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Comparison of photosynthesis and antioxidant performance of several *Citrus* and *Fortunella* species (Rutaceae) under natural chilling stress

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Abstract Citrus plants originate from southeastern Asia, in a large area with various climates characterized by a broad range of temperatures. Some species have been diversified in temperate climates, others in subtropical climates. Temperature is assumed to be a key factor in citrus species adaptation and diversification of basic cellular functions. In a field experiment, the tolerance of the three fundamental *Citrus* species *C. medica* L., *C. reticulata* Blanco and *C. maxima* (Burm.) Merr., and *Fortunella japonica* (Thunb.) Swing. to photooxidative stress caused by seasonal climatic changes was evaluated on adult trees by measuring net photosynthesis (P_{net}), stomatal conductance (G_s), maximum photosynthesis (P_{max}) and chlorophyll fluorescence (F_v/F_m). In addition, seasonal changes in oxidative status, antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase) and antioxidant metabolites (ascorbate and glutathione) were monitored. Mandarin and pummelo appeared to be the most tolerant, showing the lowest down-regulation of photosynthetic parameters, and the lowest accumulation of oxidized compounds associated with efficiency of their antioxidant system. Kumquat showed intermediate behaviour, with a large diminution of photosynthetic parameters and marked accumulation of hydrogen peroxide, whereas the malondialdehyde content remained low, with a strong induction of glutathione synthesis. Finally, citron appeared to be the most sensitive genotype with a marked decrease in photosynthetic performance, the largest accumulation of oxidative parameters, insufficient induction of antioxidant enzymes and down-regulation of ascorbate and glutathione synthesis.

Keywords Antioxidant enzymes, Antioxidant metabolites, Citrus, Oxidative status, Photosynthetic parameters, Seasonal climatic changes

Introduction

The citrus plants belong to the subtribe Citrineae of the tribe Citreae in the subfamily Aurantioideae of the Rutaceae family (Webber et al. 1967). Strictly, true citrus plants comprise six genera: *Clymenia*, *Eremocitrus*, *Microcitrus*, *Poncirus*, *Fortunella* and *Citrus* (Swingle and Reece 1967). Scora (1975) and Barrett and Rhodes (1976) considered that *Citrus medica* L.(citron), *C. maxima* (Burm.) Merr. (pummelo) and *C. reticulata* Blanco (mandarin) were the three fundamental species of *Citrus*. Nicolosi et al. (2000) added a fourth species, *C. micrantha* Wester, involved in the genesis of the lime (*C. aurantifolia* (Christm.) Swing.). Recently, this addition has gained support from various biochemical and molecular studies (Federici et al. 1998; Nicolosi et al. 2000; Barkley et al. 2006; Fanciullino et al. 2006). *Citrus*, *Poncirus* and *Fortunella* are believed to have their primary center of origin in south and southeastern Asia. In particular, *C. medica* originates from an area covering northeastern India, Burma, and western China; *C. reticulata* from Vietnam, southern China, and Japan; *C. maxima* from the tropical region of Malaysia and Indonesia; *Poncirus* from Chinese provinces close to the Himalayas and *Fortunella* from southeastern China (Swingle and Reece 1967; Scora 1975; Gmitter and Hu 1990; Mabberley 2004). This allopatric evolution has resulted in strong genetic and also phenotypic differentiation between these *Citrus* taxa (Garcia-Lor et al. 2012). Thus each species had initially to develop specific physiological and biochemical determinants, depending on climatic conditions in these areas of origin, that allowed their cultivation in different parts of the world, particularly at the northern limit of Mediterranean agriculture.

The Mediterranean climate, with its characteristic seasonality, provides a useful opportunity to study the adaptation of these species relative to their climate areas of origin. 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.

Low temperatures can cause a number of physiological and biochemical dysfunctions to occur, such as a decrease in enzymatic activities participating in the Calvin cycle and reduced photosynthetic activity (Sevillano et al. 2009). Cold temperatures during winter inhibit the enzymatic reactions underlying CO₂ fixation, but do not affect the light absorption ability of many overwintering evergreens such as citrus (Allen and Ort 2001; Ribeiro and Machado 2007). This results in photoinhibition or photooxidative damage or both (Oquist and Huner 2003). Such dysfunction has been found in citrus plants in other environmental conditions (Veste et al. 2000; Medina et al. 2002; Jifon and Syvertsen 2003), although the genetic response of true citrus species to chilling conditions is unknown.

Cold temperatures reduce CO₂ availability and may decrease the CO₂/O₂ ratio in chloroplasts (Foyer and Noctor 2000). This condition facilitates electron flow to molecular oxygen (O₂) and superoxide anion (O₂^{•-}) production by the Mehler reaction (Foyer and Noctor 2003). Overproduction of ROS in plant cells under stress can damage cell components, including DNA, proteins and membrane lipids (Mittler 2002).

Plants have evolved efficient antioxidant systems that can protect them from the damaging effects of oxidative stress (Asada 1999) even at low temperatures induced by seasonal climatic changes (Polle and Rennenberg 1992; Verhoeven et al. 2005; Wang et al. 2009). These antioxidant mechanisms employ (i) ROS-scavenging enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, 1.11.1.11), (ii) recycling enzymes of ascorbate-glutathione cycle such as monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and

glutathione reductase (GR, EC 1.6.4.2) and (iii) low molecular weight antioxidants, such ascorbic acid (Asa) and glutathione (Gsh).

In this study, seasonal climatic changes were used to analyze the response to chilling conditions of four ancestral species of *Citrus* and *Fortunella* originating from dispersed native areas in southeastern Asia, and selected for their ranging behaviour with respect to freezing injuries. Although it is considered to be very tolerant to freezing temperatures, the trifoliolate orange (*Poncirus trifoliata* (L.) Raf.) was not included in this study because, unlike other citrus species, it is deciduous in winter. This behaviour is a form of physiological adaptation to low temperatures but lies outside our study. Among the evergreen citrus species, kumquat (*Fortunella*) is thought to be the most tolerant to freezing temperatures (can withstand -15°C), while citron is considered to be one of the most vulnerable. Pummelos, diversified in tropical regions, are also known to be vulnerable to negative temperatures and mandarins to be more tolerant (Yelenosky 1985). This sample of citrus species was intended to be representative of different physiological reactions when evergreen citrus trees are exposed to chilling conditions. The aim of this study was to investigate the effect of broader seasonal temperature variation on citrus photosynthesis regulation and protective mechanisms against oxidative stress.

Materials and methods

Plant material and growth conditions

Experiments were carried out on leaves from 8-year-old trees of 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 ($42^{\circ} 18' 55'' \text{N}$, $9^{\circ} 29' 29'' \text{E}$; 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). Trees were spaced at $6 \times 4 \text{ m}$ and were subjected to homogenous 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 Penman-Monteith (1965). Fertilizers were supplied and insects and diseases controlled according to the recommendations of the local department of agriculture.

The experiment in orchards was conducted on 20–21 September 2010 (ambient air temperature 25°C) and in January 2011 (ambient air temperature 6°C) on one genotype of each of the three basic true species of the *Citrus* genus and on one genotype of the *Fortunella* genus (Table 1). Average daily minimum air temperatures for each of the months from July to January were 18.6°C , 16.9°C , 13.8°C , 10.9°C , 6.6°C , 4.1°C and 4.6°C , respectively. Average daily global radiation for each of the months from July to January at this location was 2559, 2157, 1725, 942, 685, 546 and $579 \text{ J}\cdot\text{cm}^{-2}$. For each genotype, three individual trees growing in the same field were used. For physiological parameter measurements, the same leaves were used. On each tree, three fully expanded leaves from spring of the current year's growth were selected in September (end of summer, warm period) and January (winter, cold period). Thus nine measurements per genotype were made on sunny mornings of each season (between 08:00h and 12:00h). For biochemical assays, on each of the three trees of a genotype, two samples of 15 fully expanded leaves from the current year's growth were collected on sunny mornings (between 08:00h and 12:00h) of each specific sampling date and immediately frozen in liquid nitrogen and stored at -80°C . Thus six samples of 15 leaves were separately analyzed for each genotype and for each season.

Before analysis, each leaf sample was ground to a fine powder in liquid nitrogen using a pre-chilled pestle and mortar.

The experiment under controlled conditions was performed on detached leaves from the same trees of each genotype (Table 1) in September 2011. On each tree, two fully expanded leaves of the current year's growth were collected on sunny mornings. Thus six leaves per genotype were collected and incubated independently in closed Petri dishes containing tissue paper moistened with distilled water. Petri dishes were then transferred for 72 hours before cold treatment to a growth chamber in which the temperature was set at 25 °C (equivalent to orchard ambient air temperatures during September experiments). The cold treatment was imposed by shifting the temperature of the growth chamber to 6 °C (equivalent to orchard ambient air temperatures during January experiments), 3 h after the beginning of the day period and for a period of 72 h. A similar experiment was performed on detached leaves maintained at 25 °C for at least 168 h to check the absence of changes in *P*_{net}. The relative humidity was maintained at 85% for both temperature conditions. The photoperiod was 16 h and photon flux density was provided by cool fluorescent lamps (350 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$). Three measurements per genotype were made at each temperature. Air temperature, relative humidity and light level were continuously monitored at 1 h intervals in growth chambers throughout the entire experiment.

Gas exchange measurements

Measurements of net photosynthetic rate (*P*_{net}), stomatal conductance (*G*_s) and maximum photosynthetic rate (*P*_{max}) were determined with a portable open gas exchange system (GFS 3000, WALZ, Effeltrich, Germany). Air flow rate was 750 $\mu\text{mol.s}^{-1}$. In a gas exchange chamber, photosynthetic photon flux density (PPFD) was controlled using a LED radiation source and was fixed at a PPFD of 1400 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ for *P*_{net} and *G*_s or at a saturated PPFD of 2000 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ for *P*_{max}. The use of this LED source ensured a constant, uniform light across all measurements. Carbon dioxide concentration was set at 380 $\mu\text{mol.mol}^{-1}$ for *P*_{net} and *G*_s measurements and at 2000 $\mu\text{mol.mol}^{-1}$ for *P*_{max} measurements.

Chlorophyll a fluorescence measurements

In vivo chlorophyll fluorescence was measured on sunny days on the same leaves previously used for gas exchange measurements using a portable chlorophyll fluorometer (Hansatech, Norfolk, England). Intact leaves were dark-adapted with leaf clips for 20 min to allow relaxation of fluorescence quenching associated with thylakoid membrane energization (Krause et al. 1983). Minimal fluorescence (*F*_o) and maximal fluorescence (*F*_m) were obtained by imposing a 1 s saturating flash to reduce all the PSII reaction centers. The maximum potential photochemical efficiency of PSII was expressed as the ratio *F*_v/*F*_m ($= (F_m - F_o)/F_m$). The degree of photoinhibition was evaluated by the reduction in the value of *F*_v/*F*_m. Seasonal data were collected on sunny mornings at 08:30h for each month (ambient air temperature respectively 16.4 °C and 5.3 °C).

Measurement of H₂O₂ and MDA

H₂O₂ levels were measured following the protocol described by Zhou B. et al. (2006). For extraction, 200 mg of frozen leaf powder was homogenized in 3 mL of trichloroacetic acid (TCA) 5% (w:v) containing 60 mg of activated charcoal. The mixture was then centrifuged at 5000 × g for 20 min at 4 °C.

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

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

Assay of antioxidant enzymes activities

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

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured using a modified method of Oberley and Spitz (1984). 100 µL of diluted extract was added to a solution containing 1 mM DETAPAC buffer (pH 7.8), 1.25 units of catalase, 0.07 mM NBT, 0.2 mM xanthine and 0.010 units of xanthine oxidase in a total volume of 1.0 mL. One unit of SOD was defined as the amount of enzyme causing 50% inhibition of the rate of NBT reduction at 560 nm, at 25 °C.

Catalase (CAT, EC 1.11.1.6) activity was measured according to the method of Aebi (1984). The reaction mixture (1.1 mL) contained 100 µL of crude enzyme extract, 37.8 mM sodium phosphate buffer (pH 7.0) and 4.4 mM H₂O₂. The decrease in absorbance was measured at 240 nm ($\epsilon = 39.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). One unit of CAT was expressed as 1 µmol H₂O₂ degraded per min at 25 °C.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to a modified method described by Asada (1984). The standard reaction mixture (1.0 mL) contained 0.17 mM ascorbate, 33 µL of crude enzyme extract in a 60.3 mM potassium phosphate buffer (pH 7.0). The reaction was triggered when 4.95 mM H₂O₂ was added. The rate of ascorbate oxidation was evaluated at 290 nm for 3 min ($\epsilon = 2.8 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). One unit of APX was expressed as the oxidation of 1 µmol ascorbate per min at 25 °C.

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

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

with 0.5 mM DHA added to initiate the reaction. One DHAR unit was defined as the amount of enzyme that allowed the formation of 1 μ mol ascorbate per min at 25 °C.

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

Assay of antioxidant metabolites

Total ascorbate (tAsa) and reduced ascorbate (Asa) contents were measured according to the method of Gillespie and Ainsworth (2007). For extraction, 40 mg of frozen leaf powder was homogenized in 2.0 mL of a 6% (w/v) TCA solution and centrifuged at 13,000 \times g for 5 min at 4 °C.

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

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

Statistical analyses

The experimental designs were split-plot with genotype as the main plot and season (or temperature for the second experiment) as the subplot. Differences between genotypes and seasons were analyzed with two-way ANOVA and comparisons between means were made with the least significant difference (LSD) test at $P < 0.05$ using R statistical software (<http://www.R-project.org>).

Results

Seasonal changes in *P*_{net} and *G*_s

Responses of *P*_{net} and *G*_s for the four species were established on 20–21 September 2010 (warm period) and 4–5 January 2011 (cold period) (Fig. 1; Table 2). Based on warm period results, several differences could be observed on the photosynthetic behaviour of the different varieties studied, CC and MK having the highest *P*_{net} and *G*_s and WLM and PP having the lowest *P*_{net} and *G*_s (Fig. 1a, b). In the cold period, the reduction in *P*_{net} and *G*_s was significantly greater in CC and MK compared with the warm period (\sim 64 % and \sim 78% for *P*_{net}, respectively; \sim 53 % and \sim 67% for *G*_s, respectively) than in WLM and PP (\sim 25% and \sim 31% for *P*_{net}, respectively; \sim 34% and \sim 33% for *G*_s, respectively) (Fig. 1a, b).

Comparison of *P*_{net} under orchard climatic conditions and controlled conditions

To check that seasonal changes observed in the P_{net} values under orchard conditions were due to temperature variations and not to leaf senescence, we also measured the previous parameter in the same genotypes under controlled conditions at 6 °C and 25 °C. Comparing the P_{net} values measured under controlled conditions in September 2011 at the same temperature as those obtained in September 2010 (Fig. 2a) and January 2011 (Fig. 2b) in the orchards, no significant difference was found, whatever the genotype, between the two mean values for each treatment (attached leaves vs. detached leaves). Likewise, no significant variation was observed for G_s between attached and detached leaves (data not shown).

Seasonal changes in photosynthetic traits

F_v/F_m was lower in the cold period for all genotypes, especially when temperatures were close to 0 °C (Fig. 3a; Table 2). The leaves of CC and MK had significantly lower F_v/F_m compared with the warm period (~25% and ~22%, respectively) and were consequently more photoinhibited than leaves of WLM and PP (~15% and ~10%, respectively).

P_{max} decreased significantly in the cold period in all the genotypes (Fig. 3b; Table 2), and this reduction was greater in CC and MK (~53% and ~73%, respectively) than in WLM and PP (~39%).

Seasonal changes in the oxidative status and antioxidant system

H_2O_2 and MDA content increased in all the genotypes from the warm to the cold period (Figs. 4a, b; Table 2), indicating that all the genotypes were subjected to oxidative stress. CC and MK maintained relatively higher levels of leaf H_2O_2 than WLM and PP in the cold period. CC showed higher basal values of MDA in the warm period and in the cold period, indicating a higher lipid peroxidation level. In addition, in the cold period, its values reached a very high level compared with the other genotypes and especially MK (~3 times higher).

We analyzed the activities of various enzymes acting as ROS scavengers, i.e. SOD, CAT and APX (Fig. 5; Table 2) or ensuring the supply/regeneration of primary antioxidants, i.e. MDHAR, DHAR and GR (Fig. 6; Table 2). Highly variable patterns of enzymatic activity were observed among genotypes.

SOD activity increased slightly in all genotypes from the warm to the cold period (Fig. 5a), from ~1.12 times higher for PP to ~1.27 times higher for MK. Basal CAT levels were higher in CC and MK (54.4 and 39.1 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein, respectively) than in WLM and PP (13.2 and 19.2 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein, respectively). All the genotypes studied significantly depressed CAT activity in response to cold climatic conditions (Fig. 5b). The loss of activity was more substantial in the leaves of CC, MK and WLM (~45%, ~54% and ~65%, respectively) than in PP (~24%). APX activity was enhanced significantly in all the citrus genotypes from the warm to the cold period (~2.4-, ~1.75- and ~1.77-fold increase for CC, MK and PP, respectively) except for WLM, where no significant changes were found (Fig. 5c).

Seasonal changes had a significant effect on MDHAR activity in MK and PP (~2.9 and ~1.6 times higher in the cold than in the warm period, respectively). No significant difference for CC and WLM was found (Fig. 6a). However, MDHAR activity in WLM was similar to PP and MK in the cold period and significantly higher than in CC. Considering DHAR activity, the increase between the warm and the cold period was identical whatever

the genotype concerned (from ~1.21- to ~1.59-fold increases for PP and CC, respectively) (Fig. 6b). The pattern of regulation of GR differed according to genotype. Whereas WLM showed a slight diminution (~17%) in the cold period, CC and MK showed significantly contrasting increases (~2.1 and ~1.4 times higher, respectively) (Fig. 6c). GR activity in PP showed no significant difference between the two periods, but its level was similar to CC and MK in the cold period. Overall, WLM and PP had significantly higher basal levels of the three recycling enzymes of the ascorbate-glutathione cycle during the warm period than CC and MK.

In the cold period conditions, leaves of the four studied varieties accumulated significantly higher levels of tAsa, Asa and DHA than in the warm period conditions (Table 2, Table 3). For tAsa and DHA, a significant separation of MK had been observed with larger increases (~1.78 and ~2.84 times higher, respectively) than the other genotypes (except CC for tAsa) in which the enhancements were equivalent. No greater increase in one genotype compared with another was found for Asa. Leaf redox Asa/DHA ratio decreased for all genotypes from the warm to the cold period. A significant sharp decrease was observed in MK (~57%) due to a larger amount of DHA which unbalanced redox status. In other genotypes, the decline was much lower (~29% in CC, ~27% in WLM and ~19% in PP). However, only CC exhibited a value less than 1. tGsh, Gsh and GssG contents were always higher in the cold period (Table 2, Table 4). MK showed significantly higher increases than other genotypes, particularly for tGsh and Gsh (~3.53 and ~4.43 times higher than warm period values). GssG, the oxidized form of glutathione, accumulated more intensively in CC and MK (~2.58- and ~2.70-fold increases, respectively) than in WLM and PP. However, a different pattern of regulation of the redox status was observed. Whereas MK showed a significant increase in Gsh/GssG ratio (~1.72 times higher compared with the warm period), the ratio remained unchanged in WLM and PP and was depleted in CC (~40% compared with the warm period).

Discussion

The successive cellular events that occur in chilling conditions have often been investigated in different species under controlled conditions (Lim et al. 2009; Bonnacerrere et al. 2011). The main studies have been conducted on deciduous species in which cold treatments were applied abruptly in a growth chamber, with no transition period and under conditions of constant growth. Also, there is still a debate on how best to chill sensitive plant species to model cold temperatures. Here we investigated the effect of seasonal climatic changes on the photosynthesis and antioxidant system in evergreen varieties from ancestral species of citrus in a marginal zone of their areas of origin. We found ranging chilling response among the different citrus genotypes studied.

Seasonal changes in photosynthetic components and photoinhibition

Citrus species have generally a low temperature threshold of around 13 °C, with photosynthetic metabolism being severely disrupted at lower temperatures (Ribeiro and Machado 2007). During the experimental period, minimum air temperature fluctuated around 15 °C in September and around 4.5 °C in January. Thus measurements carried out in September 2010 could correspond to the regular photosynthesis of citrus given their areas of origin. Net photosynthetic rate (P_{net}), stomatal conductance (G_s), chlorophyll fluorescence (F_v/F_m) and maximum photosynthetic rate (P_{max}) measurements have been commonly used to investigate the effect of

seasonal climatic changes on the functioning of the photosynthetic system (Maxwell and Johnson 2000; Oquist and Huner 2003). Under warm conditions, photosynthesis of MK, WLM and PP were clearly stomata-controlled, and the interspecific dissimilarities appeared to be mainly due to differences in stomatal aperture. However, photosynthesis of CC might be either stomata-controlled or limited by the external CO₂ concentration alone (380 $\mu\text{mol.mol}^{-1}$). The lower photosynthetic activity observed in all the genotypes in the cold period might be explained by both a closure of stomata and low temperature (Figs. 1, 3). According to Allen and Ort (2001), the decline in photosynthesis during chilling stress in warm-climate plants is caused by stomatal closure, which compromises gas exchange and CO₂ fixation, and the loss of enzymatic activities participating in the Calvin cycle. Low temperature, which occurs at night in both air and soil, is therefore an environmental factor that affects plant physiology (Allen and Ort 2001). In ‘Valencia’ sweet orange, Ribeiro et al. (2009) had previously observed, during seasonal climatic changes, that lower temperatures led to a decrease in stomatal conductance with a drop in photosynthetic activity. A decrease in CO₂ assimilation linearly with stomatal conductance reduction had already been observed during other abiotic stress such as drought stress in ‘Swingle’ citrumelo (de Campos et al. 2011), salt stress in Cleopatra mandarin (Anjum 2010) and chilling stress in ‘Valencia’ sweet orange (Santos et al. 2011). Seasonal results of *P*_{net} were confirmed by our experiments conducted on detached leaves at 6 °C. *P*_{net} measurements obtained on detached leaves suggested that the temperature sensitivity of Calvin cycle enzymes was probably responsible for the drop in photosynthesis (Holaday et al. 1992). It was therefore possible to exclude an effect of leaf senescence in orchards from September to January. Although trifoliate orange rootstock is known for its relative resistance to cold compared with Volkamer lemon (Yelenosky 1985), these measurements on detached leaves also enabled us to exclude an effect of rootstock on the photosynthetic parameters estimated in the orchard, as no variation was observed between attached and detached leaves. The decline of photosynthesis at low temperature has been previously observed in “Valencia” sweet orange placed in a growth chamber where air temperatures were similar to a typical winter day under a Mediterranean climate (Santos et al. 2011). Thus the greater decrease in *P*_{net}, *G*_s, *F*_v/*F*_m and *P*_{max} in CC and MK than in WLM and PP could suggest a greater sensitivity to cold, as previously shown in *Hevea*, another chill-sensitive species (Mai et al. 2010). This implies that leaves become progressively less efficient at processing the incident photon flux, possibly related to an increased proportion of closed, reversibly inactivated or destroyed photosystem II reaction centers resulting from enhanced ROS accumulation (Grover et al. 1986). During waterlogging stress, Arbona et al. (2009) demonstrated that Cleopatra mandarin, a flooding-sensitive rootstock, exhibited severe reductions in *P*_{net}, *G*_s and *F*_v/*F*_m, while Carrizo citrange, a flooding-tolerant rootstock, showed only small differences from control. Conversely, during salt stress, Brumos et al. (2009) established that Cleopatra mandarin, a salt-tolerant rootstock, exhibited a greater decrease in these three parameters than the salt-sensitive Carrizo citrange. They showed that Cleopatra mandarin induced greater stress responses in gene expression while repressing central metabolic processes such as photosynthesis. For instance, different chitinases, involved in defense responses, were strongly induced in Cleopatra mandarin and repressed in Carrizo citrange. Thus studying the response of physiological parameters is a first step in the evaluation of tolerance or sensitivity that must be extended by a biochemical analysis of antioxidant systems.

Leaf antioxidant systems and photoprotection

Excitation energy not used for photochemistry can be transferred to O₂, resulting in the production of ROS such as superoxide anion (O₂^{•-}) and hydrogen peroxide (H₂O₂). The increased level of H₂O₂ in winter, particularly in CC and MK, could be due to higher superoxide radical dismutation in the presence of a reductant or elevated synthesis and (or) decreased activity of CAT and (or) APX. It is noteworthy that leaf H₂O₂ content increased linearly with decreasing CO₂ assimilation in all the genotypes. The changes in MDA content are an indicator of lipo-peroxidation caused by ROS. The larger increase in this parameter in CC is consistent with its probably greater sensitivity and vulnerability to photooxidation previously noted. In addition, the lower accumulation of MDA content in MK suggests an efficient scavenging of ROS. There may also be a putative relationship between lower H₂O₂/MDA levels and higher tolerance to stress. The occurrence of an H₂O₂ and MDA burst had previously been observed in citrumelo under waterlogging stress (Hossain et al. 2009), and in two rice genotypes under low temperature stress (Bonnecarrere et al. 2011).

To cope with elevated levels of ROS, aerobic cells have evolved a range of enzymatic and non-enzymatic antioxidant reactions. In several studies, the antioxidant enzyme response as indicative of tolerance or sensitivity has been explored, and has shown a direct relationship between increased antioxidant activity and stress tolerance (Arbona et al. 2008; Hossain et al. 2009). Specific modifications to the enzymatic complement of cells overcome the metabolic challenge of cold temperatures *in vivo*. For instance, the cell can (i) produce more enzymes to maintain adequate activity, (ii) increase the activation state or (iii) generate new isozyme forms with enhanced catalytic function (Guy 1999). Among antioxidant enzymes, SOD forms the first line of defense against ROS and is located in many subcellular compartments (Gill and Tuteja 2010). Our results show an increase in SOD activity in all the genotypes with the same pattern as previously shown in citrumelo, Carrizo citrange and Cleopatra mandarin under waterlogging stress (Arbona et al. 2008) or in *Rhododendron* species under chilling stress (Wang et al. 2009). According to its cellular localization, H₂O₂ produced during SOD reaction can then be metabolized into oxygen and water by CAT in peroxisomes (Foyer et al. 1994) or by APX using Asa as electron donor in the chloroplasts, mitochondria and cytosol (Foyer and Noctor 2000). Unexpectedly, CAT decreased in all the genotypes studied as previously reported in ‘Sour’ pummelo and ‘Xuegan’ orange grown under magnesium-deficiency by Yang et al. (2012). The decline in CAT activity could be a consequence of its sensitivity to light and its low temperature lability (Hertwig et al. 1992). Its photoinactivation can also be caused by oxidative damage initiated in the chloroplast *via* direct absorption of light by the heme moieties of the enzyme itself (Shang and Feierabend 1999). These data suggest that the reduced CAT activity observed at low temperatures during January can account for accumulation of ROS such as H₂O₂. APX isoforms were induced to compensate for deficiencies in CAT activity (Palatnik et al. 2002). In this study, the increase in total APX activity did not compensate for the impaired CAT activity, as H₂O₂ accumulated as previously reported in *Hevea* during chilling stress (Mai et al. 2010) or in citrumelo during waterlogging stress (Hossain et al. 2009). However, it could be sufficient to prevent a lethal accumulation of H₂O₂ if we consider the lower MDA accumulation in MK, WLM and PP compared with CC. These results are consistent with previous reports on two flooding-tolerant rootstocks (Arbona et al. 2008).

The antioxidants Asa and Gsh are crucial for plant protection against oxidative stress (Noctor and Foyer 1998). It is believed that the redox status of these metabolites is essential for the proper scavenging of ROS in cells and it is therefore related to genotype tolerance. The ratio between reduced and oxidized forms of ascorbic acid is controlled by two enzymes, including monodehydroascorbate reductase (MDHAR) and dehydroascorbate

reductase (DHAR), which hold the pool of reduced ascorbic acid. High significant levels of MDHAR in MK, WLM and PP compared with CC might be sufficient to maintain the ratio above 1 in winter. However, in CC, improving only DHAR activity did not prove sufficient to fight against chilling stress, as the ratio was below 1. Thus the levels of MDHAR suggest that this enzyme may help to explain the differences in sensitivity to seasonal climatic changes. These results are in close agreement with experiments conducted on tomato fruits, where a positive correlation was observed between MDHAR activity and ascorbic acid content under chilling stress (Stevens et al. 2008). Consequently, to recycle ascorbic acid efficiently during chilling stress, an increased activity of both MDHAR and DHAR seem to be required. Generally, precise metabolic tuning of GR allows the cell to maintain the favourable Gsh/GssG ratio for cellular redox regulation. Despite a higher increase in GR activity and higher Gsh content in CC than in other genotypes, this increment might be insufficient to recycle Gsh pool efficiently, as the decrease in the ratio was very marked in the cold period. For the other genotypes, the maintenance or significant increase of Gsh/GssG ratio could additionally suggest *de novo* glutathione synthesis (Queval et al. 2007). Previous observations agree with those of Arbona et al. (2008), who demonstrated that during waterlogging stress, the most tolerant genotype, Carrizo citrange, was able to keep active forms of Asa and Gsh, while the most sensitive genotype, Cleopatra mandarin, found these responses impaired. Queval et al. (2007) demonstrated that in the *Arabidopsis* photorespiratory mutant *cat2*, glutathione appeared as a key modulator of H₂O₂ concentration. These results are consistent with the lowest accumulation of MDA in WLM, PP and MK. The higher basal levels of the three recycling enzymes in WLM and PP may explain, in part, the lower accumulation of stress indicators and oxidized forms of antioxidant metabolites observed during the warm period (essentially H₂O₂, MDA, DHA and GssG). Also, the strong production of H₂O₂ in MK and its strong ability to reduce cellular damage could be explained by an effective regulation of Asa-Gsh cycle and especially by the glutathione pool.

Relationships between genotype tolerance to cold photooxidative stress and geographical origins

As no information regarding the chilling behaviour of these genotypes was available in the literature, the extent of the decline in photosynthetic parameters, the accumulation of oxidative compounds and the increase in antioxidant performance in cold period allowed us to classify three of the four genotypes studied as 'sensitive' or 'tolerant'. Considering our results, PP and WLM showed the lowest loss of photosynthesis and photochemical performances, the lowest accumulation of oxidative stress indicators, and the effective activation of antioxidant defenses, mainly APX and glutathione (especially in PP). Hence these can be considered as the genotypes most tolerant to cold photooxidative stress. CC showed a large decrease in photosynthetic and photochemical parameters, a marked accumulation of H₂O₂ and MDA and an antioxidant system that responded to stress, but not effectively. This genotype can be classified as the one most sensitive to cold photooxidative stress. MK showed special behaviour with a large decrease in photosynthetic and photochemical parameters, a marked accumulation of H₂O₂ but not of MDA, and an efficient recycling of glutathione.

These conclusions are only partially in line with the hypothesis of a link between the geographical origins of the analyzed citrus species and the tree tolerance to chilling. The subtropical diversification area of citron could explain their greater sensitivity. The most northern origin of kumquat, and to a lesser extent of mandarin, could explain their greater adaptation to cold. However, by this argument, the tropical origin of pummelo should not

have favoured its adaptation to chilling. This theoretical classification, based on origin areas, has been noted by some authors who have monitored leaf freezing tolerance (LFT) (Yelenosky 1985; Yelenosky and Guy 1989). This parameter is generally used to highlight the very important process in plants known as cold acclimation (CA). CA involves changes in gene expression and in many cellular processes that result in modified metabolic and biosynthetic pathways enabling tissues to survive cold or freezing conditions (Guy 1990). For example, pummelo, classified by Yelenosky (1985) as cold sensitive, can cold-acclimate to survive freezing at -4°C or -5°C (Moore et al. 2000). Thus differences in classification of pummelo between geographical origins and our results could also be explained by an efficient cold acclimation process in this genotype. Additionally, photosynthetic traits and antioxidant metabolism are sensitive to many different abiotic stresses, thereby also confounding the relationship between genotype tolerance to a specific stress and geographical origins.

In conclusion, the comparison of physiological and biochemical parameters carried out on genotypes from varying areas of origin and belonging to the three basic true species of *Citrus* and to genus *Fortunella* highlighted contrasting behaviours towards cold photooxidative stress induced by seasonal climatic changes. Four conclusions may be drawn: (i) the response of photosynthesis to photooxidative stress is strongly influenced by genetic factors, (ii) lower *Fv/Fm* values can be related to a greater sensitivity of genotypes, (iii) APX activity may be insufficient in sensitive genotypes to scavenge all H_2O_2 produced in excess and (iv) MDHAR and the redox status of Gsh and Asa appear crucial in preventing cell oxidative damage in cold resistant citrus genotypes. Despite their known sensitivity to cold, our results show different cold tolerance capabilities that could be exploited in breeding programs. Taking the present work as a reference, it is now possible to investigate and screen the cultivated citrus genotypes for their performance under a Mediterranean climate or in other area with cold periods.

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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	<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

^aInternational citrus variety numbering.

Table 2 Probabilities produced by analysis of variance (ANOVA) on data sets of orchard conditions

	<i>P</i> _{net}	<i>P</i> _{max}	<i>G</i> _s	Fv/Fm	H ₂ O ₂	MDA	tAsa	Asa	DHA	Asa/DHA	tGsh	Gsh	GssG	Gsh/GssG	SOD	CAT
A (genotype)	10 ⁻¹⁵	10 ⁻¹⁶	10 ⁻⁸	10 ⁻¹⁶	10 ⁻¹³	10 ⁻¹⁴	10 ⁻¹⁶	10 ⁻¹¹	10 ⁻¹⁶	10 ⁻⁵	10 ⁻¹⁴	10 ⁻⁹	10 ⁻¹²	0.017	10 ⁻⁹	10 ⁻¹³
B (season)	10 ⁻¹⁵	10 ⁻¹⁰	10 ⁻¹⁵	10 ⁻¹⁶	10 ⁻⁶	10 ⁻¹⁶	10 ⁻⁶	10 ⁻¹¹	10 ⁻¹³	10 ⁻⁹	10 ⁻¹⁶	10 ⁻¹⁶	10 ⁻⁹	10 ⁻¹³	10 ⁻⁶	10 ⁻¹⁶
C (A × B)	10 ⁻¹¹	10 ⁻¹¹	10 ⁻⁵	10 ⁻⁷	0.057	0.0004	0.001	0.798	10 ⁻⁶	0.051	10 ⁻⁶	0.0008	10 ⁻⁵	10 ⁻⁵	0.291	10 ⁻¹⁵
	APX	MDHAR	DHAR	GR												
A (genotype)	10 ⁻¹⁵	10 ⁻⁹	10 ⁻⁷	10 ⁻⁵												
B (season)	0.0002	10 ⁻⁷	10 ⁻¹²	10 ⁻⁵												
C (A × B)	10 ⁻⁷	10 ⁻⁶	0.621	10 ⁻⁵												

Initial data were subjected to a two-way analysis of variance. The qualitative factors studied were genotype and season and their interaction. The analyses were performed on a group of three trees. The *P* values showing the level of significance of each qualitative factor are presented.

Table 3 Effects of seasonal changes on total ascorbate content (tAsa), reduced ascorbate content (Asa), oxidized ascorbate content (DHA) and redox status (Asa/DHA) in leaves of four citrus genotypes.

Genotype	tAsa ($\mu\text{mol.g}^{-1}$ FW)	Asa ($\mu\text{mol.g}^{-1}$ FW)	DHA ($\mu\text{mol.g}^{-1}$ FW)	Asa/DHA
CC				
WP	23.64 \pm 1.24 ^d	12.67 \pm 1.00 ^f	10.97 \pm 0.33 ^b	1.15 \pm 0.07 ^d
CP	37.22 \pm 0.55 ^{ab}	16.68 \pm 0.46 ^{de}	20.53 \pm 0.84 ^a	0.82 \pm 0.05 ^e
MK				
WP	22.69 \pm 0.51 ^d	15.74 \pm 1.00 ^e	6.94 \pm 0.75 ^c	2.46 \pm 0.48 ^b
CP	40.40 \pm 1.25 ^a	20.67 \pm 0.73 ^b	19.72 \pm 0.98 ^a	1.06 \pm 0.06 ^d
WLM				
WP	25.25 \pm 1.24 ^d	19.13 \pm 0.77 ^{bc}	6.11 \pm 0.68 ^c	3.27 \pm 0.30 ^a
CP	35.06 \pm 1.44 ^b	24.47 \pm 0.94 ^a	10.59 \pm 0.93 ^b	2.39 \pm 0.21 ^b
PP				
WP	20.22 \pm 0.33 ^e	13.30 \pm 0.48 ^f	6.91 \pm 0.20 ^c	1.93 \pm 0.11 ^b
CP	30.57 \pm 0.77 ^c	18.55 \pm 0.29 ^{cd}	12.02 \pm 0.68 ^b	1.57 \pm 0.10 ^c

The data are presented as mean values \pm standard errors of six independent replicated samples ($n = 6$). In the same column, statistically significant differences among genotypes at $P < 0.05$ for Fisher's LSD pairwise comparisons are indicated for each mean value in the warm period (WP) and the cold period (CP) by different letters.

Table 4 Effects of seasonal changes on total glutathione content (tGsh), reduced glutathione content (Gsh), oxidized glutathione content (GssG) and redox status (Gsh/GssG) in leaves of four citrus genotypes.

Genotype	tGsh (nmol.g ⁻¹ FW)	Gsh (nmol.g ⁻¹ FW)	GssG (nmol.g ⁻¹ FW)	Gsh/GssG
CC				
WP	751.02±41.04 ^b	634.62±31.35 ^b	116.39±11.65 ^c	5.64±0.41 ^a
CP	1279.13±70.87 ^a	978.36±77.97 ^a	300.77±21.66 ^a	3.39±0.47 ^b
MK				
WP	198.68±10.02 ^e	94.67±3.92 ^f	104.01±7.36 ^c	0.92±0.05 ^e
CP	701.61±67.87 ^b	420.31±49.83 ^c	281.29±37.11 ^a	1.59±0.20 ^d
WLM				
WP	160.73±13.73 ^e	99.58±10.64 ^f	61.15±6.53 ^d	1.71±0.22 ^d
CP	270.97±13.59 ^d	163.13±15.61 ^e	107.84±5.15 ^c	1.54±0.20 ^d
PP				
WP	333.7±26.88 ^d	230.88±22.69 ^d	102.81±9.23 ^c	2.30±0.25 ^c
CP	525.82±22.21 ^c	366.53±21.09 ^c	159.29±7.21 ^b	2.32±0.16 ^c

The data are presented as mean values ± standard errors of six independent replicated samples ($n = 6$). In the same column, statistically significant differences among genotypes at $P < 0.05$ for Fisher's LSD pairwise comparisons are indicated for each mean value in the warm period (WP) and the cold period (CP) by different letters.

Figure captions

Fig. 1 Changes in (a) net photosynthetic rate (P_{net}) and (b) stomatal conductance (G_s) in four citrus genotypes in the warm period (black bars) and cold period (white bars). All data are presented as mean values (\pm S.E.) of nine independent measurements ($n = 9$). Bars followed by different letters indicate statistically significant differences at $P < 0.05$ for Fisher's LSD pairwise comparisons

Fig. 2 Comparison of net photosynthetic rate (P_{net}) of four citrus genotypes under orchard climatic conditions (white bars) and growth chamber conditions (black bars). P_{net} was measured under (a) September orchard conditions and on detached leaves at 25 °C or (b) under January orchard conditions and on detached leaves at 6 °C. The data are presented as mean values (\pm S.E.) of 6–9 independent measurements. Bars followed by different letters indicate statistically significant differences at $P < 0.05$ for Fisher's LSD pairwise comparisons

Fig. 3 Changes of (a) maximal quantum yield of photosystem II (F_v/F_m) and (b) maximum photosynthetic rate (P_{max}) in four citrus genotypes in the warm period (black bars) and cold period (white bars). All data are presented as mean values (\pm S.E.) of nine independent measurements ($n = 9$). Bars followed by different letters indicate statistically significant differences at $P < 0.05$ for Fisher's LSD pairwise comparisons

Fig. 4 Changes in (a) hydrogen peroxide (H_2O_2) and (b) malondialdehyde (MDA) content in four citrus genotypes in the warm period (black bars) and cold period (white bars). All data are presented as mean values (\pm S.E.) of six independent measurements ($n = 6$). Bars followed by different letters indicate statistically significant differences at $P < 0.05$ for Fisher's LSD pairwise comparisons

Fig. 5 Changes in the specific activities of (a) superoxide dismutase (SOD), (b) catalase (CAT) and (c) ascorbate peroxidase (APX) in four citrus genotypes in the warm period (black bars) and cold period (white bars). All data are presented as mean values (\pm S.E.) of six independent measurements ($n = 6$). Bars followed by different letters indicate statistically significant differences at $P < 0.05$ for Fisher's LSD pairwise comparisons

Fig. 6 Changes in the specific activities of (a) monodehydroascorbate reductase (MDHAR), (b) dehydroascorbate reductase (DHAR) and (c) glutathione reductase (GR) in four citrus genotypes in the warm period (black bars) and cold period (white bars). All data are presented as mean values (\pm S.E.) of six independent measurements ($n = 6$). Bars followed by different letters indicate statistically significant differences at $P < 0.05$ for Fisher's LSD pairwise comparisons

Figure 1

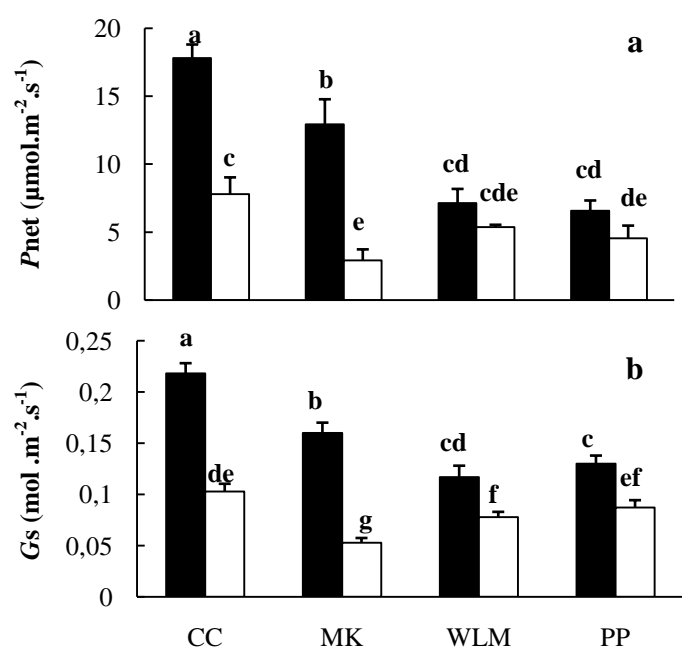


Figure 2

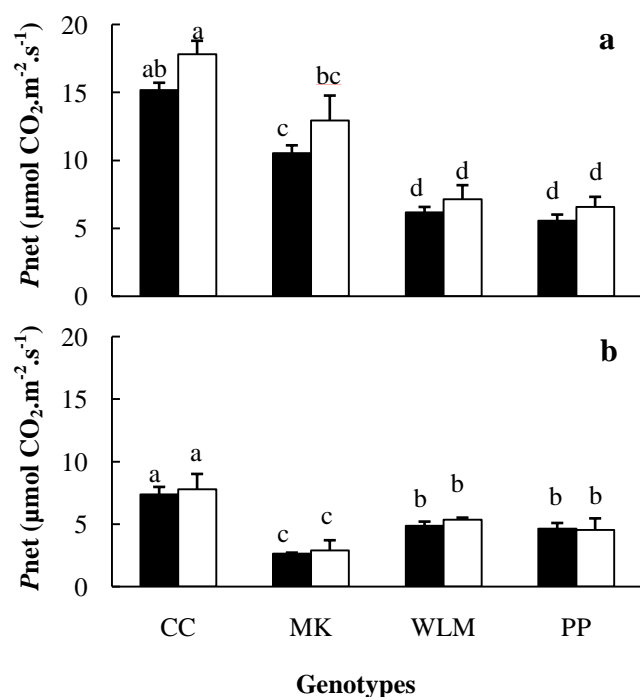


Figure 3

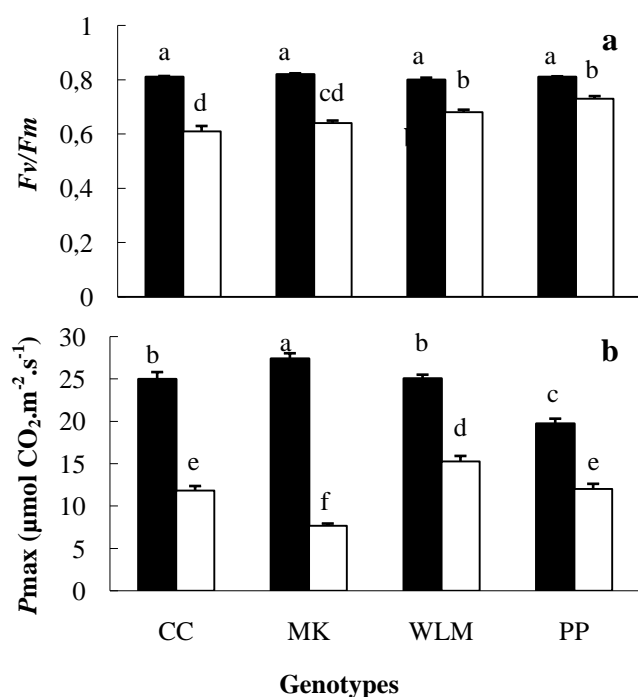


Figure 4

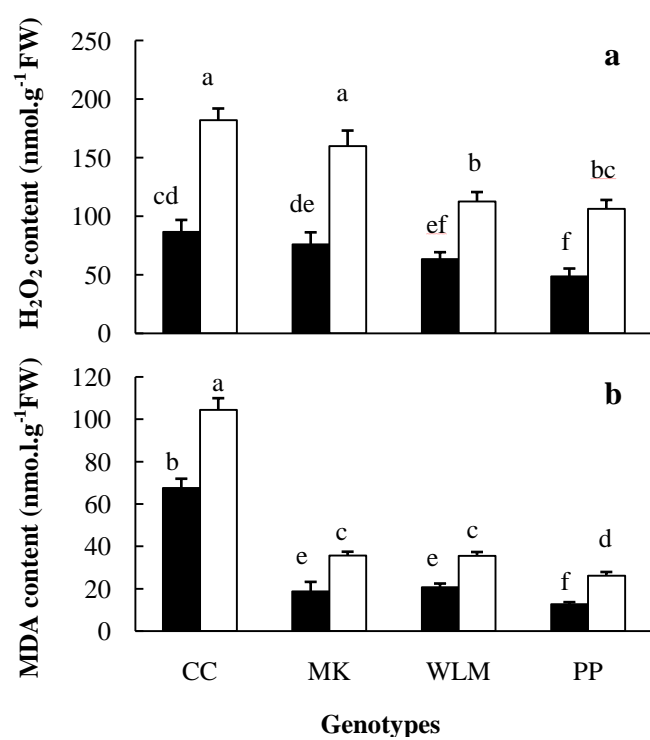


Figure 5

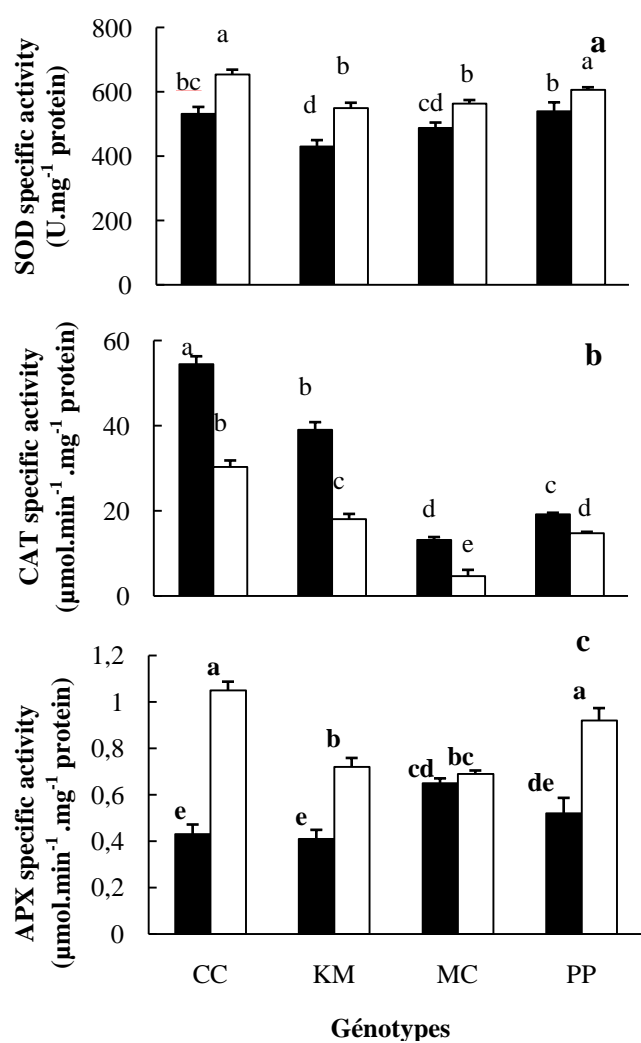


Figure 6

