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## 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

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37 ABSTRACT

38 Despite the economic importance of citrus, insights on the genetic response to stress are  
39 scarce. The aim of the present study was to compare fundamental citrus species for their  
40 response to photooxidative stress. The experiment was conducted under orchard conditions on  
41 three fundamental citrus species *C. medica* L., *C. reticulata* Blanco and *C. maxima* (Burm.)  
42 Merr., and on *Fortunella japonica* (Thunb.) Swing.. We examined their respective net  
43 photosynthesis (*P*<sub>net</sub>), stomatal conductance (*G*<sub>s</sub>) and chlorophyll fluorescence (*F*<sub>v</sub>/*F*<sub>m</sub>) on  
44 sun-acclimated leaves and shade-acclimated leaves returned under natural sunlight irradiance.  
45 To compare the respective response mechanism, we analyzed changes in oxidative status  
46 (hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and malondialdehyde (MDA)), reactive oxygen species (ROS)-  
47 scavenging enzymes (superoxide dismutase (SOD), catalase, ascorbate peroxidase), recycling  
48 enzymes (monodehydroascorbate reductase, dehydroascorbate reductase and glutathione  
49 reductase) and antioxidant metabolites (ascorbate and glutathione). Kumquat and pummelo  
50 exposed lower down-regulation and full recovery of photosynthetic parameters, lower  
51 accumulation of oxidized compounds associated with greater production of reduced  
52 glutathione (Gsh) and enhanced activity of the three ROS scavenging enzymes, especially  
53 SOD. Citron and mandarin showed a marked decrease and incomplete recovery in  
54 photosynthetic performance, mainly in *P*<sub>net</sub> and *F*<sub>v</sub>/*F*<sub>m</sub>, larger accumulation of oxidative  
55 parameters, slighter induction of antioxidant enzymes and down-regulation of reduced  
56 ascorbate (Asa) and Gsh synthesis. These results suggest that kumquat and pummelo have a  
57 greater tolerance to photooxidative stress than citron and mandarin.

58

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

61

## 62 **1. Introduction**

63

64 Citrus is the world's most economically important fruit crop. Strictly, true citrus plants  
65 comprise six genera: *Clymenia*, *Eremocitrus*, *Microcitrus*, *Poncirus*, *Fortunella* and *Citrus* .  
66 Scora (1975) and Barrett and Rhodes (1976) considered *Citrus medica* L. (citron), *C. maxima*  
67 (Burm.) Merr. (pummelo) and *C. reticulata* Blanco (mandarin) to be the three fundamental  
68 species of *Citrus*, the other species resulting from hybridization of these true species. This  
69 view has recently gained support from various biochemical and molecular studies (Federici et  
70 al., 1998; Barkley et al., 2006; Fanciullino et al., 2006). Allopatric evolution has resulted in  
71 strong genetic and also phenotypic differentiation between these *Citrus* taxa (Garcia-Lor et al.,  
72 2012).

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

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

92 A common effect of most environmental factors is an increased production of reactive  
93 oxygen species (ROS) in green plant cells, a situation called photooxidative stress, driven by  
94 the light energy absorbed in excess of assimilatory requirements (Foyer et al., 1994). These  
95 harmful ROS such as singlet oxygen ( $^1O_2$ ), superoxide anion ( $O_2^{\bullet-}$ ), hydrogen peroxide  
96 ( $H_2O_2$ ), and hydroxyl radical ( $OH^{\bullet}$ ) are involved in the mechanism of photoinhibition (Asada,  
97 1999). The production of ROS in plant cells is enhanced by conditions that limit  $CO_2$  fixation,  
98 such as drought, salt, heat and cold stresses, and by the combination of these conditions with  
99 strong light (Foyer and Noctor, 2003). Because aerobic organisms, such as plants, live in a  
100 highly oxidative environment, they have evolved efficient antioxidant systems protecting  
101 them from the damaging effects of ROS (Asada, 1999) such as decreased protein synthesis,  
102 damage to DNA and membrane lipids (Frohnmeier and Staiger, 2003; Mackerness et al.,  
103 2001). These antioxidant mechanisms employ (i) ROS-scavenging enzymes, such as  
104 superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and ascorbate  
105 peroxidase (APX, 1.11.1.11), (ii) recycling enzymes of the ascorbate-glutathione cycle, such  
106 as monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase  
107 (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2), and (iii) low molecular  
108 weight antioxidants, such as reduced ascorbic acid (Asa) and reduced glutathione (Gsh).  
109 Some authors have reported that antioxidative systems play a major role in protecting plants  
110 from the harmful effects of excess light energy (Foyer et al., 1994; Favaretto et al., 2011).

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

113 Some authors have shown that citrus physiology is adversely affected by abiotic stresses,  
114 such as drought (Avila et al., 2012), waterlogging (Hossain et al., 2009) and salinity (Balal et  
115 al., 2012; Brumos et al., 2009). Currently, experiments have been mainly performed on the  
116 most common rootstocks under the superimposition of a specific stress with strong light. For  
117 instance, it was found that the Cleopatra mandarin was very sensitive to flooding stress and  
118 tolerant to salt stress, whereas Carrizo citrange showed the opposite behavior (Arbona et al.,  
119 2008; Brumos et al., 2009). Thus, a heterogeneous response to oxidative stress between  
120 rootstocks exists under homogeneous cultural conditions. To date, no study has focused on the  
121 possible differences of stress response that could exist between citrus species and, especially,  
122 for the species at the origin of the broad genetic diversity of cultivated citrus.

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

133

## 134 **2. Materials and methods**

135

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,

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

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

171

## 172 2.2. Gas exchange measurements

173

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

180

## 181 2.3. Chlorophyll *a* fluorescence measurements

182

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



186 relaxation of fluorescence quenching associated with thylakoid membrane energization  
187 (Krause et al., 1983). Minimal fluorescence ( $F_o$ ) and maximal fluorescence ( $F_m$ ) were  
188 obtained by imposing a 1 s saturating flash to reduce all the PSII reaction centers. The  
189 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  
190  $F_v/F_m$ .  
191

192

#### 193 *2.4. Measurement of $H_2O_2$ and MDA levels*

194

195  $H_2O_2$  levels were measured following the protocol described by Zhou et al. (2006). For  
196 extraction, 200 mg of frozen leaf powder was homogenized in 3 mL of trichloroacetic acid  
197 (TCA) 5% (w:v) containing 60 mg of activated charcoal. The mixture was then centrifuged at  
198  $5000 \times g$  for 20 min at 4 °C.

199 The MDA concentration of leaves was determined using a thiobarbituric acid (TBA)  
200 reaction described by Hodges et al. (1999). For extraction, 100 mg of frozen leaf powder was  
201 homogenized with inert sand in 2.5 mL of 80% ethanol (v/v), followed by centrifugation at  
202  $3000 \times g$  for 10 min at 4 °C.

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

205

#### 206 *2.5. Assay of antioxidant metabolites*

207

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

211 5 min at 4 °C. Oxidized ascorbate (DHA) was calculated by subtracting Asa concentration  
212 from the tAsa concentration.

213 Total glutathione (tGsh) and oxidized glutathione (GssG) contents were measured  
214 according to the DTNB-GR recycling procedure of Rahman et al. (2006). For extraction,  
215 50 mg of frozen leaf powder was homogenized in 2.0 mL of mixed buffer (100 mM  
216 potassium phosphate, pH 7.5, 5 mM EDTA, 0.1% (v:v) Triton X-100 and 23 mM  
217 sulfosalicylic acid) and centrifuged at  $8000 \times g$  for 10 min at 4 °C. Gsh concentration was  
218 calculated by subtracting GssG concentration from the tGsh concentration.

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

221

## 222 *2.6. Assay of antioxidant enzyme activities*

223

224 For all enzymatic assays, frozen leaf powder was homogenized in extraction medium  
225 (100 mM potassium phosphate buffer, pH 7.5, containing 0.1% (v/v) TritonX-100 and 1%  
226 (w/v) polyvinylpyrrolidone (PVP)) using 27 mg FW per mL of buffer. The homogenate was  
227 then centrifuged at  $13,000 \times g$  for 30 min at 4 °C. The supernatant was used for the protein  
228 and enzyme analysis (except for SOD, where the extract was diluted 20-fold). Protein  
229 concentration was determined by the method of Bradford (1976). All kinetic measurements  
230 were made using a V-630 spectrophotometer (Jasco Inc., Tokyo, Japan).

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

235 was defined as the amount of enzyme causing 50% inhibition in the rate of NBT reduction at  
236 560 nm, at 25 °C.

237 Catalase (CAT, EC 1.11.1.6) activity was measured according to the method of Aebi  
238 (1984). The reaction mixture (1.1 mL) contained 100 µL of crude enzyme extract, 37.8 mM  
239 sodium phosphate buffer (pH 7.0) and 4.4 mM H<sub>2</sub>O<sub>2</sub>. The decrease in absorbance was  
240 measured at 240 nm ( $\epsilon = 39.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ ). One unit of CAT was expressed as 1 µmol H<sub>2</sub>O<sub>2</sub>  
241 degraded per min at 25 °C.

242 Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to a  
243 modified method described by Asada (1984). The standard reaction mixture (1.0 mL)  
244 contained 0.17 mM ascorbate, and 33 µL of crude enzyme extract in a 60.3 mM potassium  
245 phosphate buffer (pH 7.0). The reaction was triggered when 4.95 mM H<sub>2</sub>O<sub>2</sub> was added. The  
246 rate of ascorbate oxidation was evaluated at 290 nm for 3 min ( $\epsilon = 2.8 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ ). One unit  
247 of APX was expressed as the oxidation of 1 µmol ascorbate per min at 25 °C.

248 Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity was established by  
249 monitoring the MDHA-dependent oxidation of NADH according to the slightly modified  
250 method of Drew et al. (2007). 100 µL of crude enzyme extract was added to a solution  
251 containing 9.7 mM potassium phosphate, 0.125% Triton X100 (pH 8), 2.5 mM ascorbate,  
252 0.128 units of ascorbate oxidase in a total volume of 1.0 mL. The reaction was started by  
253 adding 0.2 mM NADH. The decrease in absorbance was measured at 340 nm ( $\epsilon = 6.3 \text{ mM}^{-1}$   
254  $\cdot \text{cm}^{-1}$ ). One MDHAR unit was defined as the amount of enzyme required to oxidize 1 µmol  
255 NADH per min at 340 nm at 25 °C.

256 Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was assayed by measuring the  
257 rate of appearance of ascorbate measured at 265 nm ( $\epsilon = 14.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ ) (Asada, 1984). The  
258 standard reaction mixture (1.0 mL) contained 41 mM potassium phosphate buffer (pH 6.5),  
259 5 mM Gsh, 0.11 mM EDTA, and 75 µL of crude enzyme extract, with 0.5 mM DHA added to

260 initiate the reaction. One DHAR unit was defined as the amount of enzyme that allowed the  
261 formation of 1  $\mu\text{mol}$  ascorbate per min at 25 °C.

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

269

## 270 2.7. Statistical analyses

271

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

279

## 280 3. Results

281

282 In order to minimize the effects of changes in environmental conditions during the  
283 experiment, the results were expressed as relative data. Thus, only the effect of the light  
284 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

310 light-acclimated leaves. Highly variable accumulations of H<sub>2</sub>O<sub>2</sub> and MDA were found  
311 between genotypes after exposure to full light (Figs. 3a, b). CC and WLM maintained  
312 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>  
313 and MDA remained high and stable in CC compared with the control (more than 1.5 times  
314 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  
315 MDA levels was also transitory with a maximum value at 6 h (~1.5 times higher than the  
316 control). PP displayed a very specific pattern with no changes in H<sub>2</sub>O<sub>2</sub> and MDA levels along  
317 the time course.

318

### 319 *3.3. Effect of photooxidative stress on the antioxidant system*

320

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

331 For the glutathione concentration, GssG was the most significantly increased in CC (~3.5  
332 times higher at 48 h compared with control) and WLM (~2.5 times higher at 6 h compared  
333 with control), whereas in MK and PP little difference was observed (Fig. 5). Conversely, MK  
334 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.

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

368

#### 369 **4. Discussion**

370

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

381

##### 382 *4.1. Differences in the sensitivity to photooxidative stress between fundamental citrus species*

383



384 To date no relationship between taxonomic affiliations and ability to tolerate  
385 photooxidative stress has ever been evidenced. The biochemical and physiological parameters  
386 studied enabled us to highlight the contrasting strategies implemented by ancestral genotypes  
387 of citrus to cope with photooxidative stress. When plants are exposed to high irradiation, the  
388 stomata normally close to prevent water loss, resulting in a decrease in the intercellular CO<sub>2</sub>  
389 concentration and a depression of photosynthesis (Favaretto et al., 2011). Although *P*net  
390 followed the same pattern as *G*s in all the genotypes up to 24 h, light stress-induced changes  
391 in photosynthesis were primarily caused by non-stomatal factors, as they were accompanied  
392 by similar CO<sub>2</sub> intercellular concentrations (data not shown), as previously shown in ‘Xuegan’  
393 orange (*Citrus sinensis*) or in ‘Sour’ pummelo (*Citrus grandis*) during boron or magnesium  
394 deficiency (Han et al., 2009; Yang et al., 2012). Our results also show that whereas *G*s  
395 recovered 48 hours after exposure to full light in all the genotypes, *P*net did not, in either CC  
396 or WLM. This suggests that factors additional to stomatal closure must limit photosynthetic  
397 activity in the latter. Arbona et al. (2009) demonstrated that Carrizo citrange, a flooding-  
398 tolerant rootstock, had a better *P*net and *G*s recovery after subsequent drainage, unlike  
399 Cleopatra mandarin, a flooding-sensitive rootstock, in which *G*s recovered, but *P*net did not.

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

409 1989). In addition, the incomplete recovery after 48 h of treatment in CC and WLM could be  
410 attributable to an increased proportion of closed, reversibly inactivated or destroyed PSII  
411 reaction centers, probably caused by enhanced ROS accumulation (Foyer and Noctor, 2000).  
412 These results were confirmed by the greatest increase of  $F_o$  in CC and WLM compared to  
413 MK and PP (data not shown). An increase in  $F_o$  is considered to be the characteristic of  
414 inhibition of the acceptor side of PSII (Setlik et al., 1990) and is interpretable in terms of  
415 photodamages (Wingler et al., 2004). Previous studies showed that PSII, but not PSI, was a  
416 target during high temperature stress in Satsuma mandarin (*Citrus unshiu*) and Navel orange  
417 (*Citrus sinensis*) (Guo et al., 2006). Genotype differences in stress sensitivity was further  
418 demonstrated by parameters that estimate oxidative stress. Oxidative damage is caused by  
419 increased production of ROS. Among the most abundant ROS,  $H_2O_2$  produced in  
420 peroxisomes and chloroplasts might diffuse to the cytosol, where it reacts with transition  
421 metal ions ( $Fe^{2+}$ ) during the Fenton reaction, yielding hydroxyl radical ( $OH^\cdot$ ), considered as  
422 the main cell-damaging product responsible for lipid peroxidation (Foyer et al., 1994).  
423 Considering MDA as an important indicator of lipid peroxidation, the concomitant and  
424 significant accumulation of  $H_2O_2$  and MDA in CC and WLM indicated that these two  
425 genotypes suffered a higher oxidative pressure than MK and PP. These results were consistent  
426 with their probably greater sensitivity and vulnerability to the light stress discussed above.  
427 The occurrence of an  $H_2O_2$  and MDA burst had previously been observed in flooding-  
428 sensitive Cleopatra mandarin, or in a chilling-sensitive rice genotype IR50, whereas smaller  
429 amounts of these two compounds had been highlighted in flooding-tolerant Carrizo citrange,  
430 or chilling-tolerant rice genotype L2825CA (Arbona et al., 2008; Bonnacerrere et al., 2011).  
431 In response to photooxidative stress, CC also exhibited high amounts of DHA and GssG, the  
432 oxidized forms of ascorbate and glutathione, implying that the cells had undergone greater  
433 oxidative pressure. Whereas PP and WLM displayed different patterns with a large amount of

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

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

448

449

450 *4.2. Could Antioxidant system explain the differences of response to photooxidative stress*  
451 *between citrus species ?*

452

453 The antioxidant system is fundamentally important in protecting the photosynthetic  
454 apparatus, and it was assumed that higher antioxidant protection would be needed to  
455 compensate for higher light-mediated oxidative stress (Hansen et al., 2002). Various studies  
456 have highlighted the importance of antioxidant in tolerance to stress. Here, the complexity of  
457 the antioxidant system regulation is highlighted by the number of antioxidant components and  
458 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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**

509

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)  $F_v/F_m$  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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523

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683

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686

**Table 1**

Genotypes used for physiological and biochemical analysis and their corresponding rootstocks

Abbreviation	Genotype			Corresponding rootstock		
	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**

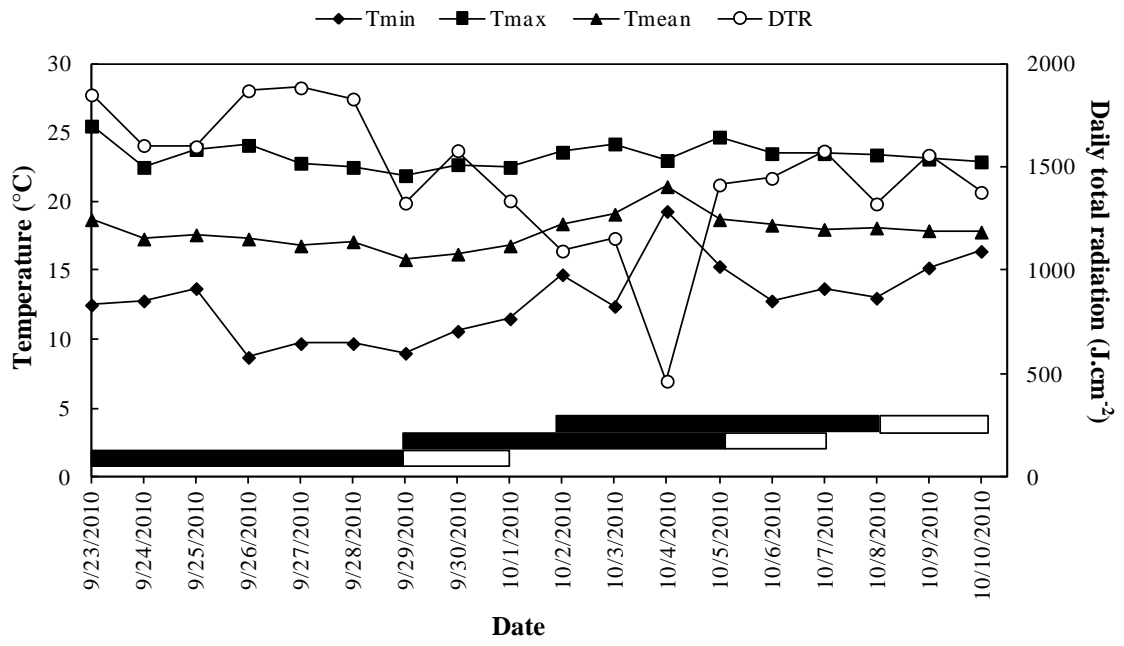
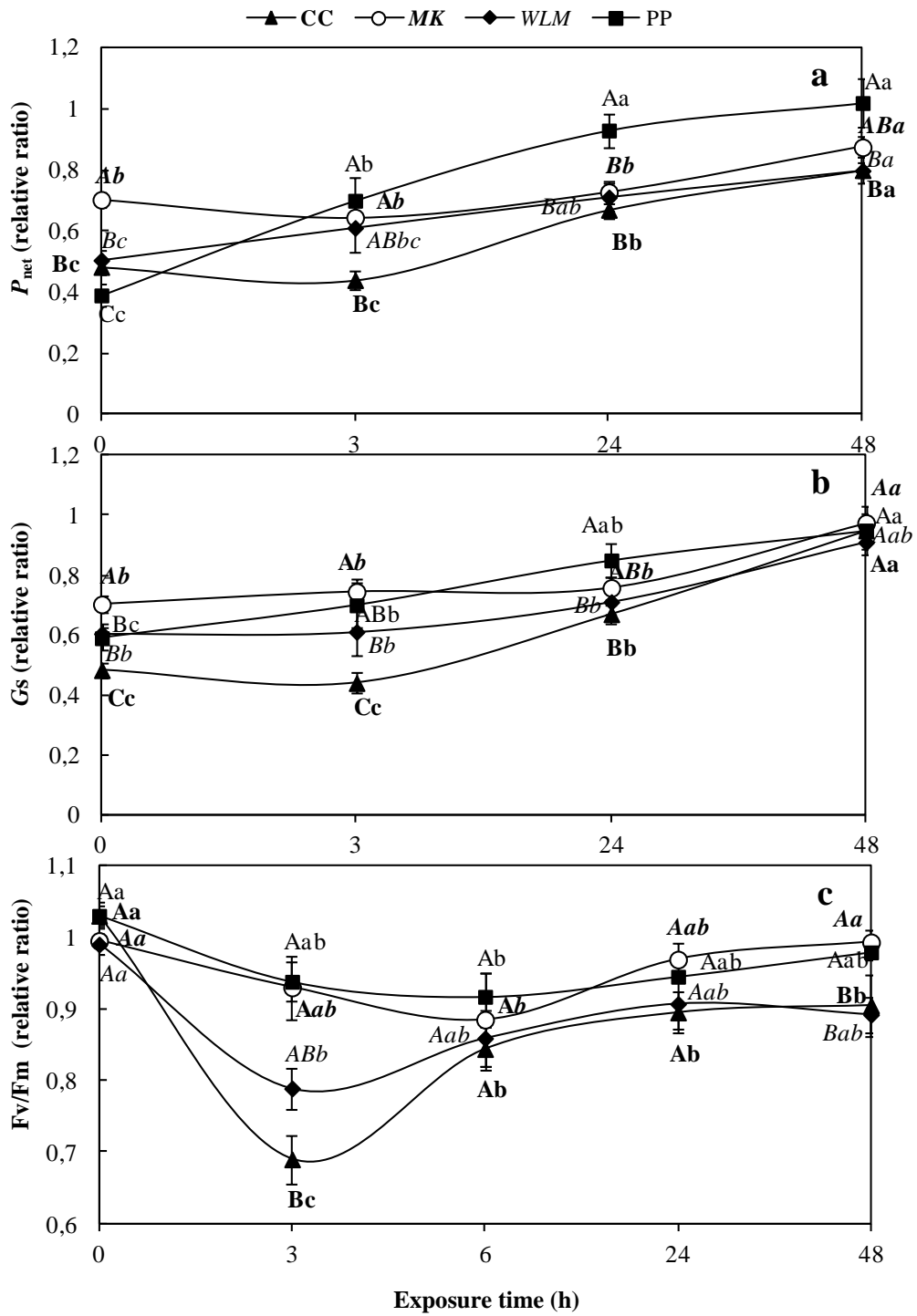
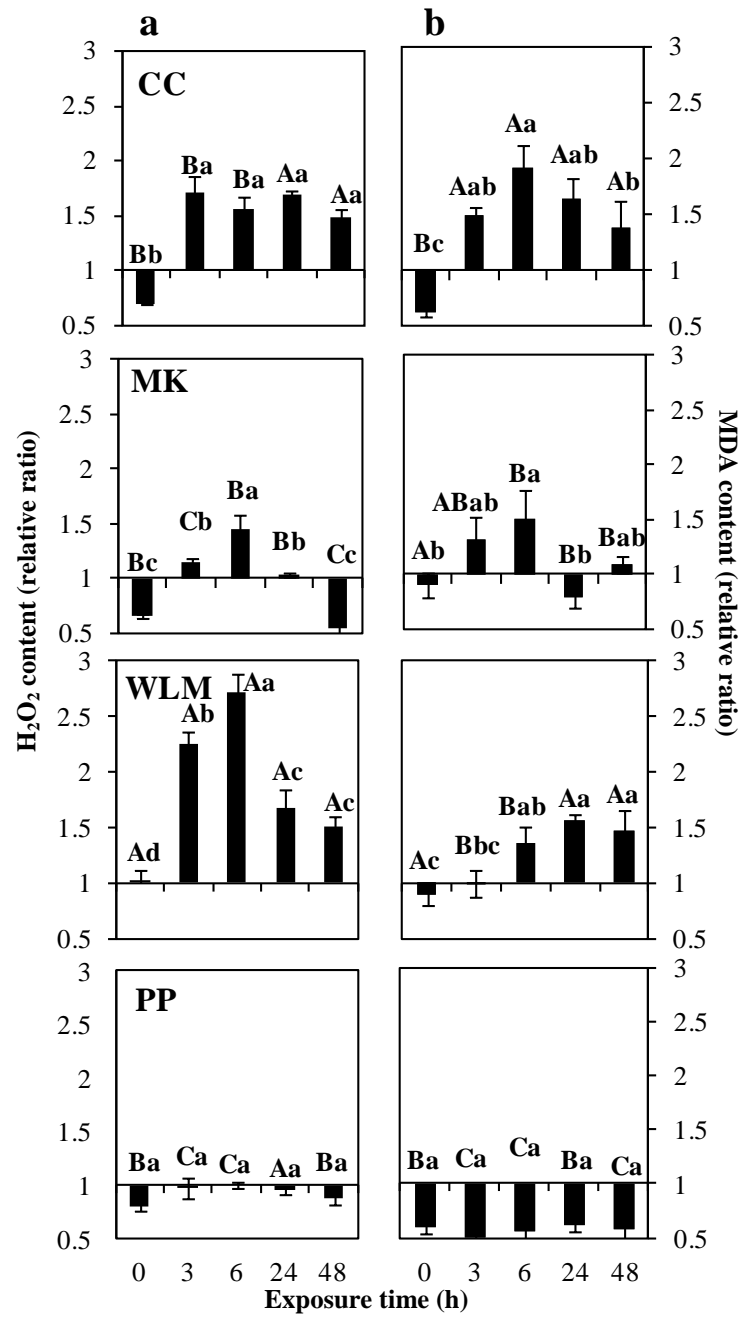




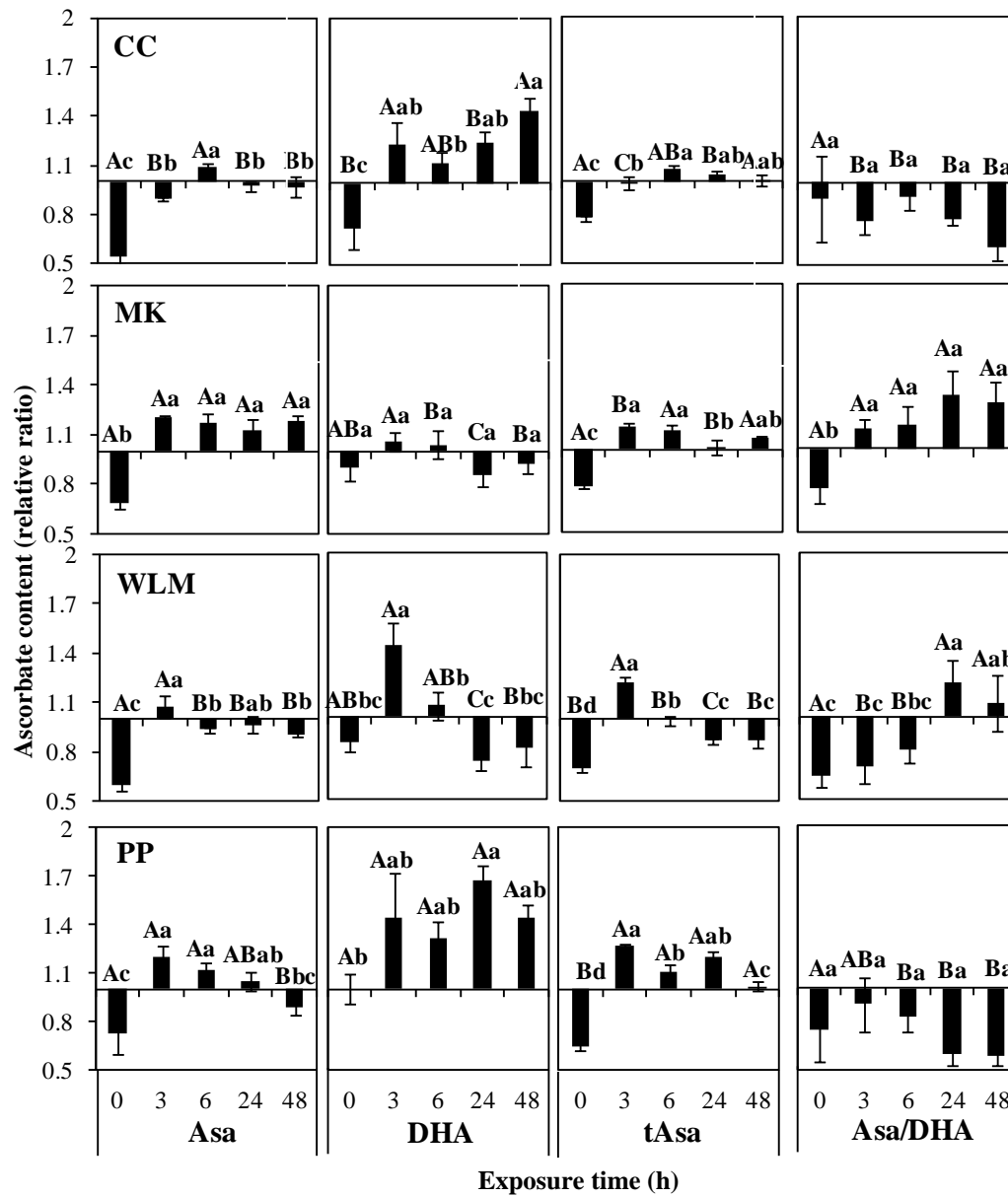
Figure 2



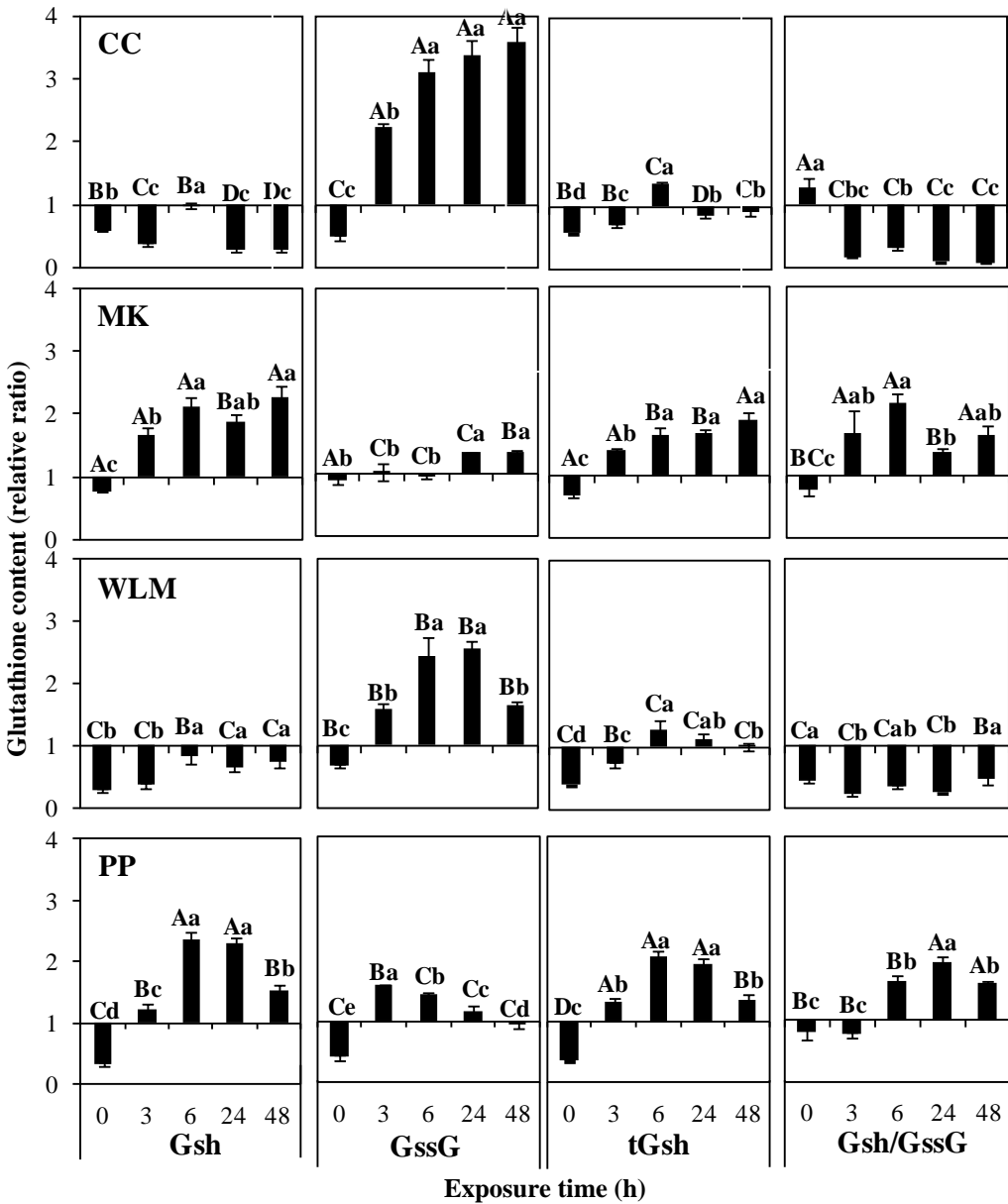
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**

