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1 **Physiological and biochemical response to photooxidative stress of the fundamental**

2 **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.

1. Introduction

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

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

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 (Fv/Fm) (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 (Frohnmeyer 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

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,

6

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 °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 (Pnet) and stomatal conductance (Gs) were made

175   with a portable open gas exchange system (GFS 3000, WALZ, Effeltrich, Germany). Air flow

176   rate was 750 $\mu mol.s^{-1}$. 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 $\mu mol.m^{-2}.s^{-1}$.

178   The use of this LED source ensured a constant, uniform light across all measurements.

179   Carbon dioxide concentration was set at 380 $\mu mol.mol^{-1}$.

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 (Fo) and maximal fluorescence (Fm) 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 Fv/Fm (=

190 (Fm − Fo)/Fm). The degree of photoinhibition was evaluated by the reduction in the value of

191 Fv/Fm.

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 × 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 × 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 × 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) polyvinylpyrolidone (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 μL 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_2O_2. The decrease in absorbance was

240 measured at 240 nm ($\varepsilon = 39.4$ mM^{-1}.cm^{-1}). One unit of CAT was expressed as 1 μmol H_2O_2

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_2O_2 was added. The

246 rate of ascorbate oxidation was evaluated at 290 nm for 3 min ($\varepsilon = 2.8$ mM^{-1}.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 ($\varepsilon = 6.3$ mM^{-}

254 ^{1}.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 ($\varepsilon = 14.5$ mM^{-1}.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 μmol 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 μL 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 ($\varepsilon = 14.15$ mM^{-1}.cm^{-1}). One

267 GR unit was defined as the amount of enzyme that allowed the formation of 1 μmol 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

3.1. Effect of light stress on net photosynthesis (Pnet), stomatal conductance (Gs) and

maximum photochemical efficiency of PSII (Fv/Fm)

288

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

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

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

304

3.2. Effect of light stress on the oxidative status

306

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

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

3.3. Effect of photooxidative stress on the antioxidant system

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

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

~1.5 times higher for MK compared with control), caused essentially by a higher incremental

Gsh concentration (~2 times higher from 6 h compared with control) than in GssG

concentration, which remained very low. Throughout the experiment, a significant increase in

Gsh/GssG compared with the control was observed in response to photooxidative stress in

MK (~2.1-fold increase at 6 h) and PP (~2-fold increase at 24 h). By contrast, CC and WLM

displayed a very marked decrease in the ratio values compared with the control (~−84% and

~−77% at 3 h, respectively), and these values continued to decrease in CC to 48 h, but

remained essentially unchanged along the time course in WLM.

We analyzed the activities of various enzymes acting as ROS scavengers, i.e. SOD, CAT

and APX, or ensuring the supply/regeneration of primary antioxidants, i.e. MDHAR, DHAR

and GR (Fig. 6). For all the genotypes, SOD activity increased rapidly after 3 h of exposure to

photooxidative stress and more intensively in CC, MK and PP. A decline was observed in CC

and WLM at 24 h to reach values equivalent to the control (ratio values close to 1). By

contrast, in MK and PP, SOD remained very active at 48 h (~1.5 times and ~1.3 times higher

than the control, respectively). CC was the only genotype studied with a specific CAT pattern.

From 3 h, CAT activity increased significantly compared with 0 h, but remained depressed

relative to control in the light section (ratio values below 1). In MK and PP, a peak of activity

was observed at 24 h (~1.6 and ~2.2 times higher than control, respectively), whereas this

peak was present at 3 h in WLM, and was followed by a significant loss of activity. Overall,

APX activity was rapidly increased, with a peak at 3 h in all the genotypes. At the end of the

kinetics, the activity became equivalent to control (ratio values close to 1) in MK and PP

whereas in CC and WLM its activity was blocked (ratio values below 1). MDHAR was the

antioxidant enzyme whose activity was the most strongly increased. We observed an early

peak of activity from 3 h in all the genotypes (~3 times higher than control), followed by a

slow decrease up to 48 h except for WLM, where the decrease was very marked from 6 h.

14

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_2

389   concentration and a depression of photosynthesis (Favaretto et al., 2011). Although Pnet

390   followed the same pattern as Gs 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_2 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 *Gs*

395   recovered 48 hours after exposure to full light in all the genotypes, Pnet 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 Pnet and Gs recovery after subsequent drainage, unlike

399   Cleopatra mandarin, a flooding-sensitive rootstock, in which Gs recovered, but Pnet did not.

400   We measured photoinhibition and oxidative damage to investigate potential mechanisms

401   for tolerance to photooxidative stress. PSII maximum quantum efficiency (Fv/Fm) was the

402   best indicator for photoinhibition (Maxwell and Johnson, 2000). Photoinhibition can be

403   considered as a photoprotective process only when decreases in Fv/Fm are slight (Adams et

404   al., 2006). We found that Fv/Fm 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 Fv/Fm 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 Fo in CC and WLM compared to

413 MK and PP (data not shown). An increase in Fo 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 ($OH^{\bullet}$), 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; Bonnecarrere 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_2O_2 and MDA. These results are consistent

489 with their previously observed greater sensitivity. Some authors had already observed a slight

490 increase in these two enzymes in *Hevea* chilling-sensitive clones (Mai et al., 2010) or even a

491 depression in the flooding-sensitive rootstock, Cleopatra Mandarin (Arbona et al., 2008). CC

492 presented the peculiarity of significantly inactivating CAT compared with WLM. Favaretto et

493 al. (2011) and Yang et al. (2012) also respectively reported a decline in CAT activity in

494 pioneer tree species and in magnesium-deficient leaves of 'Sour' pummelo (*Citrus grandis*),

495 probably because this enzyme is light-sensitive and suffers from photoinactivation caused by

496 oxidative damage initiated *via* direct absorption of light by the heme moieties of the enzyme

497 itself (Shang and Feierabend, 1999). It was also postulated that inactivation of CAT could

498 also be mediated by photo-oxidative events initiated through light absorption by chlorophyll

499 (Feierabend and Engel, 1986). Earlier induction of CAT and APX in WLM could explain why

500 this genotype tended to decrease the accumulation of H_2O_2 from 6 h compared with CC, in

501 which these two enzymes were completely inactivated from 6 h. The significant decrease in

502 Gsh/GssG and Asa/DHA ratios suggested that the increased activity of the recycling enzyme

503 was insufficient to produce enough glutathione/ascorbate to regulate the redox status, and that

504 no new synthesis was occurring as previously reported by Yang et al. (2012) in citrus

505 magnesium-deficient leaves or by Arbona et al. (2008) in the flooding-sensitive rootstock

506 Cleopatra mandarin.

507

508 **5. Conclusions**

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

522 **Acknowledgments**

523

528 **References**

529

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

[a]International citrus variety numbering.

Figure captions

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

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

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

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

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

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

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

Fig. 6. Time course of changes in antioxidant enzyme specific activities (SOD, CAT, APX, MDHAR, DHAR, GR) in leaves of four citrus genotypes during photooxidative stress. The results are expressed as ratios relative to control values. Leaves acclimated under shade conditions for one week were suddenly exposed to full light conditions. Activities were assayed just before exposure to full light (0h), and 3, 6, 24 and 48 h after full light exposure. The results are expressed as ratios relative to the values obtained on control leaves acclimated to full light conditions. All data are presented as mean values (±S.E.) of six independent measurements ($n = 6$). Data were analyzed using ANOVA and Fisher LSD tests ($P < 0.05$). Different upper case letters indicate significant differences between genotypes at a point of the time course and different lower case letters indicate significant differences along the time course for one genotype. See the Table 1 for abbreviations information.
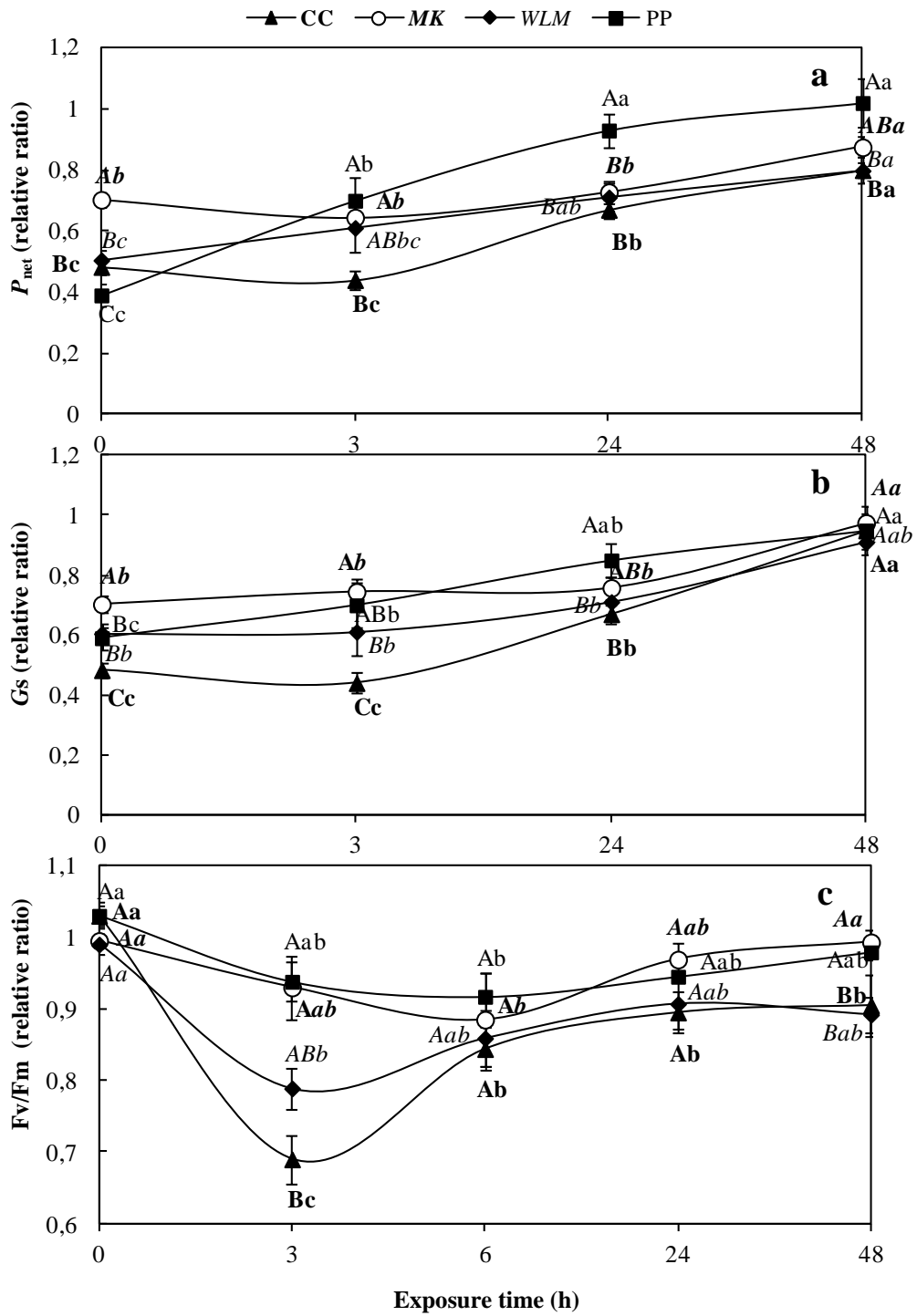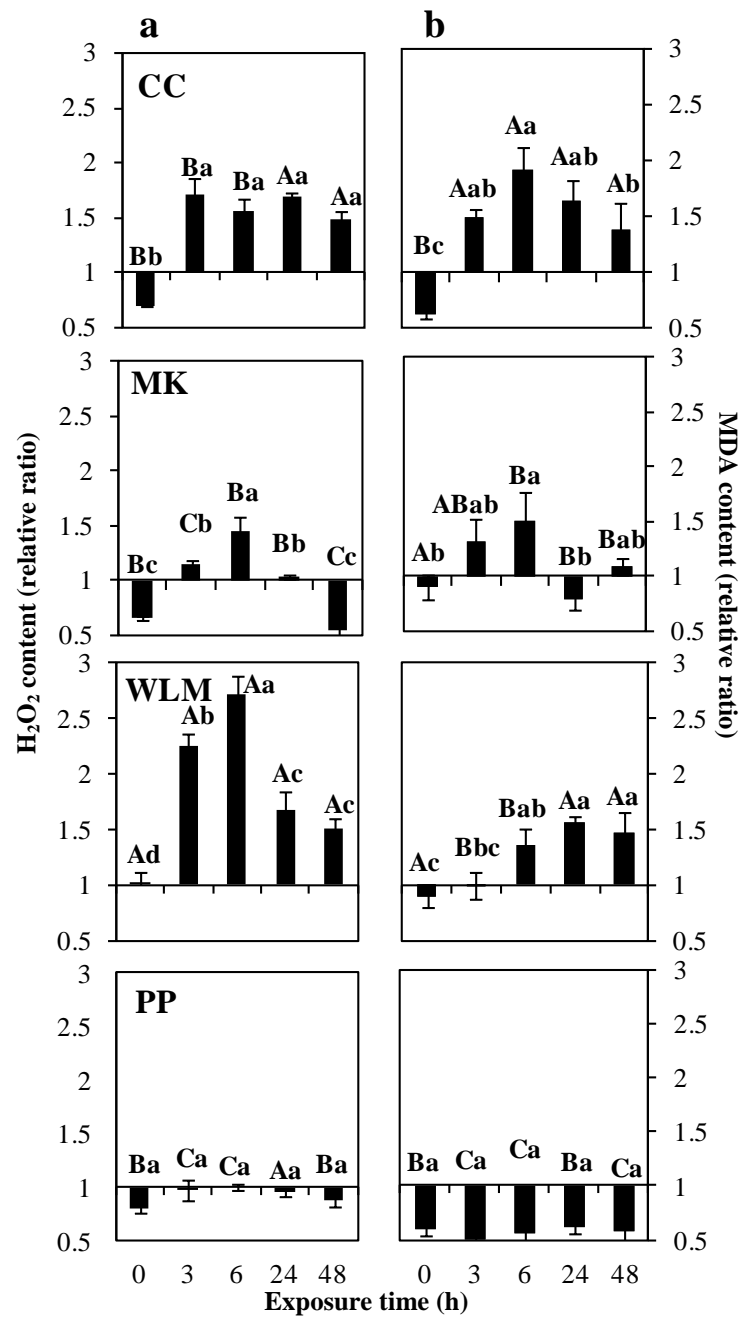
Figure 1
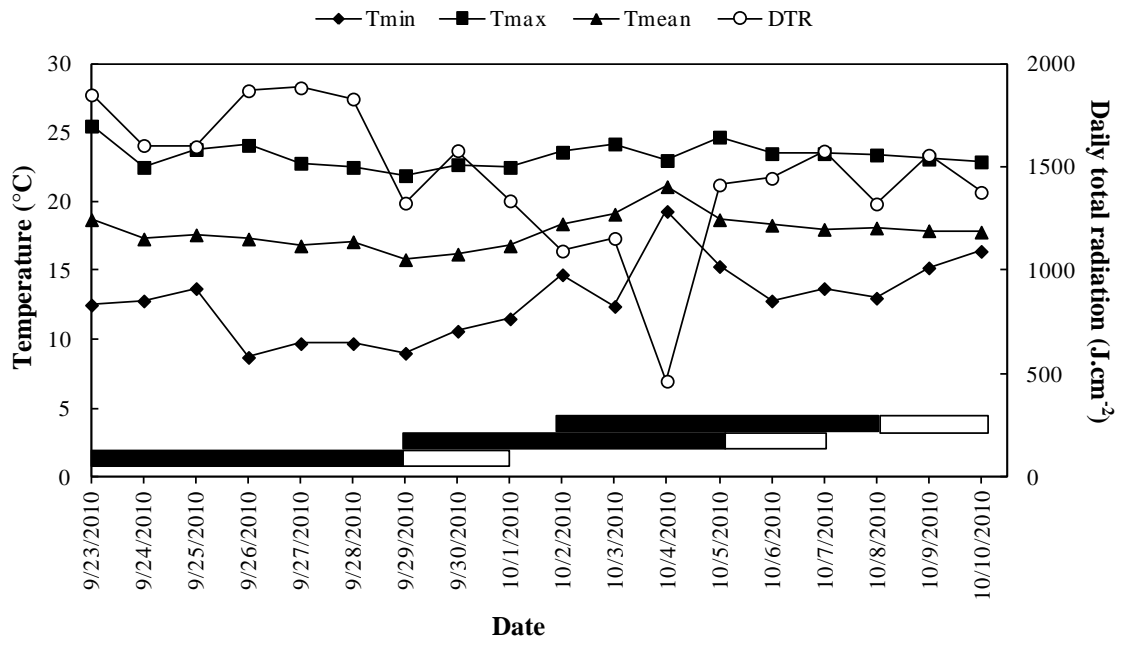
Figure 2

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Figure 4

Figure 5

Figure 6