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

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## 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  $\mu\text{mol.m}^{-2}\text{s}^{-1}$  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 (Frohnmeier 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**

## 2.1. Plant material and growth conditions

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

The experiment was conducted from September 23, 2010 to October 10, 2010 on clear days. For each of the basic true species of the *Citrus* genus and of the *Fortunella* genus (Table 1), three trees were analyzed. We isolated two independent sections on each of the three trees. The first section did not undergo any special treatment, and served as control. On this section, the leaves were kept uncovered throughout the experimental period to receive 100% sunlight irradiance. The leaves of the second section were shaded using a 90% shade cloth. This shade cloth allowed the actual transmission of 9.2% sunlight irradiance (90% shade cloth). We checked that spectra were not modified by shading, using a Li-Cor Li-1800 spectrometer. After one week of adaptation, the photooxidative stress was applied. The leaves were completely uncovered and received full light. Each treatment was allocated among the three selected trees of each genotype of the trial at three different periods (Fig. 1). At each period, one tree of each genotype was studied. In this way, the variability associated with different 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  $-80^{\circ}\text{C}$ . 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 ( $P_{\text{net}}$ ) and stomatal conductance ( $G_s$ ) were made with a portable open gas exchange system (GFS 3000, WALZ, Effeltrich, Germany). Air flow rate was  $750\text{ }\mu\text{mol.s}^{-1}$ . In a gas exchange chamber, photosynthetic photon flux density (PPFD) was controlled using a LED radiation source, and was fixed at a PPFD of  $1400\text{ }\mu\text{mol.m}^{-2}.\text{s}^{-1}$ . The use of this LED source ensured a constant, uniform light across all measurements. Carbon dioxide concentration was set at  $380\text{ }\mu\text{mol.mol}^{-1}$ .

## *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  $5000 \times g$  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  $3000 \times g$  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  $13,000 \times g$  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) polyvinylpyrrolidone (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  $\mu$ L 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<sub>2</sub>O<sub>2</sub>. The decrease in absorbance was measured at 240 nm ( $\epsilon = 39.4 \text{ mM}^{-1}.\text{cm}^{-1}$ ). One unit of CAT was expressed as 1 µmol H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> was added. The rate of ascorbate oxidation was evaluated at 290 nm for 3 min ( $\epsilon = 2.8 \text{ mM}^{-1}.\text{cm}^{-1}$ ). 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 ( $\epsilon = 6.3 \text{ mM}^{-1}.\text{cm}^{-1}$ ). 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 ( $\epsilon = 14.5 \text{ mM}^{-1}.\text{cm}^{-1}$ ) (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  $\mu\text{mol}$  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  $\mu\text{L}$  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 ( $\varepsilon = 14.15 \text{ mM}^{-1}.\text{cm}^{-1}$ ). One GR unit was defined as the amount of enzyme that allowed the formation of 1  $\mu\text{mol}$  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.

285  
286 *3.1. Effect of light stress on net photosynthesis ( $P_{net}$ ), stomatal conductance ( $G_s$ ) and*  
287 *maximum photochemical efficiency of PSII ( $F_v/F_m$ )*  
288

289 In all the genotypes,  $P_{net}$  and  $G_s$  values were lower under the shade treatment (ratio values  
290 below 1 at 0 h) (Figs. 2a, b). Leaves acclimated to shade conditions showed a reduction in  
291  $P_{net}$  of ~30%, ~50%, ~50% and ~60% in MK, WLM, CC and PP, respectively, compared to  
292 control leaves acclimated to full light conditions.

293 Likewise, the decrease in  $G_s$  (Fig. 2b) was very marked in CC (~52%), moderate in  
294 WLM and PP (~40%) and much lower in MK (~30%). At the end of the time course,  $P_{net}$   
295 was totally recovered in PP and MK (ratio values close to 1), whereas in CC and WLM, this  
296 recovery was incomplete (for  $P_{net}$ : ~80% of the control value).  $G_s$  was completely recovered  
297 in all genotypes after 48 h.

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

304  
305 *3.2. Effect of light stress on the oxidative status*  
306

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

light-acclimated leaves. Highly variable accumulations of H<sub>2</sub>O<sub>2</sub> and MDA were found between genotypes after exposure to full light (Figs. 3a, b). CC and WLM maintained relatively high levels of leaf H<sub>2</sub>O<sub>2</sub> and MDA contents. Along the time course, the rate of H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> was transitory. In MK, increase in H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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

335 ~1.5 times higher for MK compared with control), caused essentially by a higher incremental  
336 Gsh concentration (~2 times higher from 6 h compared with control) than in GssG  
337 concentration, which remained very low. Throughout the experiment, a significant increase in  
338 Gsh/GssG compared with the control was observed in response to photooxidative stress in  
339 MK (~2.1-fold increase at 6 h) and PP (~2-fold increase at 24 h). By contrast, CC and WLM  
340 displayed a very marked decrease in the ratio values compared with the control (~84% and  
341 ~77% at 3 h, respectively), and these values continued to decrease in CC to 48 h, but  
342 remained essentially unchanged along the time course in WLM.

343 We analyzed the activities of various enzymes acting as ROS scavengers, i.e. SOD, CAT  
344 and APX, or ensuring the supply/regeneration of primary antioxidants, i.e. MDHAR, DHAR  
345 and GR (Fig. 6). For all the genotypes, SOD activity increased rapidly after 3 h of exposure to  
346 photooxidative stress and more intensively in CC, MK and PP. A decline was observed in CC  
347 and WLM at 24 h to reach values equivalent to the control (ratio values close to 1). By  
348 contrast, in MK and PP, SOD remained very active at 48 h (~1.5 times and ~1.3 times higher  
349 than the control, respectively). CC was the only genotype studied with a specific CAT pattern.  
350 From 3 h, CAT activity increased significantly compared with 0 h, but remained depressed  
351 relative to control in the light section (ratio values below 1). In MK and PP, a peak of activity  
352 was observed at 24 h (~1.6 and ~2.2 times higher than control, respectively), whereas this  
353 peak was present at 3 h in WLM, and was followed by a significant loss of activity. Overall,  
354 APX activity was rapidly increased, with a peak at 3 h in all the genotypes. At the end of the  
355 kinetics, the activity became equivalent to control (ratio values close to 1) in MK and PP  
356 whereas in CC and WLM its activity was blocked (ratio values below 1). MDHAR was the  
357 antioxidant enzyme whose activity was the most strongly increased. We observed an early  
358 peak of activity from 3 h in all the genotypes (~3 times higher than control), followed by a  
359 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<sub>2</sub> concentration and a depression of photosynthesis (Favaretto et al., 2011). Although *P*<sub>net</sub> followed the same pattern as *G*<sub>s</sub> 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<sub>2</sub> 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 *G*<sub>s</sub> recovered 48 hours after exposure to full light in all the genotypes, *P*<sub>net</sub> 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*<sub>net</sub> and *G*<sub>s</sub> recovery after subsequent drainage, unlike Cleopatra mandarin, a flooding-sensitive rootstock, in which *G*<sub>s</sub> recovered, but *P*<sub>net</sub> did not.

We measured photoinhibition and oxidative damage to investigate potential mechanisms for tolerance to photooxidative stress. PSII maximum quantum efficiency (*F*<sub>v</sub>/*F*<sub>m</sub>) was the best indicator for photoinhibition (Maxwell and Johnson, 2000). Photoinhibition can be considered as a photoprotective process only when decreases in *F*<sub>v</sub>/*F*<sub>m</sub> are slight (Adams et al., 2006). We found that *F*<sub>v</sub>/*F*<sub>m</sub> 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*<sub>v</sub>/*F*<sub>m</sub> 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; Bonnacarrere 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).

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

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

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

The two sensitive genotypes CC and WLM showed sharply contrasting behaviors compared with the tolerant ones. The slight increases in SOD and APX activity could probably explain the very high accumulation of H<sub>2</sub>O<sub>2</sub> and MDA. These results are consistent with their previously observed greater sensitivity. Some authors had already observed a slight increase in these two enzymes in *Hevea* chilling-sensitive clones (Mai et al., 2010) or even a depression in the flooding-sensitive rootstock, Cleopatra Mandarin (Arbona et al., 2008). CC presented the peculiarity of significantly inactivating CAT compared with WLM. Favaretto et al. (2011) and Yang et al. (2012) also respectively reported a decline in CAT activity in pioneer tree species and in magnesium-deficient leaves of ‘Sour’ pummelo (*Citrus grandis*), probably because this enzyme is light-sensitive and suffers from photoinactivation caused by oxidative damage initiated *via* direct absorption of light by the heme moieties of the enzyme itself (Shang and Feierabend, 1999). It was also postulated that inactivation of CAT could also be mediated by photo-oxidative events initiated through light absorption by chlorophyll (Feierabend and Engel, 1986). Earlier induction of CAT and APX in WLM could explain why this genotype tended to decrease the accumulation of H<sub>2</sub>O<sub>2</sub> from 6 h compared with CC, in which these two enzymes were completely inactivated from 6 h. The significant decrease in Gsh/GssG and Asa/DHA ratios suggested that the increased activity of the recycling enzyme was insufficient to produce enough glutathione/ascorbate to regulate the redox status, and that no new synthesis was occurring as previously reported by Yang et al. (2012) in citrus magnesium-deficient leaves or by Arbona et al. (2008) in the flooding-sensitive rootstock Cleopatra mandarin.

## 5. Conclusions

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

521

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**Table 1**

Genotypes used for physiological and biochemical analysis and their corresponding rootstocks

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

<sup>a</sup>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 ( $T_{\text{mean}}$ ). 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 ( $P_{\text{net}}$ ), (b) stomatal conductance ( $G_s$ ) and (c) maximum quantum yield of photosystem II ( $F_v/F_m$ ) 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 ( $\pm$ 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 ( $\text{H}_2\text{O}_2$ ) 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 ( $\pm$ 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 ( $\pm$ 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 ( $\pm$ 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 ( $\pm$ 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**

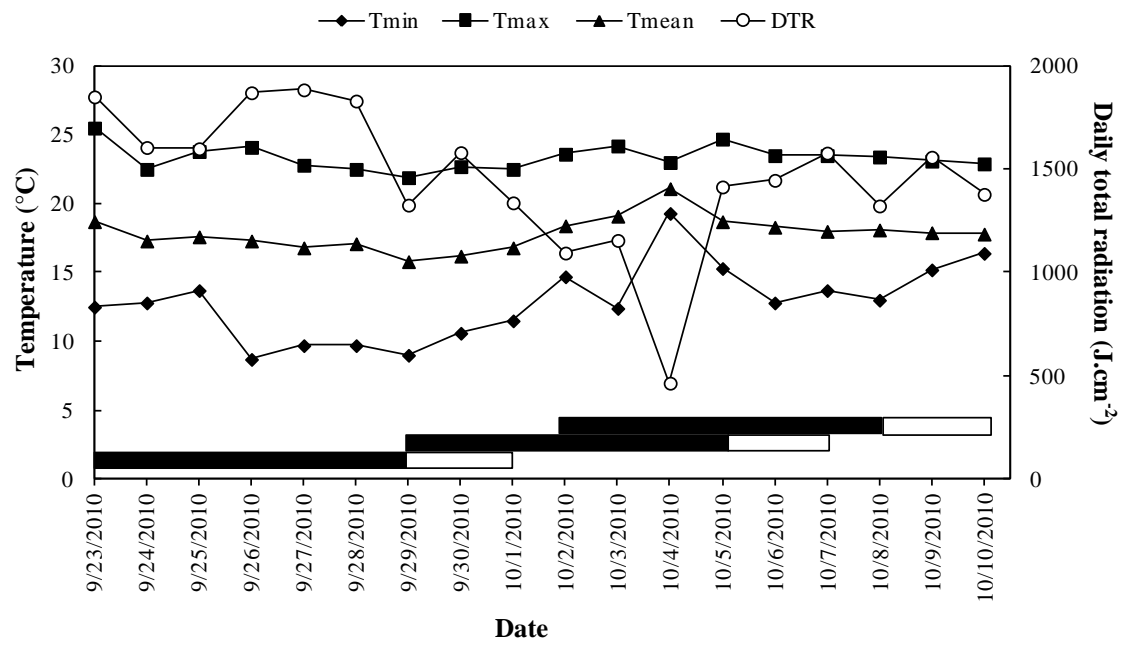
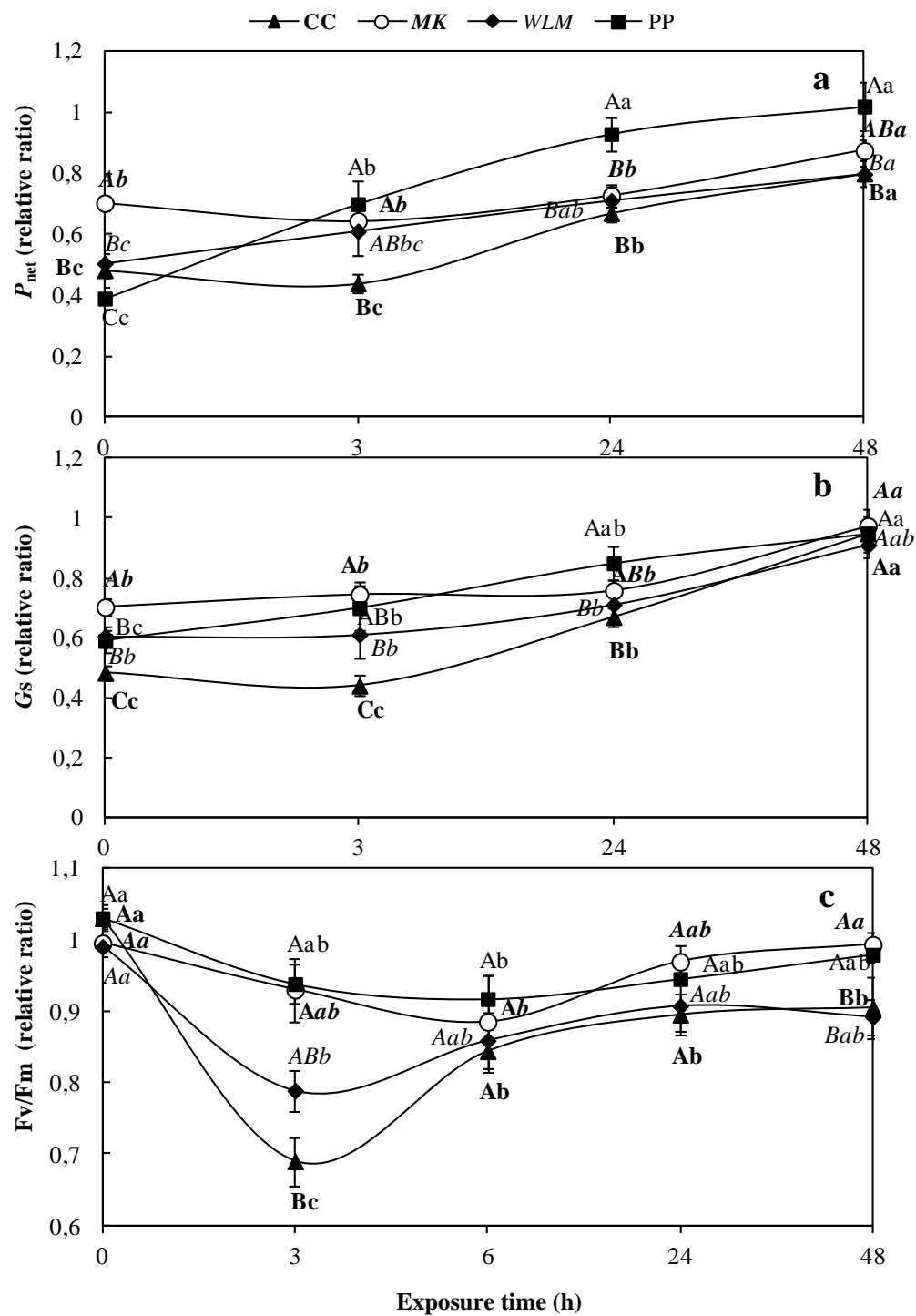




Figure 2



**Figure 3**

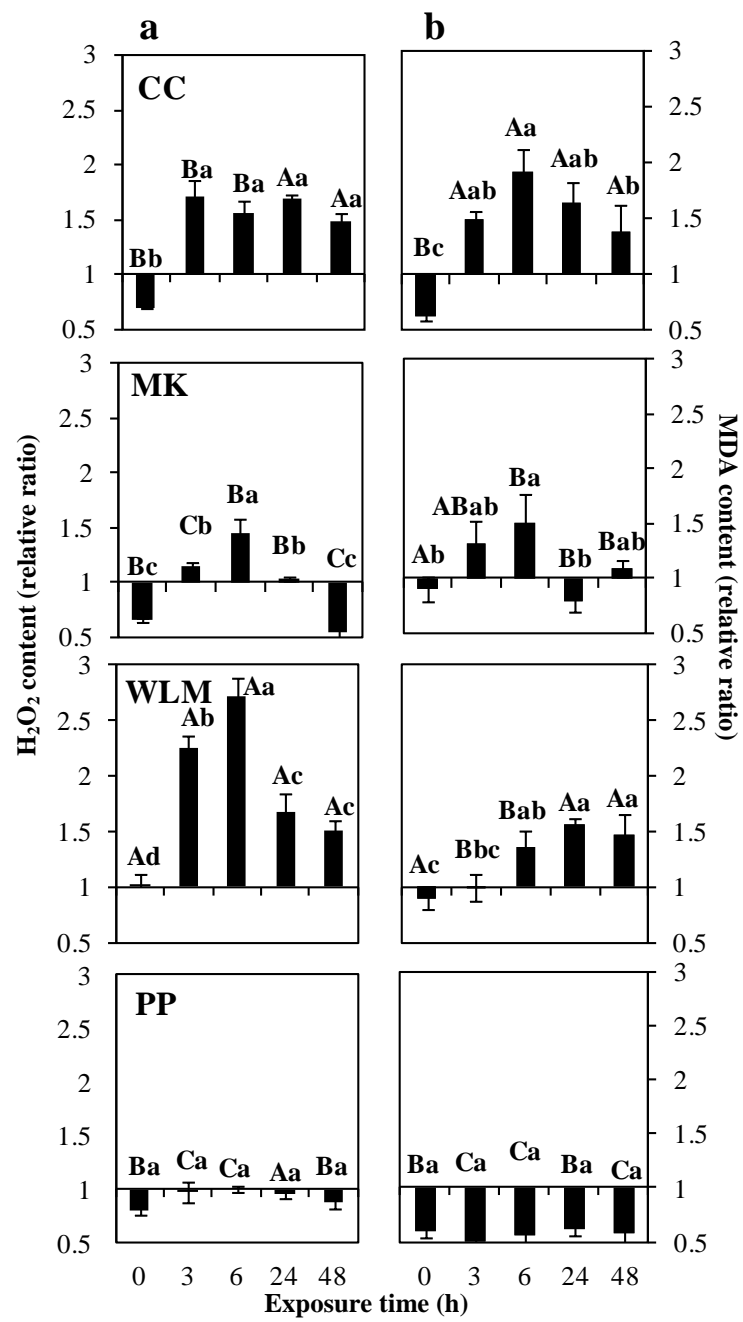


Figure 4

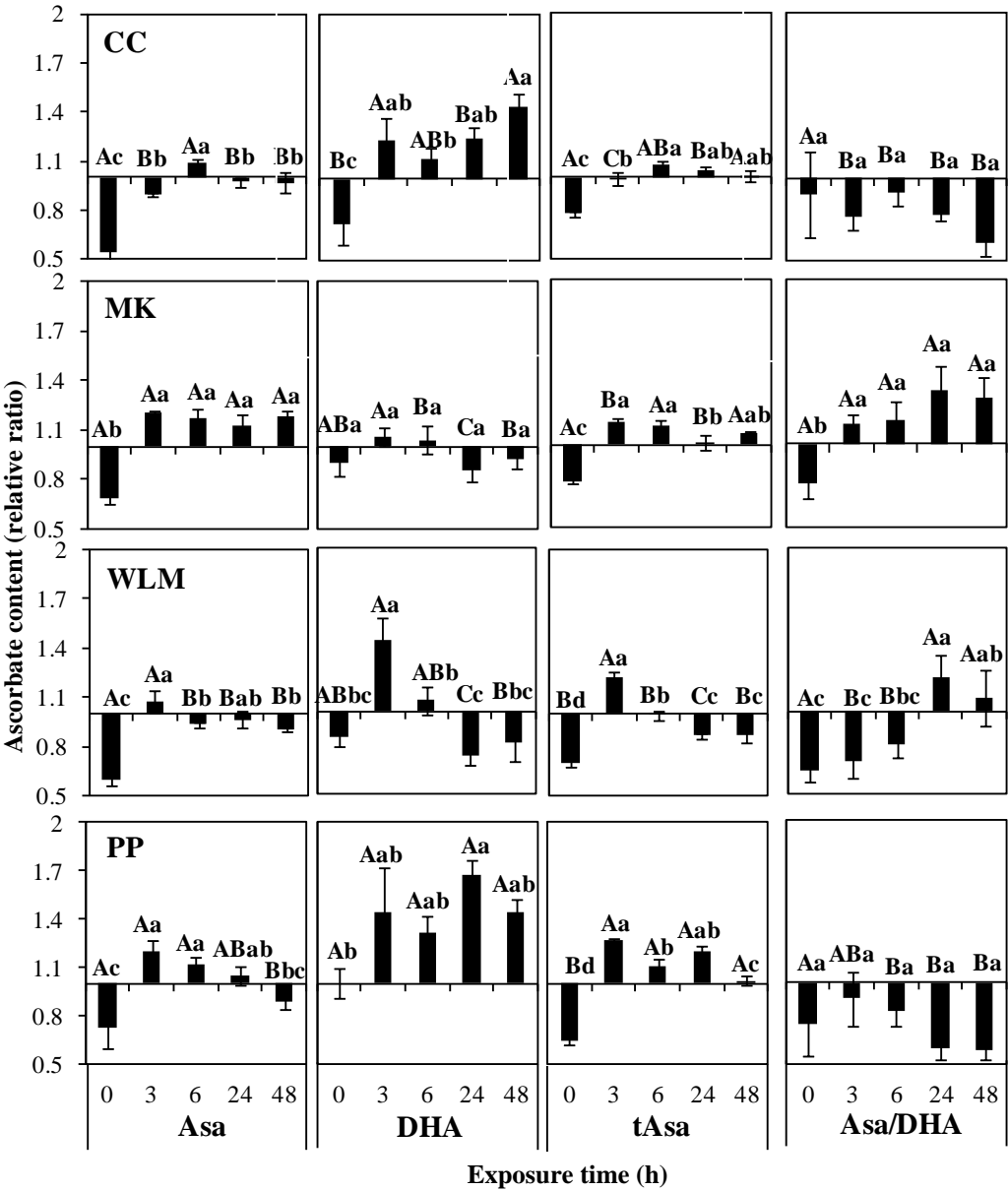
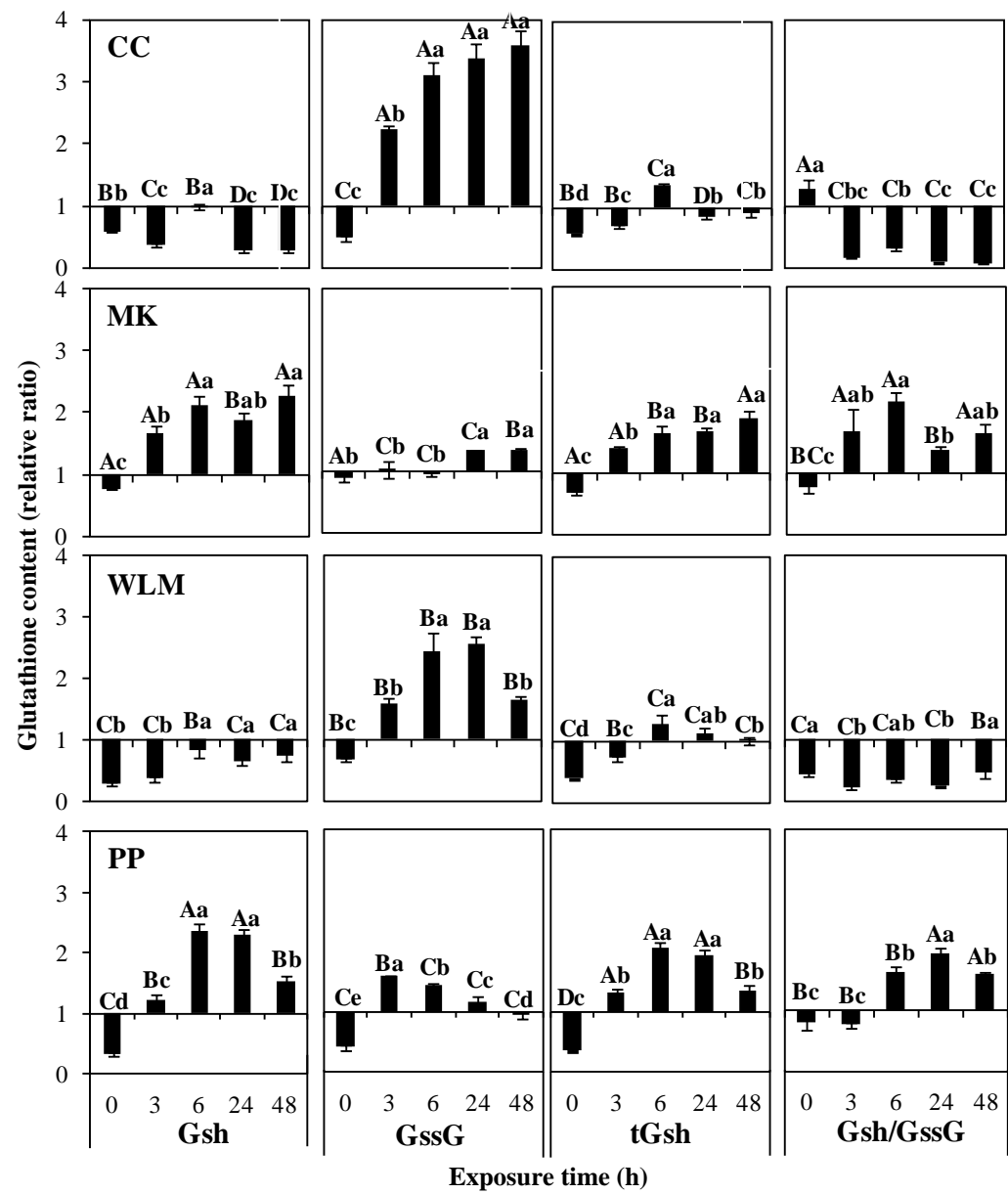


Figure 5



**Figure 6**

