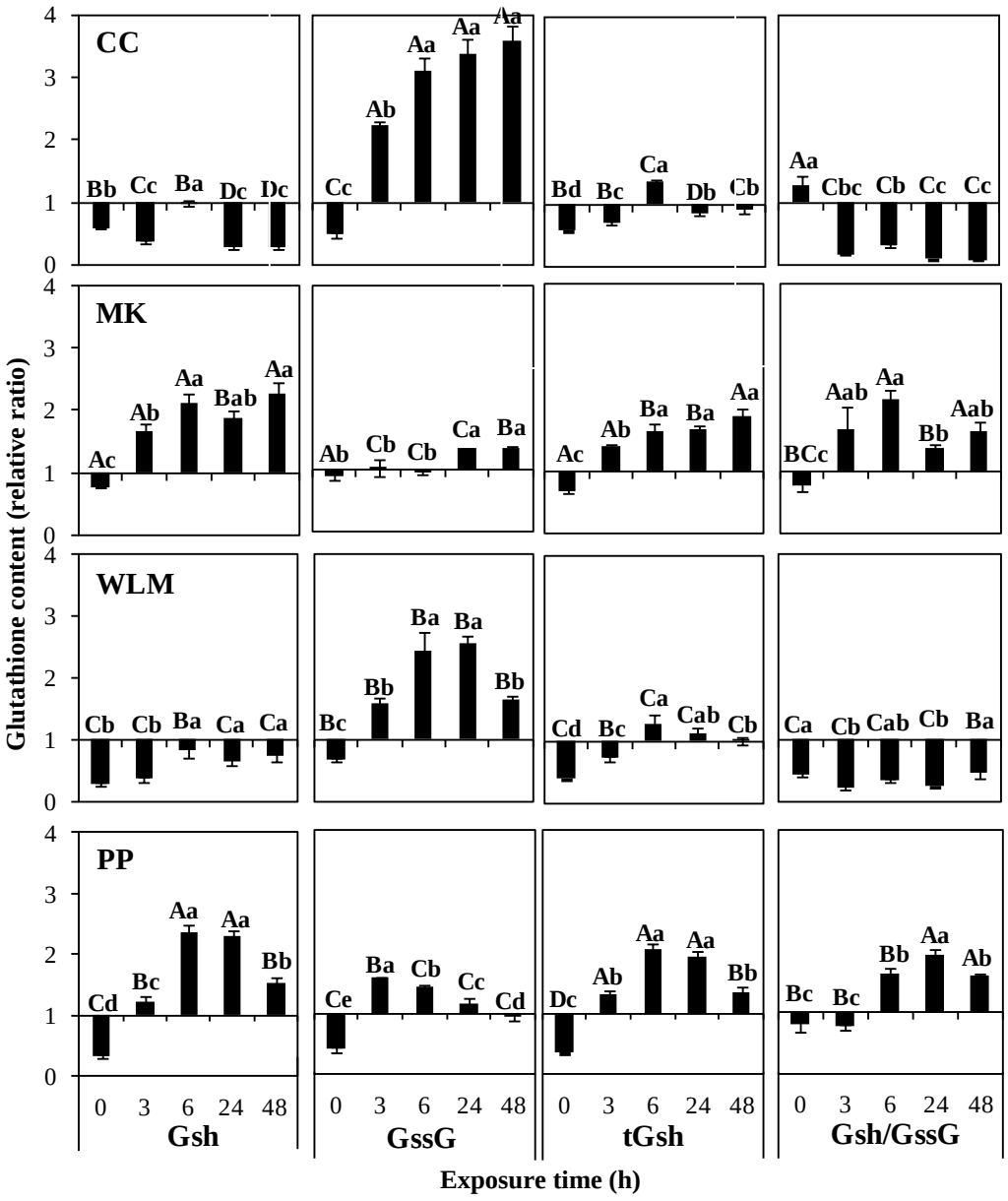
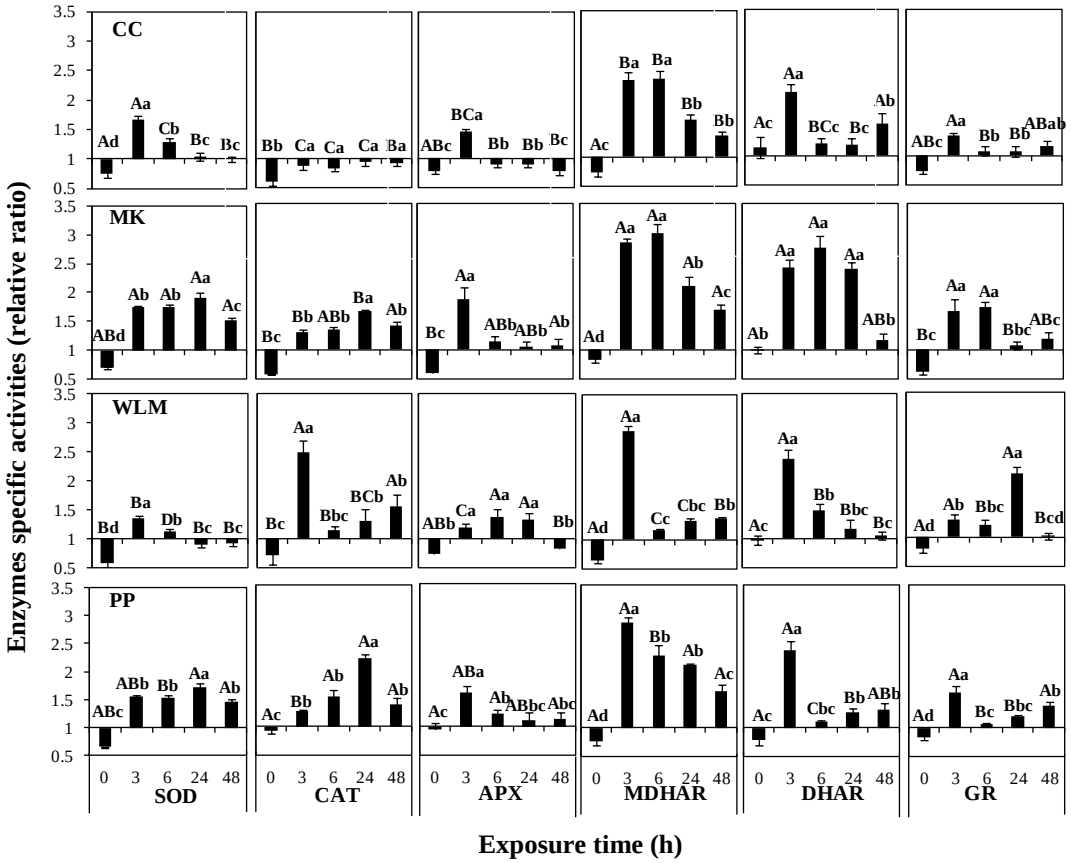

CC
MK
WLM
PP
Glutathione content (relative ratio)
Exposure time (h)
Gsh
GssG
tGsh
Gsh/GssG
0 3 6 24 48

Figure 6


CC
MK
WLM
PP
Enzymes specific activities (relative ratio)
Exposure time (h)
0 3 6 24 48
SOD
CAT
APX
MDHAR
DHAR
GR