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Article

# Effect of Propagation Method and Ploidy Level of Various Rootstocks on the Response of the Common Clementine (*Citrus clementina* Hort. ex Tan) to a Mild Water Deficit

Julie Oustric <sup>1,\*</sup>, Radia Lourkisti <sup>1,†</sup>, Stéphane Herbette <sup>2</sup>, Raphaël Morillon <sup>3,4</sup>, Gilles Paolacci <sup>5</sup>, Noémie Gonzalez <sup>1</sup>, Liliane Berti <sup>1</sup> and Jérémie Santini <sup>1</sup>

<sup>1</sup> Unité Mixte de Recherche (UMR) 6134 Sciences Pour l'Environnement (SPE), Centre National de la Recherche Scientifique (CNRS), Laboratoire Biochimie and Biologie Moléculaire du Végétal, Université de Corse, 20250 Corte, France; lourkisti\_r@univ-corse.fr (R.L.); gonzalez.noemie@gmail.com (N.G.); berti@univ-corse.fr (L.B.); santini\_j@univ-corse.fr (J.S.)

<sup>2</sup> Institut National de Recherche Pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), Laboratoire de Physique et Physiologie Intégratives de l'Arbre Fruitier et Forestier (PIAF), Université Clermont Auvergne (UCA), 63000 Clermont-Ferrand, France; stephane.herbette@uca.fr

<sup>3</sup> UMR-Amélioration Génétique et Adaptation des Plantes Méditerranéennes et Tropicales (AGAP), Centre de Coopération Internationale en Recherche Agronomique Pour le Développement (CIRAD), Equipe Structure Évolutive des Agrumes, Polyploïdie et Amélioration Génétique (SEAPAG), F-97170 Petit-Bourg, Guadeloupe, France; raphael.morillon@cirad.fr

<sup>4</sup> AGAP, Université de Montpellier, CIRAD, INRAE, Institut Agro, F-34398 Montpellier, France

<sup>5</sup> Association de Recherche et d'Expérimentation sur les Fruits et Légumes en Corse (AREFLEC), F-20230 San Giuliano, France; gilles.paolacci@areflec.fr

\* Correspondence: oustric\_j@univ-corse.fr; Tel.: +33-495-450-616

† These authors have contributed equally to this work.

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**Abstract:** Current climatic upheavals reduce water availability which impacts the growth and fruit quality of plants. In citrus crops, scion/rootstock combinations are used to ensure high fruit production and quality and a stress tolerance/resistance. Our objective was to assess the effect on the clementine scion (C) under natural mild water deficit of (i) polyploid rootstocks by comparing the allotetraploid FlhorAG1 (C/4xFLs; trifoliolate orange + Willowleaf mandarin) with its diploid parents, trifoliolate orange (C/2xTOs), and Willowleaf mandarin (C/2xWLs), and with a diploid genotype used as reference (Carrizo citrange, C/2xCCs), (ii) rootstock propagation methods by comparing trifoliolate orange seedling (C/2xTOs) with cutting (C/2xTOc). A mild water deficit observed under orchard conditions during the summer period (July–August) induced a significant change in yield (except in C/2xTOs), fruit size, and quality. C/2xCCs, C/2xTOs, and C/2xWLs appeared less affected by water deficit as indicated by their lower reduction of predawn leaf water potential ( $\Psi_{pd}$ ), relative water content (RWC), transpiration (E), and photosynthetic parameters ( $P_{net}$  and  $g_s$ ). Their greater redox balance was probably due to their better antioxidant efficiency. Seedling rootstocks lead to a better adaptation of clementine scions to water deficit than cutting or allotetraploid rootstock. Improving the tolerance to water deficit requires taking into consideration the rootstock genotype, propagation method, and ploidy level.

**Keywords:** antioxidant; *Citrus*; fruit parameters; oxidative stress; photosynthetic capacity; water deficit

## 1. Introduction

Citrus fruits belong the Rutaceae family and are among the best-selling fruits in the world, both fresh and processed. Today, climate change and emerging environmental concerns are new challenges to which citrus crops must adapt to maintain their production systems and competitiveness. Increases in temperature and reduction in precipitation in most regions of the world due to global warming have occurred over the past decade [1], impacting the available freshwater resources. The current drought periods in most production areas are hampering the production and quality of citrus fruits and are increasing the costs associated with water consumption. For example, the production of citrus fruits projected for 2019/2020 in Mexico, Australia, and even Europe will be lower than in previous years [2]. Consequently, a more rational use of water would make it possible to respond (i) to the future water constraints induced by climatic upheavals, (ii) to the economic constraints generated by the high costs of fresh water, and above all (iii) to ecological constraints with the negative impact of overconsumption of fresh water for the planet.

Water deficit reduces growth and development by altering the photosynthetic system, cell division and elongation, the root systems water-nutrient relation and redox balance [3]. The responses to water deficit are complex and include an increase in water uptake through root proliferation or deep rooting, reduction of water losses through stomata closure, as well as osmotic adjustments, and control of the accumulation of reactive oxygen species (ROS) and by-products such as malondialdehyde (MDA) [4–6]. These reactive species include superoxide ( $O_2^-$ ), hydroxyl radical ( $OH^\bullet$ ), hydrogen peroxide ( $H_2O_2$ ), and singlet oxygen ( $^1O_2$ ) that are thought to be detrimental to proteins, carbohydrates, nucleic acids and lipids [7–9]. These molecules inhibit plant growth and development if not carefully regulated. Lipids are the most susceptible to oxidative stress. Lipid peroxidation can lead to changes in the permeability and fluidity of the membrane phospholipid bilayer and can significantly alter cellular integrity. MDA is the main secondary product of lipid peroxidation. During water deficit stress, MDA levels increase significantly in plants, which makes it an interesting oxidative stress marker [10,11]. To avoid damage caused by ROS accumulation, plants produce a number of compatible enzymatic antioxidant and non-enzymatic molecules that confer secondary protection against oxidative stress [12–14]. Enzymatic antioxidant systems, including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and dehydroascorbate reductase (DHAR), have a crucial role in the drought tolerance of plants by scavenging mainly ROS. The tolerance of plants to water deficits in dry regions and under low water regimens could also come from the accumulation of low molecular mass compounds like ascorbate and proline [15]. These molecules act as cellular osmotic regulators and as antioxidants [16,17].

Modern citrus cultivation is based on composite trees associating a rootstock and a scion. The scion depends on the rootstock for water and mineral nutrients, while the rootstock depends on the scion for photosynthetic assimilation [18]. Scions are chosen based on criteria related to fruit production (i.e., size, yield and quality parameters) in grafted plants [19]. Rootstocks are selected based on (i) their tolerance or resistance to diseases (*Phytophthora*, tristeza, exocortis) and pests (nematodes), (ii) the pedoclimatic conditions (soil type, salinity, excess water, nutrient deficiency, etc.), and (iii) their agronomic performance in association with the grafted variety.

Over the past 20 years, there has been an increase in the popularity of easy peeler fruits including mandarins and its related varieties like clementines at the expense of oranges. Numerous studies have shown a positive influence of rootstocks on clementine scions under salt stress [20], chilling [21], drought stress [22], or against the citrus tristeza virus [23]. Many rootstocks exist for clementine cultivation such as trifoliolate orange (*Poncirus trifoliata* [L.] Raf., cv 'Pomeroy') and Carrizo citrange (*Citrus sinensis* [L.] Osbeck x *Poncirus trifoliata* [L.] Raf) [24]. Trifoliolate orange is widely used in acidic soil because of its resistance to tristeza and cold although it is quite tolerant to drought when studied as a seedling [25] and quite sensitive when grafted [24]. Grafting onto trifoliolate range increases fruit productivity and quality. Carrizo citrange is better adapted to acidic and neutral soil and inherited tristeza tolerance from its trifoliolate orange parent. The use of Carrizo citrange as a rootstock helps to

increase productivity (without reducing fruit size) but its poor performance in drought conditions limits its use in dry areas. Willowleaf (*Citrus deliciosa* Ten.) mandarin is not used as a rootstock because of its sensitivity to phytophthora. With trifoliolate orange, Willowleaf is one of the parents of the allotetraploid FlhorAG1.

Most commercial citrus rootstocks are polyembryonic and thus propagated by seeds. Although these seeds produce most of the time, zygotic or polyploid citrus seedlings may arise. Work performed in the past decade has shown that diploid (2x trifoliolate orange and Willowleaf mandarin, as well as the allotetraploid somatic hybrid FlhorAG1 obtained from the protoplast fusion of trifoliolate orange and Willowleaf mandarin, are highly polyembryonic and have limited zygotic genotype among seedlings [26]. However, some rootstocks used in the citrus industry such as C35 citrange or newly created tetraploids such as 4475 citrumelo, have a relatively high percentage (>20%) of off types among seedlings [27,28]. Comparing a genotype propagated by seeds and that has a high polyembryonic rate (and thus a high genetic conformity) to the same genotype propagated by cutting (which provides strict conformity of the genetic material) will indicate if the phenotypic traits associated with the propagation method have an impact on stress tolerance. Indeed, rootstocks obtained from seeds lead to the formation of a pivotal root that contributes to better soil anchoring while the lateral roots provide better absorption of water and nutrients from the soil [29,30]. In addition to being quick and easy, propagation from cuttings has the advantage of offering uniform rootstocks; however, it promotes the development of a root system composed of adventitious or lateral roots [31] whereas plants grown from seeds have a pivotal root system that is responsible for growth in depth [32]. Thus, we believed that it was relevant to evaluate the impact of these two propagation methods using a rootstock with a very low rate of off-types (trifoliolate orange) when propagated by seeds [26] to evaluate the impact of both kinds of root systems on the adaption to water deficit.

Currently, most of the genetic resources of citrus rootstocks are diploid, i.e., they have two sets of chromosomes in their genetic heritage. However, a doubling of the number of chromosomes can appear naturally or be induced artificially and lead to the formation of tetraploid rootstocks (4x). Two types of polyploids exist: autopolyploids which come from chromosome doubling within the same species, and allopolyploids, which are formed from the combination of at least two sets of chromosomes from two different species. Previous studies have shown that the allotetraploid FlhorAG1 has better tolerance than its respective diploid parents when subjected to salt stress or cold [33,34]. When grafted with common clementine, it has high productivity and yields good quality fruit [24]. The study conducted by [35] on 4x trifoliolate orange 'Pomeroy' grafted with clementine highlighted that using 4x rootstocks modifies the plant's physiology through the indication of a dwarf tree phenotype without altering the fruit quality criteria. Generally, antioxidant system efficiency is enhanced in tetraploid rootstock, but also in scion grafted with a tetraploid rootstock [21,34]. However, its tolerance to water deficit has not been studied even though this adverse condition is highly relevant in the future. The search for rootstock/scion combinations that reduce water consumption while maintaining attractive production and optimal fruit quality is key to responding to climatic upheavals.

Based on the literature, we can assume that polyploidization increases the resistance to water deficits. We can also hypothesize that tolerance to stress, as well as fruit yield and quality could be related to better tolerance of the photosynthetic activities and efficient control of oxidative stress.

The objective of this study was to test the effect of polyploidy and the rootstock propagation method of during mild water deficit by comparing five scion/rootstock combinations. Tolerance to water deficiency was assessed by looking at photosynthetic properties, oxidative and antioxidant metabolisms, and the impact on fruit yield parameters.

## 2. Materials and Methods

### 2.1. Plant Material, Growth and Sampling Conditions

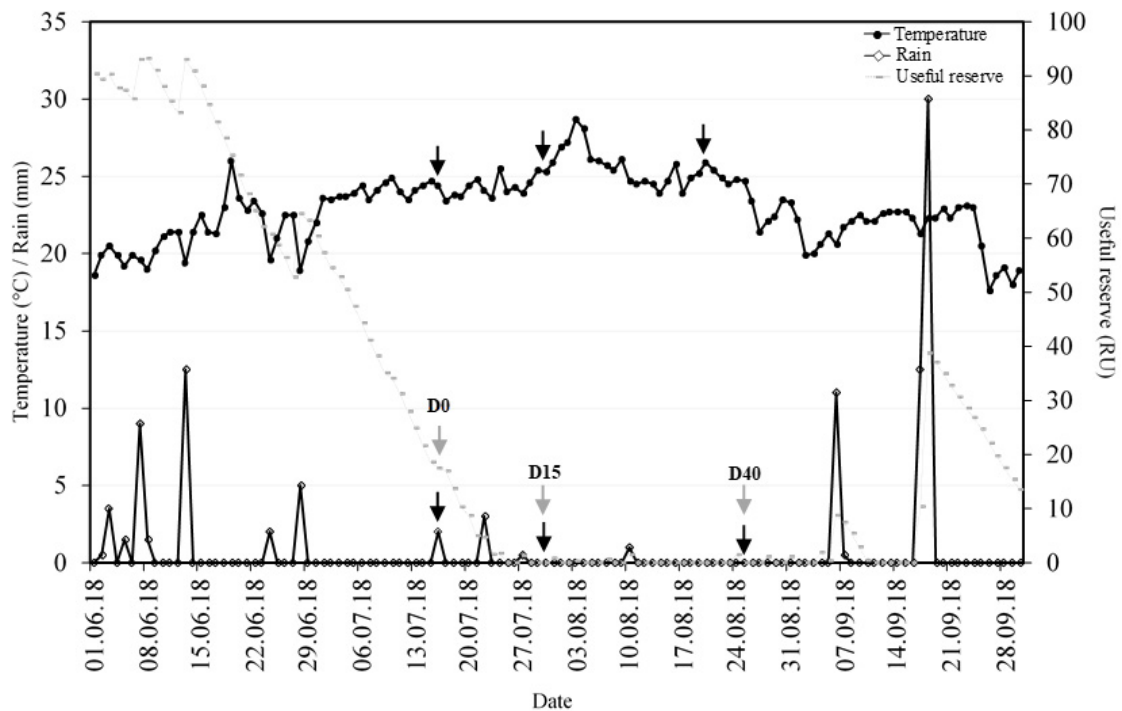
The experiment was carried out in experimental orchards at the agronomic research station INRAE-Cirad of San Giuliano, Corsica, France (42°18'55" N, 9°29'29" E; 51 m above sea level). The soil is alluvial and fersiallitic and acidic with a pH varying from 6 to 6.5. Seedlings of diploid trifoliolate orange (*Poncirus trifoliata* L. Raf, cv: Poncirus Pomeroy) (2xTOs), Willowleaf mandarin (*Citrus deliciosa* Blanco) (2xWLs), Carrizo citrange (*Citrus sinensis* (L.) Obs. × *Poncirus trifoliata* (L.) Raf) (2xCCs) and allotetraploid FlhorAG1 (4xFLs) were propagated as described by [33]. Propagation by cuttings of TO (2xTOc) was obtained using 10 cm soft branch sections of the current year soaked in commercial hormonal solution and then placed in perlite with high humidity. Only one half-leaf was maintained by cutting. After three weeks, cuttings were transferred in soil substrate. All 1-year-old seedlings were then grafted with a bud of diploid clementine scion (*Citrus clementina* Hort. ex Tan; SRA 92). For each scion/rootstock combination, trees were planted in the field and grown for 20 years following classical orchard management.

Six homogenous (pruning, size, and leaf area) trees of each scion/rootstock combination were selected for further investigations. Bark samples were collected (1 cm<sup>2</sup> area cut from below the bud union) from each single rootstock to confirm its ploidy level using flow cytometry [36]. A total of 30 trees was distributed on two irrigation blocks (control and water deficit) equipped with an automatic microjet system (90 L/h) for 40 days. Water deficit conditions were induced during stage II (initial fruit enlargement) which corresponds to the period when clementines are most sensitive to drought [37]. The first irrigation block (control) was composed of 15 “well-watered” trees (3 randomized trees per scion/rootstock combination) with 100% of their water requirement. The second irrigation block was composed of 15 trees (3 randomized trees for each combination) grown with only 30% of their water requirement. For reference, the water requirement was calculated using the water balance method:  $((ET_p \times K_c) - \text{rain})$ , where  $ET_p$  was the potential evapotranspiration in mm,  $K_c$  was the crop coefficient, and rain was the quantity of rain in mm for each day. Fertilizers were supplied, and insects and diseases were controlled according to the recommendations of the local Department of Agriculture.

The useful water reserve (RU) was surveyed daily on the Office de développement agricole et rural de la corse (ODARC) GéODARC cartographic tool ([www.odarc.fr/geodarc](http://www.odarc.fr/geodarc)). Temperatures and daily total rain were recorded throughout the experiment by a meteorological station located on the orchard (Figure 1).

Measurements and samplings were performed from 16 July to 20 August 2018 (summer) at three different time points (0 (D0), 15 (D15), and 40 (D40) days after the onset of the water treatment) on control and stressed trees. The experiment (D0) was initiated when the RU was around 20%. The experiment was then stopped (D40) before the first rains of September (Figure 1).

Physiological parameters were measured between 7:00 a.m. and 11:00 a.m. on 3 leaves per tree (i.e., 9 replicates per modality). The measurements were carried out on fully expanded leaves from the spring of the current year's growth with south exposure. For biochemical assays, 3 samples of 8 leaves were collected at the same time from each scion/rootstock combination and water treatment (3 replicates per modality). Harvested leaves were immediately immersed in liquid nitrogen and then stored at −80 °C. Each leaf sample was ground to a fine powder using a cryogenic mortar with liquid nitrogen just before the analyses. Fruit yield and quality parameters were collected on mature fruits harvested from 29 December 2018 to 9 January 2019. Fruit yield and size were recorded on all the fruits for each scion/rootstock combination. The physicochemical results of agronomic relevance were analysed on 20 mature fruits selected from around the canopy of each scion/rootstock combination (20 replicates per modality).



**Figure 1.** Meteorological data from June to 10 September 2018 in the orchard. Black circle symbols represent the mean daily temperature (°C). White diamond symbols correspond to the daily total rain (mm). Grey dash symbols correspond to the daily useful reserve (RU). The black and grey arrows correspond to the days of measurements and sampling: day 0 (D0) for the control, days 15 (D15) and 40 (D40) after the beginning of the experiment.

## 2.2. Predawn Leaf Water Potential Measurements

The predawn leaf water potential ( $\Psi_{pd}$ ) was measured using a pressure chamber (known as a Scholander) (PMS 1000, PMS Instruments, Corvallis, OR, USA) equipped with a compressed nitrogen source, a flow regulator and a precision pressure gauge (0.001 MPa).

$\Psi_{pd}$  was carried out at the end of the night between 5:00 a.m. and 6:00 a.m. on 3 leaves per tree (i.e., 9 replicates per modality).

## 2.3. Relative Water Content Measurements

Relative water content (RWC) was measured as described by [38]. Between 10:00 a.m. and 11:00 a.m., three discs were cut with a cork borer from three different fully expanded leaves for each scion/rootstock combination and immediately weighed to determine fresh weight (FW). The leaf discs were re-hydrated overnight, and the turgid weight was measured (TW). Then the leaves were dried in oven at 80 °C for 24 h to obtain their dry weight (DW). Relative water content was calculated with the following formula:  $RWC (\%) = [(FW - DW)/(TW - DW)] \times 100$ .

## 2.4. Gas Exchange Measurements

Leaf net photosynthetic rate ( $P_{net}$ ,  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), transpiration ( $E$ ,  $\text{mmol}\cdot\text{H}_2\text{O}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) and stomatal conductance ( $g_s$ ,  $\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) were measured using a portable photosynthetic system (LC Pro, ADC Bioscientific, Hoddesdon, UK) between 7:00 a.m. and 10:00 a.m. During the experiment, photosynthetically active radiation (PAR) was applied at the leaf surface and fixed at  $1400 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Leaf temperature was set at 25 °C and ambient carbon dioxide concentration ( $\text{CO}_2$ ) was used ( $390 \mu\text{mol}\cdot\text{mol}^{-1}$ ) with an air flow rate of  $500 \mu\text{mol}\cdot\text{s}^{-1}$ .

### 2.5. Chlorophyll *a* Fluorescence Measurements

Chlorophyll *a* fluorescence was measured using an OS1p chlorophyll fluorimeter (Opti-Sciences, Inc. Hudson, United States, USA) between 7:00 a.m. and 10:00 a.m. Leaves were first dark-acclimated with a “leafclip” for at least 30 min to inhibit all light-dependent reactions by completely oxidizing PSII electron acceptor molecules. Then, leaves were illuminated with a light intensity of 3000  $\mu\text{mol photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  produced by an array of three light-emitting diodes (650 nm) for a duration of 1 s. Variable fluorescence ( $F_v$ ) was obtained by subtracting the minimal values of chlorophyll *a* fluorescence ( $F_0$ ) from maximum fluorescence ( $F_m$ ). The maximum quantum efficiency of photosystem II (PSII) ( $F_v/F_m$ ) [39] was used as an estimate of the maximum portion of absorbed quanta used in PSII reaction centers [40].

In the presence of actinic illumination, the current fluorescence yield ( $F_t$ ) and the actual light-adapted fluorescence ( $F_m'$ ) were determined. From this, the effective PSII quantum yield ( $Y(\text{II}) = [F_m' - F_t]/F_m' = \Delta F/F_m'$ ) was calculated according to [41]. Electron transport rate ( $\text{ETR} = Y(\text{II}) \times \text{PAR} \times 0.5 \times \text{PAR absorptivity}$ ) was estimated automatically by OS1p. The coefficient of 0.5 is a factor that assumes equal distribution of absorbed photons between photosystem II (PSII) and I (PSI) [42].

### 2.6. Determination of Oxidative Markers and Antioxidant Levels

Malondialdehyde (MDA), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), ascorbate, and antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase and dehydroascorbate reductase) assays were performed as described by [43]. Proline content was measured as described by [44] A V-630 spectrophotometer was used for all measurements (Jasco Inc., Tokyo, Japan).

### 2.7. Fruit Yield and Quality Parameters

Depending on the amount of fruits harvested, the yield was the first parameter measured and corresponded to the average of the total number of fruits per scion/rootstock combination. Fruit diameter was determined with a mechanical calibrator (Mitutoyo, Beauvais, France) and the percentages of different sizes (0–8) were calculated based on the total fruit harvested. Three physicochemical attributes of agronomic relevance were also determined. The juice yield was evaluated based on the juice mass/fruit mass ratio (%). The content of total soluble solids (TSS) in juice was assessed with a refractometer ( $^{\circ}\text{Brix}$ ). The titratable acidity (TA), which was expressed in grams of citric acid/100 mL juice, was measured by titration with 0.1 N NaOH in approximately 0.5 mL of each juice sample diluted in 40 mL distilled water. Ratio maturation index was calculated as the TSS/TA ratio.

### 2.8. Statistical Analyses

Statistical analyses were performed with R statistical software (v.2.13.1) (<http://www.R-project.org>) and Rstudio (Rstudio Inc., Boston, MA, USA, version v.1.2.5042). The qualitative factors studied were sampling date (days) (D0, D15 and D40 after start of water deficit) and scion/rootstock combination subjected to water deficit (C/2xTOc, C/2xTOs, C/2xWLs, C/2xCCs, and C/4xFLs). The influence of sampling date and scion/rootstock combination were analysed using a two-way analysis of variance (ANOVA) followed by least significant difference (LSD) test at  $P < 0.05$ .

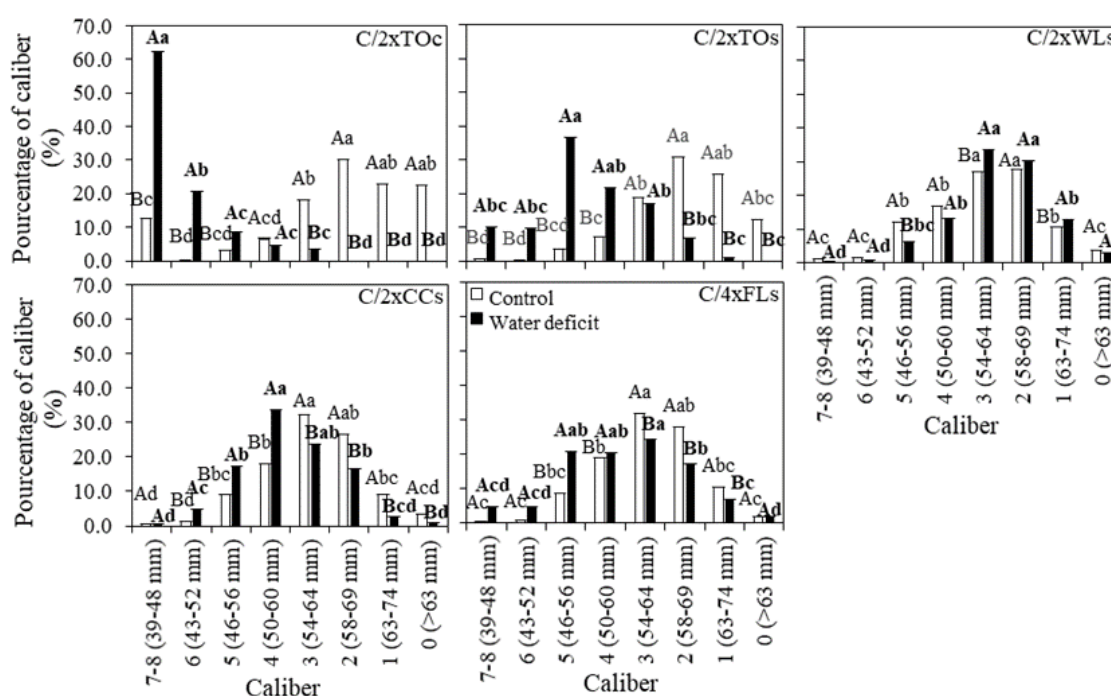
A heatmap was made to determine the differences between all scion/rootstock combinations and treatments (control and water deficit) for gas exchange, chlorophyll fluorescence and fruit yield and quality parameters.

The data obtained on D40 of the water deficit period for the 5 combinations were expressed as ratios with respect to the values obtained in the controls and analysed by principal component analysis (PCA) of centred and reduced variables with the FactomineR package bundled with R statistical software. PCA was conducted to define a clear relationship between physiological, biochemical, fruit yield, and quality parameters and scion/rootstock combinations after D40 of water deficit. PCA provided us with a better understanding of similarities between the many measured variables and individuals.

### 3. Results

#### 3.1. Fruit Yield and Quality Parameters

Under control conditions, the fruit size was close between C/2xTOc and C/2xTOs and between C/2xWLs, C/2xCCs and C/4xFLs with a majority of sizes among 0 and 3 and 1 to 5, respectively (Figure 2). However, the yield was greater in C/2xCCs than in C/2xTOc and C/2xWLs and to a greater extent in C/2xTOs and C/4xFLs (Table 1).



**Figure 2.** Influence of water deficit on fruit caliber of clementine trees grafted onto different rootstocks. The tested diploid rootstocks grafted with common clementine were trifoliate orange cutting (C/2xTOc), trifoliate orange seedling (C/2xTOs), Carrizo citrange (C/2xCCs) and Willowleaf mandarin (C/2xWLs) and FlhorAG1, an allotetraploid somatic hybrid (C/4xFLs). The white square and the black square correspond to the values obtained under control and water deficit, respectively. All the fruit caliber of clementine trees grafted onto different rootstocks was measured during the harvesting period (December–January). All data are presented as mean ( $\pm$  standard errors (S.E.)) of 3 independent measurements ( $n = 3$ ). Data were analysed using ANOVA and Fisher LSD tests ( $P < 0.05$ ). Different upper case letters indicate significant differences between control and water deficit for each scion/rootstock combination for a single day and different lower case letters indicate significant differences between the days for the control or for the water deficit conditions.

The 40 days of water deficit during the summer period (July–August) modified the fruit caliber and decreased the yield (except for C/2xTOs) during the harvesting period (December–January) in all scion/rootstock combinations (Figure 2; Table 1). The increase in yield in C/2xTOs was associated with a larger share of small fruits (8, 7, 6, and 5) and no larger fruits in water-stressed trees (Figure 2; Table 1). Larger sizes (3, 2, 1, and 0) decreased in favour of the smaller sizes (8, 7, 6, 5, and 4) in C/2xTOc

and C/4xFLs following water deficit stress (Figure 2). Conversely, C/2xWLs had a high quantity of large sizes (4, 3, 2, and 1) in comparison to smaller sizes (8, 7, 6 and 5). In C/2xCCs, the middle fruit sizes (6, 5, and 4) increased at the expense of the other sizes (8, 7, 3, 2, 1, and 0) (Figure 2).

On the whole, scion/rootstock combination presented similar average fruit weight (AFW), percentage of juice (except C/2xWLs), total soluble solids (TSS), titratable acidity (TA), and TSS/TA under control conditions (Table 1).

The AFW of C/2xTOs, C/2xWLs, C/2xCCs and C/4xFLs was identical between the control and water deficit conditions. In C/2xTOc, AFW slightly increased (Table 1).

The percentage of juice decreased in C/2xTOc and C/2xCCs and increased in C/2xTOs and C/2xWLs because of the water deficit (Table 1). Only C/4xFLs did not show changes in percentage of juice. Water deficit stress decreased the maturity in C/2xTOc, C/2xTOs and C/2xWLs as shown by a decrease in TSS/TA. Conversely, C/2xCCs had an increase in maturity linked to the TA decrease (Table 1).

### 3.2. Tree Water Status and Photosynthesis-Related Parameters

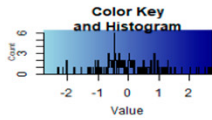
The water deficit induced a decrease in  $\Psi_{pd}$  and in RWC over time when compared to control conditions for all scion/rootstocks combinations (Table 2). However, the dynamics of this decrease differed depending on the combinations. This decrease was greater for C/2xTOc when compared to C/2xTOs and it was greater for C/4xFLs when compared to other scion/rootstock combinations. C/2xTOs and C/2xWLs had the smallest decrease. Also, E decreased in all combinations, particularly in C/4xFLs (Table 2).

$P_{net}$  varied across time, but it was still lower in water deficit conditions when compared to controls for all scion/rootstock combinations (Table 3). However, a higher photosynthetic capacity was observed in C/2xCCs for  $P_{net}$  and  $g_s$  and in C/2xTOc and C/2xTOs for  $P_{net}$  at D40. An important decrease in photosynthetic capacity was also observed in C/4xFLs under water deficit stress for  $P_{net}$  and  $g_s$  at D40 in comparison to their controls.

A slight decrease in  $F_v/F_m$  was observed in C/2xCCs at D40 under water deficit stress. Similar results were observed for C/4xFLs, while its diploid parents C/2xTOc, C/2xTOs and C/2xWLs had no significant difference in  $F_v/F_m$  between the two modalities throughout the experiment (Table 3).

Y(II) decreased slowly in all scion/rootstock combinations whereas it did not change in C/2xCCs from D15. Conversely, Y(II) remained stable in all scion/rootstock combinations during water deficit stress at D40 except for C/2xTOc and C/2xWLs in which Y(II) decreased and increased, respectively (Table 3).

ETR decreased in C/2xTOc, C/2xTOs, C/2xCMs, and C/4xFLs from D15 but it remained constant in the control and water deficit conditions at D40 except in C/2xTOc (Table 3). C/2xCCs showed no differences between the two modalities throughout the experiment.

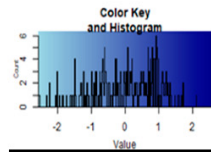


**Table 1.** Influence of water deficit on fruit yield and quality parameters of clementine trees grafted onto different roots.

Parameters	C/2xTOc		C/2xTOs		C/2xWLs		C/2xCCs		C/4xFLs	
	Control	Water deficit	Control	Water deficit	Control	Water deficit	Control	Water deficit	Control	Water deficit
Yield	52.861 ± 2.901	42.997 ± 0.763	42.324 ± 4.502	55.628 ± 1.075	50.766 ± 1.560	38.020 ± 2.985	56.539 ± 2.335	31.350 ± 1.600	45.787 ± 1.970	26.200 ± 3.080
AFW (g)	69.509 ± 1.010	71.160 ± 0.502	73.134 ± 2.725	72.643 ± 3.732	71.506 ± 0.175	70.092 ± 1.756	71.176 ± 1.517	72.144 ± 2.555	70.905 ± 1.015	70.173 ± 1.391
Juice (%)	42.500 ± 0.034	39.600 ± 0.028	41.400 ± 0.032	42.400 ± 0.045	36.400 ± 0.042	40.800 ± 0.160	40.400 ± 0.046	38.800 ± 0.042	39.700 ± 0.023	39.100 ± 0.036
TSS (°Brix)	9.725 ± 0.678	9.425 ± 0.535	9.667 ± 0.257	9.350 ± 0.550	9.200 ± 0.400	9.300 ± 0.122	9.800 ± 0.494	9.600 ± 0.200	9.900 ± 0.402	9.550 ± 0.515
TA (g/100 ml)	0.645 ± 0.040	0.671 ± 0.101	0.583 ± 0.101	0.596 ± 0.134	0.558 ± 0.084	0.600 ± 0.056	0.578 ± 0.046	0.461 ± 0.179	0.635 ± 0.046	0.626 ± 0.100
TSS/TA	15.077 ± 1.743	14.046 ± 1.431	16.581 ± 2.575	15.687 ± 2.748	16.487 ± 1.298	15.500 ± 1.664	16.955 ± 2.052	20.824 ± 1.200	15.590 ± 1.724	15.255 ± 1.848

The tested rootstocks were trifoliate orange cutting (C/2xTOc), trifoliate orange seedling (C/2xTOs), Carrizo citrange (C/2xCCs) and Willowleaf mandarin (C/2xWLs) diploids and FlhorAG1 an allotetraploid somatic hybrid (C/4xFLs). Fruit yield and quality parameters were measured on clementine fruits. Values are means of 30 independent for average fruit weight (AFW), juice, total soluble solids (TSS), titrable acidity (TA) and TSS/TA ratio of fruit. Color scale shows the intensity of the normalized mean values of different parameters. The heatmap shows the differences between all scion/rootstock combinations and treatments for each parameters. Values are associated with color ranging from white (low) to blue (high).

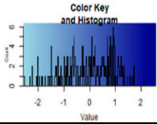
**Table 2.** Influence of water deficit on water status of clementine trees grafted onto different rootstock genotypes.



Parameters Days	C/2xTOc		C/2xTOs		C/2xWLs		C/2xCCs		C/4xFLs	
	Control	Water deficit	Control	Water deficit	Control	Water deficit	Control	Water deficit	Control	Water deficit
RWC (%)										
D0	81.529 ± 0.278	78.628 ± 1.685	82.122 ± 1.615	82.946 ± 1.297	82.642 ± 0.544	79.861 ± 180	79.786 ± 0.343	80.330 ± 1.707	82.850 ± 0.768	80.035 ± 1.478
D15	87.819 ± 1.968	77.617 ± 1.649	87.138 ± 0.986	84.646 ± 0.360	88.006 ± 0.175	79.984 ± 1.327	84.791 ± 0.320	87.934 ± 1.528	85.780 ± 1.308	83.434 ± 0.394
D40	85.219 ± 0.909	70.224 ± 1.009	85.033 ± 1.120	79.002 ± 0.894	85.136 ± 0.948	74.422 ± 0.846	85.405 ± 0.539	75.995 ± 1163	85.348 ± 0.960	72.364 ± 0.830
$\Psi_{pd}$ (MPa)										
D0	-0.675 ± 0.025	-0.917 ± 0.044	-0.613 ± 0.012	-0.708 ± 0.023	-0.650 ± 0.001	-0.660 ± 0.001	-0.642 ± 0.041	-0.558 ± 0.031	-0.680 ± 0.012	-0.860 ± 0.024
D15	-0.500 ± 0.020	-1.210 ± 0.033	-0.483 ± 0.021	-0.810 ± 0.01	-0.617 ± 0.010	-0.833 ± 0.044	-0.475 ± 0.031	-0.790 ± 0.04	-0.460 ± 0.024	-1.138 ± 0.062
D40	-0.667 ± 0.040	-2.317 ± 0.011	-0.483 ± 0.035	-0.933 ± 0.061	-0.567 ± 0.066	-0.983 ± 0.047	-0.567 ± 0.049	-1.067 ± 0.055	-0.633 ± 0.070	-1.617 ± 0.072
E (mmol H <sub>2</sub> O. m <sup>-2</sup> .s <sup>-1</sup> )										
D0	0.707 ± 0.049	0.157 ± 0.013	0.928 ± 0.059	0.359 ± 0.013	0.584 ± 0.017	0.326 ± 0.006	0.521 ± 0.031	0.398 ± 0.015	1.256 ± 0.095	0.211 ± 0.010
D15	1.738 ± 0.033	0.990 ± 0.044	1.991 ± 0.092	1.226 ± 0.011	1.887 ± 0.044	1.649 ± 0.021	2.152 ± 0.074	1.369 ± 0.042	1.950 ± 0.091	1.074 ± 0.037
D40	1.335 ± 0.035	0.871 ± 0.058	1.660 ± 0.057	1.401 ± 0.010	2.246 ± 0.068	1.521 ± 0.013	2.143 ± 0.060	1.936 ± 0.038	2.294 ± 0.076	0.940 ± 0.047

The tested diploid rootstocks grafted with common clementine were trifoliolate orange cutting (C/2xTOc), trifoliolate orange seedling (C/2xTOs), Carrizo citrange (C/2xCCs) and Willowleaf mandarin (C/2xWLs) and FlhorAG1, an allotetraploid somatic hybrid (C/4xFLs). Relative content (RWC), predawn leaf water potential ( $\Psi_{pd}$ ) and transpiration (E) were measured on leaves after different period: day 0 (D0) (control), day 15 (D15) and day 40 (D40) after the beginning of the experiment. Values are means of 9 independent measurements ( $n = 9$ ) for each parameter. Color scale shows the intensity of the normalized mean values of different parameters. The heatmap shows the differences between all scion/rootstock combinations and treatments for each parameter. Values are associated with color ranging from white (low) to blue (high).

**Table 3.** Influence of water deficit on gas exchanges of clementine trees grafted onto different rootstock genotypes.

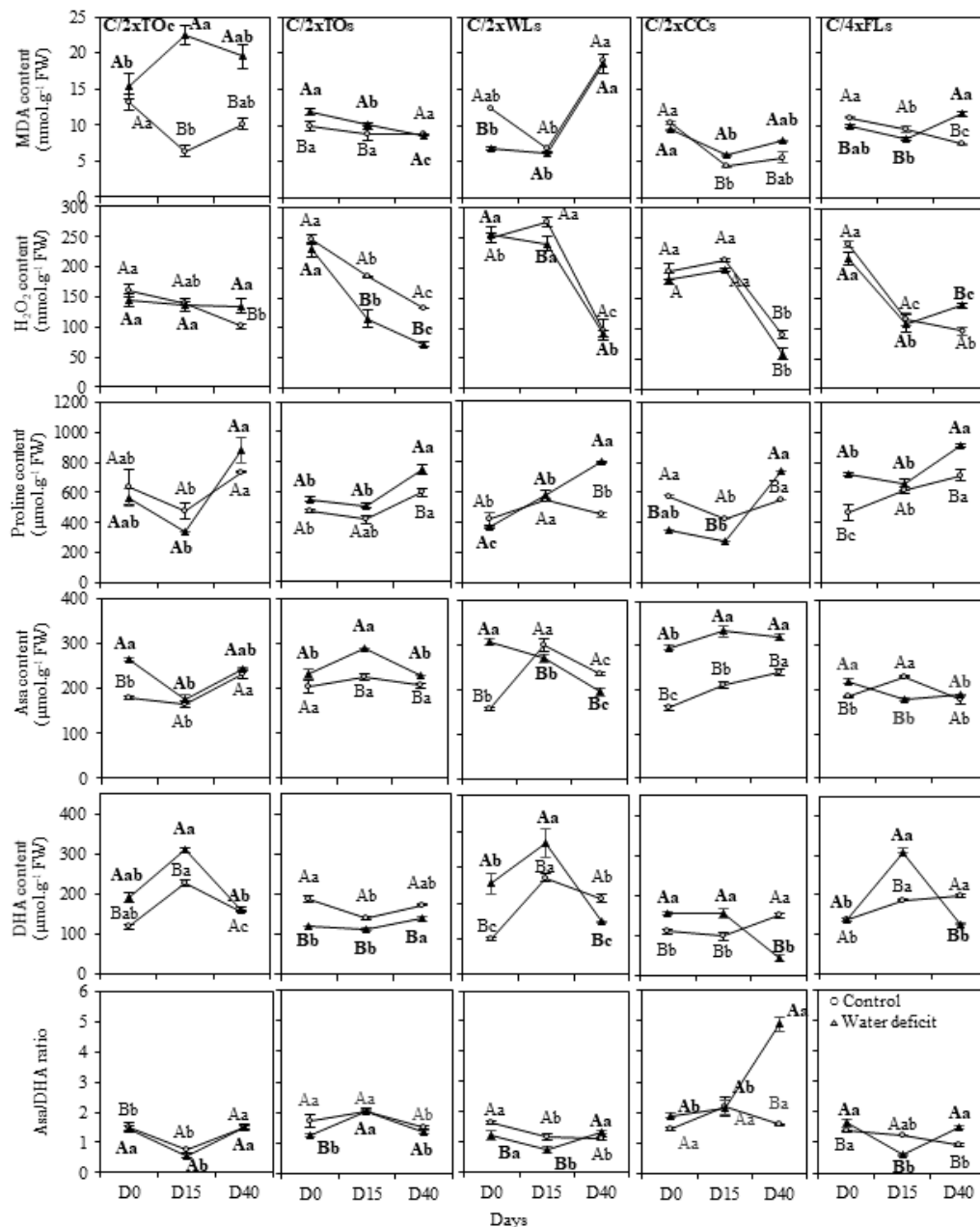


Parameters	Days	C/2xTOc		C/2xTOs		C/2xWLs		C/2xCCs		C/4xFLs	
		Control	Water deficit	Control	Water deficit	Control	Water deficit	Control	Water deficit	Control	Water deficit
$g_s$ (mol $CO_2 \cdot m^{-2} \cdot s^{-1}$ )	D0	0.061 ± 0.005	0.012 ± 0.001	0.089 ± 0.007	0.035 ± 0.001	0.043 ± 0.002	0.032 ± 0.001	0.042 ± 0.002	0.039 ± 0.002	0.111 ± 0.008	0.020 ± 0.001
	D15	0.157 ± 0.002	0.071 ± 0.004	0.157 ± 0.004	0.080 ± 0.000	0.179 ± 0.006	0.108 ± 0.002	0.226 ± 0.015	0.090 ± 0.002	0.182 ± 0.014	0.062 ± 0.003
	D40	0.071 ± 0.001	0.033 ± 0.002	0.150 ± 0.010	0.098 ± 0.001	0.126 ± 0.002	0.063 ± 0.001	0.117 ± 0.004	0.092 ± 0.001	0.134 ± 0.006	0.037 ± 0.002
$P_{net}$ ( $\mu mol CO_2 \cdot m^{-2} \cdot s^{-1}$ )	D0	5.011 ± 0.110	3.488 ± 0.094	6.132 ± 0.207	4.360 ± 0.168	6.456 ± 0.192	5.290 ± 0.180	7.437 ± 0.243	7.636 ± 0.137	9.613 ± 0.523	4.288 ± 0.247
	D15	9.319 ± 0.205	6.760 ± 0.158	11.19 ± 0.442	6.456 ± 0.243	11.834 ± 0.168	9.681 ± 0.244	11.884 ± 0.312	7.154 ± 0.064	11.855 ± 0.477	7.366 ± 0.469
	D40	7.102 ± 0.090	3.007 ± 0.200	9.144 ± 0.204	4.899 ± 0.108	11.022 ± 0.125	6.500 ± 0.059	10.084 ± 0.329	7.511 ± 0.186	10.609 ± 0.282	3.512 ± 0.292
$F_v/F_m$	D0	0.778 ± 0.008	0.782 ± 0.090	0.764 ± 0.041	0.816 ± 0.009	0.797 ± 0.019	0.815 ± 0.022	0.793 ± 0.023	0.807 ± 0.021	0.802 ± 0.026	0.818 ± 0.016
	D15	0.822 ± 0.014	0.810 ± 0.012	0.803 ± 0.024	0.801 ± 0.027	0.823 ± 0.014	0.774 ± 0.057	0.823 ± 0.013	0.818 ± 0.016	0.822 ± 0.013	0.766 ± 0.087
	D40	0.823 ± 0.008	0.826 ± 0.013	0.808 ± 0.020	0.808 ± 0.036	0.824 ± 0.017	0.822 ± 0.007	0.848 ± 0.009	0.784 ± 0.102	0.809 ± 0.017	0.781 ± 0.128
Y(II)	D0	0.682 ± 0.050	0.684 ± 0.018	0.694 ± 0.013	0.628 ± 0.036	0.714 ± 0.018	0.685 ± 0.081	0.633 ± 0.019	0.618 ± 0.054	0.703 ± 0.034	0.668 ± 0.076
	D15	0.655 ± 0.103	0.437 ± 0.013	0.707 ± 0.025	0.615 ± 0.082	0.692 ± 0.048	0.585 ± 0.055	0.735 ± 0.026	0.732 ± 0.018	0.722 ± 0.047	0.585 ± 0.085
	D40	0.767 ± 0.007	0.614 ± 0.050	0.739 ± 0.052	0.719 ± 0.027	0.604 ± 0.012	0.767 ± 0.017	0.754 ± 0.033	0.735 ± 0.023	0.736 ± 0.023	0.744 ± 0.019
ETR ( $\mu mol \cdot e^- \cdot m^{-2} \cdot s^{-1}$ )	D0	34.317 ± 1.840	24.333 ± 2.912	34.900 ± 2.599	16.500 ± 1.811	35.933 ± 2.297	19.317 ± 1.199	35.833 ± 1.481	18.550 ± 1.074	35.383 ± 1.726	28.583 ± 1.897
	D15	32.950 ± 1.210	21.950 ± 2.594	35.550 ± 1.286	30.950 ± 1.211	34.817 ± 2.411	29.417 ± 1.863	36.983 ± 1.357	36.883 ± 0.921	36.317 ± 1.424	29.400 ± 1.390
	D40	38.617 ± 0.360	34.90 ± 2.603	37.200 ± 2.633	36.183 ± 1.379	38.762 ± 0.546	38.617 ± 0.893	37.950 ± 1.705	36.967 ± 1.167	37.050 ± 1.179	37.433 ± 1.009

The tested diploid rootstocks grafted with common clementine were trifoliolate orange cutting (C/2xTOc), trifoliolate orange seedling (C/2xTOs), Carrizo citrange (C/2xCCs) and Willowleaf mandarin (C/2xWLs) and FlhorAG1, an allotetraploid somatic hybrid (C/4xFLs). Stomatal conductance ( $g_s$ ), leaf net photosynthetic rate ( $P_{net}$ ), maximum quantum efficiency of photosystem II ( $F_v/F_m$ ), effective PSII quantum yield (Y(II)) and electron transport rate (ETR) were measured on leaves after different period: day 0 (D0) (control), day 15 (D15) and day 40 (D40) after the beginning of the experiment. Values are means of 9 independent measurements ( $n = 9$ ) for each parameter. Color scale shows the intensity of the normalized mean values of different parameters. The heatmap shows the differences between all scion/rootstock combinations and treatments for each parameter. Values are associated with color ranging from white (low) to blue (high).

### 3.3. Contents of Redox-Related Compounds

Some significant differences in MDA and H<sub>2</sub>O<sub>2</sub> were observed between water treatments or across time points depending on the scion/rootstock combinations (Figure 3). Water deficit induced a sharp increase in MDA and H<sub>2</sub>O<sub>2</sub> in C/2xTOc and C/4xFLs and in MDA in C/2xCCs at D40 compared to control. Proline content increased for all scion/rootstock combinations because of water deficit at D40 when compared to controls, except for C/2xTOc (Figure 3).



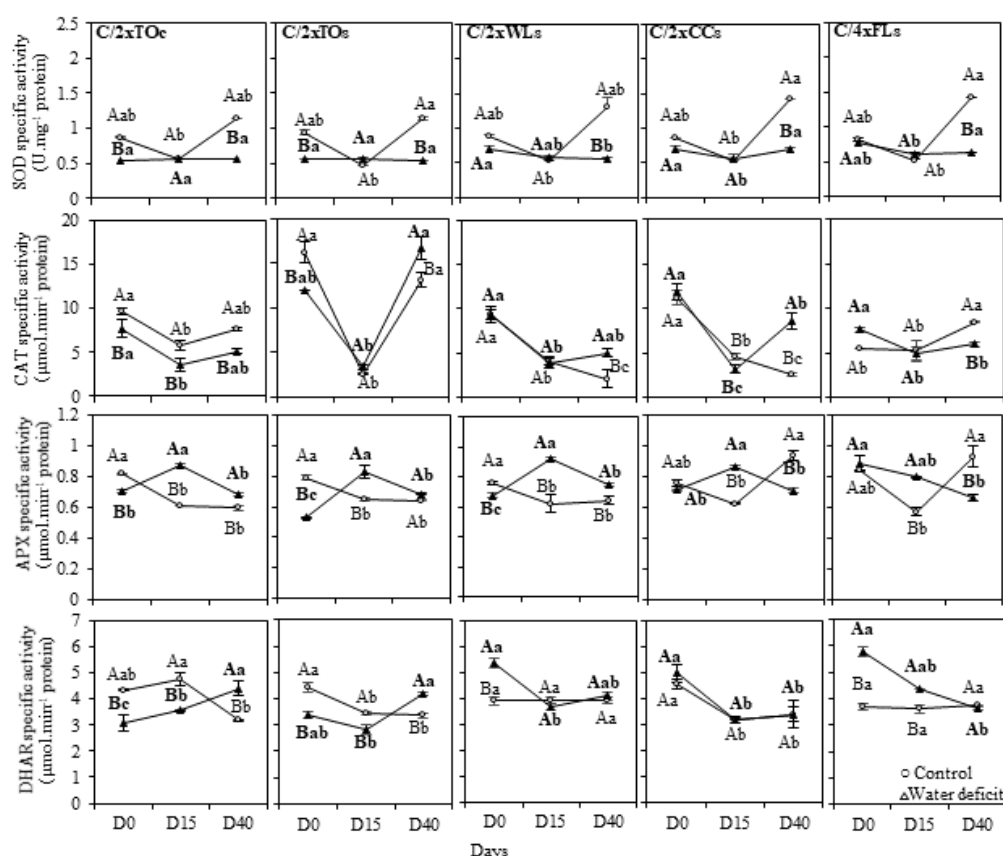
**Figure 3.** Influence of water deficit on malondialdehyde (MDA), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), proline, reduced ascorbate (Asa), oxidized ascorbate (DHA) concentration and redox status (Asa/DHA) of clementine trees grafted onto different rootstocks. The tested diploid rootstocks grafted with common clementine were trifoliate orange cutting (C/2xTOc), trifoliate orange seedling (C/2xTOs), Carrizo citrange (C/2xCCs) and Willowleaf mandarin (C/2xWLS) and FilhorAG1, an allotetraploid somatic hybrid (C/4xFLs). The white circles and the black triangles correspond to the values obtained

under control and water deficit, respectively. All concentrations were measured on leaves after different period: day 0 (D0) (control), day 15 (D15) and day 40 (D40) after the beginning of the experiment. All data are presented as mean ( $\pm$ S.E.) of 3 independent measurements ( $n = 3$ ). Data were analysed using ANOVA and Fisher LSD tests ( $P < 0.05$ ). Different upper case letters indicate significant differences between control and water deficit for each scion/rootstock combination for a single day and different lower case letters indicate significant differences between the days for the control or for the water deficit conditions.

Some small differences in Asa/DHA ratio were observed. There was no clear effect of the drought stress on this parameter, except for C/2xCCs at D40. The increase in Asa/DHA was associated with a decrease in DHA content, the oxidized form of ascorbate, and an increase in Asa, the reduced form, for C/2xCCs at D40 (Figure 3).

### 3.4. Activity of Antioxidant Enzymes

For all combinations, SOD activity was similar or lower in water-stressed trees compared to controls (Figure 4). C/2xTOs, C/2xWLs, and C/2xCCs had a greater CAT activity in the water deficit conditions at D40 when compared to control. APX activity increased significantly in all scion/rootstock combinations experiencing water deficit at D15 but only remained higher than control in C/2xTOc and C/2xWLs at D40 (Figure 4). DHAR activity was significantly increased in C/2xTOc and C/2xTOs at D40 of water deficit compared to the control conditions.

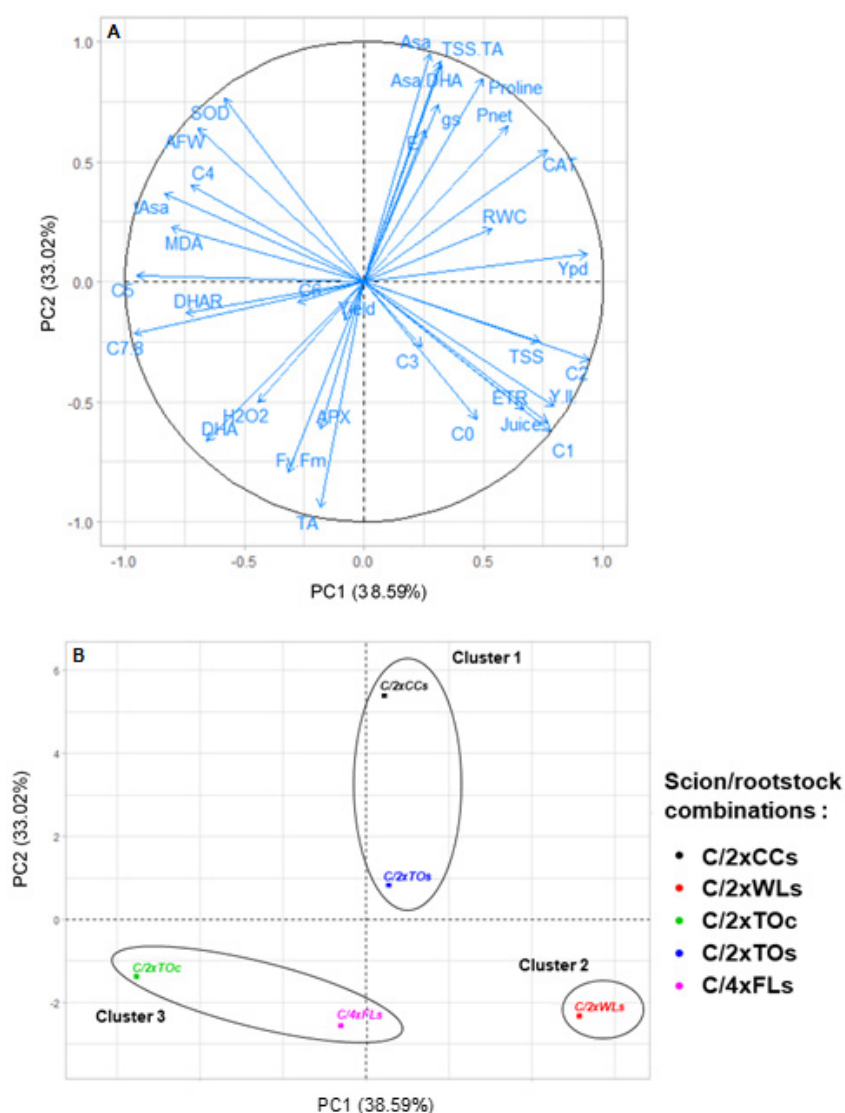


**Figure 4.** Influence of water deficit on antioxidant enzyme specific activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) of clementine trees grafted onto different rootstocks. The tested diploid rootstocks grafted with common clementine were trifoliolate orange cutting (C/2xTOc), trifoliolate orange seedling (C/2xTOs), Carrizo citrange (C/2xCCs) and Willowleaf mandarin (C/2xWLs) and FlhorAG1, an allotetraploid somatic hybrid (C/4xFLs). The white circles and the black triangles correspond to the values obtained

under control and water deficit, respectively. All specific activities were measured on leaves after different period: day 0 (D0) (control), day 15 (D15) and day 40 (D40) after the beginning of the experiment. All data are presented as mean ( $\pm$ S.E.) of 3 independent measurements ( $n = 3$ ). Data were analysed using ANOVA and Fisher LSD tests ( $P < 0.05$ ). Different upper case letters indicate significant differences between control and water deficit for each scion/rootstock combination for a single day and different lower case letters indicate significant differences between the days for the control or for the water deficit conditions.

### 3.5. Responses of Clementines Grafted onto Various Rootstocks after a Water Deficit

Based on the PCA, the first two principal axes explained 71.61% for D40 (Figure 5). Clementine trees grafted onto different rootstocks were split in three distinct groups based on their response to water deficit at D40.



**Figure 5.** Biplot obtained from principal component analysis (PCA) performed with all the parameters studied after 40 days of water deficit on clementine trees grafted onto different rootstocks. The tested rootstocks were trifoliolate orange cutting (C/2xTOc), trifoliolate orange seedling (C/2xTOs), Carrizo citrange (C/2xCCs) and Willowleaf mandarin (C/2xWLS) diploids and FlhorAG1 an allotetraploid somatic hybrid (C/4xFLs). Dispersion of scion/rootstock combinations (A) and contribution of the variables to the dispersion (B).

PC1 separated cluster 1 (C/2xTOs and C/2xCCs) and cluster 2 (C/2xWLs) from cluster 3 (C/2xTOc and C/4xFLs). PC2 separated cluster 1 (C/2xTOs and C/2xCCs) from cluster 2 (C/2xWLs) and cluster 3 (C/2xTOc and C/4xFLs).

Thus, C/2xCCs and C/2xTOs (cluster 1) were differentiated by higher water deficit parameters ( $\Psi_{pd}$  and RWC), photosynthetic capacity ( $P_{net}$ ,  $g_s$ , and E) and antioxidant activities (Asa/DHA, Asa, proline and CAT) and lower oxidative marker accumulation ( $H_2O_2$  and DHA) than in C/2xTOc and C/4xFLs (cluster 3). C/2xWLs (cluster 2) was similarly associated with water deficit parameters ( $\Psi_{pd}$  and RWC) like C/2xTOs and C/2xCCs but had better chlorophyll *a* fluorescence parameters (Y(II) and ETR) than the other scion/rootstock combinations. Fruit yield and quality parameters were dispersed into the three clusters. The higher TSS, juice, and C0-C2 sizes were associated with C/2xWLs (cluster 2). Higher TA and C5 and C7-8 sizes were observed in C/2xTOc and C/4xFLs (cluster 3), respectively. C/2xTOs and C/2xCCs (cluster 1) were also positively correlated to TSS/TA.

The variables analyzed are predawn leaf water potential ( $\Psi_{pd}$ ), relative water content (RWC), net photosynthesis rate ( $P_{net}$ ), stomatal conductance ( $g_s$ ), transpiration (E), chlorophyll fluorescence ( $F_v/F_m$ ), effective PSII quantum yield (Y(II)), electron transport rate (ETR), malondialdehyde (MDA), hydrogen peroxide ( $H_2O_2$ ), antioxidant enzymes (SOD, CAT, APX, and DHAR), reduced ascorbate (Asa), oxidized ascorbate (DHA), ascorbate redox status (Asa/DHA), proline, caliber (C0–C8), yield, average fruit weight (AFW), juice, total soluble solids (TSS), titrable acidity (TA), and ratio maturation index (TSS/TA).

## 4. Discussion

### 4.1. Effect of a Mild Summer Water Deficit on Clementine Fruit Production

It is now well-established that Clementine producers can reduce the implementation of watering between 8% and 20% without significantly reducing yield [45]. In our study, the 70% reduction in water inputs for 40 days during the summer season (July–August) impacted fruit quality and yield (Table 1) of all scion/rootstock combinations. This would imply that the changes in soil water availability induced general adjustments in the trees. The effect of water deficit was probably reinforced by the high internal drainage of the fersiallitic soil. Yet, the hydric status of the soil decreased slightly according to the  $\Psi_{pd}$  values. Indeed, these reached minimal values ranging from  $-0.93$  to  $-1.62$  MPa in the driest soil conditions at D40 for rootstocks prepared using seedlings. The rootstock prepared using cuttings (C/2xTOc) was an exception with a  $\Psi_{pd}$  reaching  $-2.3$  MPa. According to [46], stomatal closure occurs between  $-1$  and  $-2$  MPa and cavitation events accumulates below  $-2$  MPa for C/2xCCs or C/2xTOs, highlighting that severe drought can happen in drier conditions, i.e., below  $-2$  MPa. The slight decrease in  $\Psi_{pd}$  and the enhanced proline content in all scion/rootstock combinations indicate that the decrease in water supply was sufficient to induce a water deficit (Figure 3; Table 2) [47]. Furthermore, the mild water deficit induced a progressive decrease in net photosynthesis ( $P_{net}$ ) and stomatal conductance ( $g_s$ ). This phenomenon was observed in all scion/rootstock combinations although there was no change in chlorophyll *a* fluorescence parameters, such as  $F_v/F_m$  and Y(II) and ETR (except in C/2xTOc) (Table 3). These findings suggest that the rate of reduction in photosynthetic activity was reversible and only linked to stomata closure, not to non-stomatal (biochemical) factors.

In addition, a low accumulation of oxidative markers (MDA,  $H_2O_2$  and DHA) was observed in the driest conditions. Altogether, our experiment induced a mild summer water stress, but it was sufficient to affect fruit yield and quality during winter [48,49]. Therefore, regardless of the climate change scenarios, drier summer would surely affect citrus crops. This can be related to the sub-tropical and tropical areas of native Citrus in eastern Asia. Our observations imply that searching for adapted practices or rootstock that would have a better resistance to water deficit is relevant for improving or maintaining the citrus crop.

#### 4.2. Could the Scion/Rootstock Combinations Improve the Citrus Crop?

Mild water deficit had a negative impact on the yield of all scion/rootstock combinations, except in C/2xTOs, and led to large changes in fruit size but not systematically in fruit quality (Figure 2; Table 1). The yield increases in C/2xTOs and decreases in C/2xTOc strongly suggest a beneficial effect of the seedling propagation method for this scion/rootstock combination in mild water deficit conditions. However, like in C/2xTOc, C/2xCCs and C/4xFLs, the increase in small fruit sizes (4 to 8 approximately) in C/2xTOs to the detriment of large fruit sizes (1, 2, and 3) could negatively impact its economic performance. Although C/2xWLs had a lower yield, the mild water deficit triggered an increase in economically attractive sizes. In addition, the lower yield and smaller fruit production of clementines grafted with FlhorAG1 (C/4xFLs) than in clementines grafted with its diploid parents (C/2xTOs and C/2xWLs) suggests that allopolyploidy does not improve tolerance to mild water deficit.

The fruit quality was slightly modified in C/2xTOc, C/2xWLs and C/2xCCs but not in C/2xTOs and C/4xFLs (Table 1). The juice rate in all scion/rootstock combinations remained close to 40%, which corresponds to the minimum required for the sale of clementines [50]. C/2xCCs was the only one to have a slight increase in the TSS/TA value which is an advantage for the commercial value of the juice (Table 1) [50].

#### 4.3. Effect of Scion/Rootstock Combinations on the Response to Mild Water Deficit

As expected, RWC in the leaves is correlated with the availability of water in the soil as well as with the plant's efficiency at assimilating water in case of mild water deficit and at limiting water losses in the plant. The smallest decrease in  $\Psi_{pd}$  observed in C/2xCCs and C/2xTOs (cluster 1) and C/2xWLs (cluster 2) (Figure 5A,B; Table 2) indicates that they had access to soil moisture reserves, which can be attributed to their taproot. On the contrary, the lower  $\Psi_{pd}$  in C/2xTOc and C/4xFLs (Cluster 3) (Figure 5A,B; Table 2) shows that they did not have access to a deeper source of water. The C/2xTOc results are probably be due to their superficial lateral roots and thus to more limited access to soil water supply. These results are in agreement with previous studies by [31] who showed that citrus with a superficial ramified root system such as the ones propagated by cutting are more susceptible to drought stress. Although the allotetraploid FlhorAG1 was obtained by seedlings, studies with 4x citrus rootstocks found bigger secondary roots with a much more limited number of thin roots compared to diploid [51,52]. Thus, we speculate that, in the field, the allotetraploid FlhorAG1 develops lateral roots instead of a pivotal root system making this rootstock more sensitive to mild water deficit than its parents.

In our study, reduced water availability decreases the RWC and therefore the E rate, in particular in C/2xTOc and C/4xFLs and to a lesser extent in C/2xTOs, C/2xCCs and C/2xWLs (Table 2). These results make sense given their respective roots system. The E rate observed in all scion/rootstock combinations was correlated with the decrease in  $g_s$  from D15 (Table 2). Reduced transpiration helped to reduce water losses and contributed to maintaining the water status. Usually, plants reduce their transpiration before to reach a cellular stress as reported by [53] in Carrizo citrange and Cleopatra mandarin (*Citrus resnhi* Hort. ex Tanaka; Cleo).

C/2xCCs and C/2xTOs and to a lesser extent C/2xWLs appeared to have less disturbance of their photosynthetic process after D40 than C/2xTOc and C/4xFLs as indicated by their higher  $P_{net}$ ,  $g_s$  and E (Figure 5A,B; Table 3). These results suggest that stomata closure limited the E rate and reduced water loss, especially in C/2xCCs and C/2xTOs, and in C/2xWLs versus C/2xTOc and C/4xFLs (Figure 5A,B; Table 2). The greater decline of  $P_{net}$  and  $g_s$  observed in C/4xFLs at D40 implies that the allotetraploid rootstock does not result in better photosynthetic activity of the scion during a mild water deficit stress (Figure 5A,B; Table 3). In C/2xTOs, C/2xCCs, and C/4xFLs, the decrease in photosynthetic capacity did not affect Y(II) and ETR (Figure 5A,B; Table 3) suggesting that rootstock allopolyploidy may provide a physiological adaptation to mild water deficit but not better than its diploid parents with the same propagation method. The increase in Y(II) and ETR in C/2xWLs indicates an activation of protective mechanisms that allows the plant to limit the damage caused by mild water deficit

conditions (Figure 5A,B; Table 3). The marked decrease in ETR and Y(II) only observed for C/2xTOc (Figure 5A,B; Table 3) would suggest that the mild water deficit negatively influenced the efficiency of  $e^-$  capture for this rootstock combination. The disruption of the  $e^-$  chain can cause a transfer of one or several  $e^-$  to oxygen and create ROS. According to these results, and although C/2xTOc and C/4xFLs were in the same cluster, it appears that C/2xTOc would be more sensitive than C/4xFLs.

The fact that C/2xTOc had greater sensitivity than C/2xTOs and the other scion/rootstock combinations obtained by seedling propagation seems to indicate that the propagation method has a major effect on the sensitivity to the mild water deficit stress in trifoliate orange. These results are unlike those reported by [54] who had highlighted a similar sensitivity to water deficit stress in sour orange (*Citrus aurantium* L.) and Carrizo citrange rootstocks obtained by seedling and cutting propagation methods suggesting that the impact of the propagation method is genotype dependant. Increased sensitivity of cuttings to water deficit compared to seedlings for Lovell peach (*Prunus persica* (L.) Batsch) and inversely for Nemaguard (*Prunus persica* (L.) Batsch) peach was also observed in the same study [54]. Altogether, these results point to an effect of the rootstock's propagation method depending on both the water-deficit conditions and the species. Here, the impact of a mild water deficit on chlorophyll fluorescence parameters for rootstocks propagated by cuttings suggests the rootstock's propagation method has a strong effect on the response of grafted clementine.

The tolerance of scion/rootstock combinations to water deficit is not only due to the adapted plant's ability to maintain water balance by water retention but also due to a powerful antioxidant system. Enzymatic and non-enzymatic antioxidant systems are known to eliminate ROS under stress [55]. Better antioxidant enzymatic defence is associated with drought tolerance [56]. According to [57], the alteration of antioxidant enzyme activity contributes to increase oxidative stress and is partially responsible for the higher sensitivity of Cleopatra mandarin trees when faced with both drought and heat stress. In our study, C/2xTOc and C/4xFLs had high level of oxidative markers (MDA and  $H_2O_2$ ) at D40 (Figure 3; Figure 5A,B). Conversely, lower levels of  $H_2O_2$  in C/2xCCs, C/2xTOs and C/2xWLs were observed at D40 and for MDA in C/2xTOs and C/2xWLs (Figure 3; Figure 5A,B).

Regarding antioxidant enzyme systems, the SOD activity does not play a prominent role during a mild water deficit under applied stress conditions for all scion/rootstock combinations (Figure 4). Indeed, a study in canola (*Brassica napus* L.) also showed that SOD was not affected by water deficit stress. In addition, similar or better DHAR activity after D40 of the mild water deficit conditions was associated with a constant or an increase of Asa/DHA ratio above 1 at the same time point. This result suggests there is enough DHAR activity for Asa-recycling (Figures 3 and 4) effectively limiting the induced oxidative stress over time especially in C/2xCCs. It is interesting to note that the activity of CAT and APX, known for their significant role in plant defence against drought-induced oxidative stress, was reduced, maintained, or increased under mild water deficit (Figure 4). The lower  $H_2O_2$  and MDA in C/2xTOs, C/2xCCs and C/2xWLs and only  $H_2O_2$  in C/2xWLs can be explained by the greater increase in CAT activity, which is efficient at removing  $H_2O_2$  (Figures 3–5). This low MDA level indicates that membrane integrity is maintained in mild drought conditions because of an adequate response of the antioxidant system in C/2xCCs and C/2xTOs and C/2xWLs. These results agree with the higher photosynthetic capacity observed in C/2xCCs, C/2xTOs and C/2xWLs than in C/2xTOc and C/4xFLs.

In C/2xTOc and C/4xFLs, we might expect that the  $H_2O_2$  excess has not been eliminated by CAT and APX. Such accumulation of  $H_2O_2$  could lead to  $OH^\bullet$  production and therefore to lipid membrane damage. This cellular damage is underlined by the accumulation of MDA observed at D40 (Figures 3–5). Furthermore, the higher MDA contents observed in C/2xTOc could be linked to previously observed disruption of the  $e^-$  chain (Figure 3). This last finding suggests the antioxidant defence was not induced enough in mild water deficit conditions to counter the increased production of ROS (Figure 3). In this study, clementine grafted on FlhorAG1 does not appear to have a better antioxidant system than its rootstock parents with the same type of propagation. Moreover, the less efficient antioxidant system of C/2xTOc compared to C/2xTOs and the other rootstock genotypes obtained by seedling propagation

is consistent with physiological results obtained previously. However, although C/2xTOc had a more significant disturbance of its photosynthetic and antioxidant system than C/2xWLs, C/2xCCs, and C/4xFLs, its yield was less affected.

## 5. Conclusions

A 70% water deprivation induced various effects on fruit quality and the yield of clementine depending of the rootstock genotype, propagation method and ploidy level. These results suggest that no matter how the climate changes, citrus production will be impacted, even under mild drought conditions. Among the five scion/rootstock combinations, trifoliolate orange, Carrizo citrange, and to a lesser extent Willowleaf mandarin seedlings were the most tolerant to mild water deficit. However, this tolerance had a positive impact in yield in trifoliolate orange seedlings only. In comparison to the taproot of trifoliolate orange cuttings, the superficial lateral roots of orange trifoliolate cuttings contribute to reduced tolerance to mild water deficit and negatively impact yield. The allotetraploidy of FlhorAG1 does not improve the clementine tree's tolerance to mild water deficit relative to its parents obtained by seedling propagation but also relative the other genotypes, as previously shown in non-grafted FlhorAG1 for other abiotic stresses. FlhorAG1 did not counter the harmful effects of its lateral roots in mild water deficit conditions resulting in a large reduction in yield. To go further, it would be interesting to test the propagation by seedling and cutting of several other rootstocks (including tetraploid) under more severe water stress conditions. The rootstocks identified as being the most resistant could then be grafted with new emerging varieties, such as some triploid varieties. Indeed, new citrus breeding programs have focused mainly on these because they produce seedless fruit with a different maturity period and interesting pomological, agronomical, and organoleptic traits.

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## Abbreviations

2x	diploid
4x	tetraploid
APX	ascorbate peroxidase
Asa	reduced ascorbate
Asa/DHA	redox status of ascorbate
C/2xCCs	Clementine trees grafted onto diploid Carrizo citrange rootstock
C/2xWLs	Clementine trees grafted onto diploid common mandarin rootstock
C/2xTOc	Clementine trees grafted onto diploid trifoliolate orange cuttings rootstock
C/2xTOs	Clementine trees grafted onto diploid trifoliolate orange seedlings rootstock
C/4xFLs	Clementine trees grafted onto allotetraploid FlhorAG1 rootstock
CAT	catalase
DHA	oxidized ascorbate
DW	dry weight
E	transpiration
ETR	electron transport rate
$F_v/F_m$	maximum quantum efficiency of PSII
FW	fresh weight
$g_s$	stomatal conductance
MDA	malondialdehyde
$P_{net}$	leaf net photosynthetic rate

PSII	photosystem II
ROS	reactive oxygen species
RWC	relative water content
SOD	superoxide dismutase
Y(II)	effective photosystem II quantum yield
$\Psi_{pd}$	predawn leaf water potential.

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