Improved response of triploid citrus varieties to water deficit is related to anatomical and cytological properties

Radia Lourkisti[¤1], Julie Oustric[¤1], Yann Quilichini[2], Yann Froelicher[3], Stéphane Herbette[4], Raphael Morillon[5], Liliane Berti[1], Jérémie Santini*[1]

[1]CNRS, Equipe « Biochimie et Biologie moléculaire du végétal », UMR 6134 SPE, Université de Corse, Corsica, France ; [2]CNRS, Equipe « Parasites et Ecosystèmes méditerranéens, UMR 6134 SPE, Université de Corse, Corsica, France ; [3]CIRAD UMR AGAP, Station INRA, San Giuliano, Corsica, France; [4]UCA, INRA, PIAF, Clermont-Ferrand, France ; [5]Equipe « Amélioration des Plantes à Multiplication Végétative », UMR AGAP, Département BIOS, CIRAD, Petit-Bourg, Guadeloupe

***Corresponding author:** Dr. Jérémie Santini, santini_j@univ-corse.fr, Université de Corse Pascal Paoli, CNRS UMR 6134 SPE, Bat PPDB, 1er étage, Campus Grimaldi, 20250 Corte, France.

¤These authors have contributed equally to this work.

Abstract

Polyploidy plays a major role in citrus plant breeding to improve the adaptation of polyploid rootstocks as well as scions to adverse conditions and to enhance agronomic characteristics. In *Citrus* breeding programs, triploidy could be a useful tool to react to environmental issues and consumer demands because the produced fruits are seedless. In this study, we compared the physiological, biochemical, morphological, and ultrastructural responses to water deficit of triploid and diploid citrus varieties obtained from 'Fortune' mandarin and 'Ellendale' tangor hybridization. One diploid clementine tree was included and used as a reference. All studied scions were grafted on C-35 citrange rootstock. Triploidy decreased stomatal density and increased stomata size. The number of chloroplasts increased in 3x varieties. These cytological properties may explain the greater photosynthetic capacity (P_{net}, g_s, F_v/F_m) and enhanced water-holding capacity (RWC, proline). In addition, reduced degradation of ultrastructural organelles (chloroplasts and mitochondria) and thylakoids accompanied by less photosynthetic activity and low oxidative damages were found in 3x varieties. Triploid varieties, especially T40-3x, had a better ability to limit water loss and dissipate excess energy (NPQ) to protect photosystems. Higher starch reserves in 3x varieties suggest a better carbon and energy supply and increases in plastoglobuli size suggest less oxidative damage (H_2O_2, MDA), especially in T40-3x, and preservation of photosynthetic apparatus. Taken together, our results suggest that desirable cytological and ultrastructural traits induced by triploidy improve water stress response and could be a useful stress marker during environmental constraints.

Keywords: chloroplast ultrastructure, leaf gas exchange, polyploidy, stomatal response, oxidative status, water deficit

1. Introduction

Polyploidy plays a major role in plant evolution and is considered an effective tool for crop improvement (Adams & Wendel, 2005; Comai, 2005). Polyploidy can occur spontaneously from unreduced gametes of somatic hybridization. Allopolyploids can thus combine polyploid advantages and heterosis that refers to the phenomenon that hybrid offspring have greater biomass and faster growth than the average of both parents (Birchler et al., 2010). Polyploid plants are widely associated with desirable traits such as large leaves and flowers, bigger leaf thickness, darker green coloration of leaves and changes in stomatal cell size and density (Allario et al., 2013; Santana-Vieira et al., 2016; Xue et al., 2017). Such anatomical, morphological, physiological and gene expression changes confer superior adaptation to a wider range of environmental stresses (Tan et al., 2015). Thus, examining cytological properties related to physiological and biochemical parameters may be an effective tool for explaining the better tolerance to abiotic stresses including drought. Some studies have shown the relationship between morphological variations and physiological changes with better tolerance of polyploid plants, especially tetraploids, under stressful conditions relative to their diploid counterparts (Allario et al., 2013; Bondada & Syvertsen, 2003; Oustric et al., 2019). However, the tolerance of seedlings achieved by other economically relevant ploidy levels, such as triploidy, has not been studied.

Drought is the main environmental factor impacting crop yield and fruit quality (Chaves & Oliveira, 2004; LAWLOR, 2002). As climate prediction models show increased occurrences of drought episodes (IPCC 2014), global crop production will continue to be challenged. This trend is particularly worrying in the Mediterranean region which will experience more frequent periods of intensive drought, resulting in the extension of arid areas (Gao & Giorgi, 2008). Drought-sensitive plants exhibit various anatomical features including leaf wilting and leaf shedding that disrupt plant growth. Stomatal closure is among the first response of plants upon exposure to water-deficit and aims to limit water loss through transpiration (Farooq et al., 2012). However, it also limits CO_2 influx resulting in a lower photosynthesis rate. Although stomatal limitations may be the key factor responsible for the lower photosynthesis rate, non-stomatal limitations can occur and may be more important during severe water deficit (Farooq et al., 2009; Flexas et al., 2004). These limitations include the decline of major components of photosynthetic capacity, as the maximum carboxylation rate related to Rubsico activity, the light-saturated rate of electron transport and triose-phosphate used for starch and sucrose synthesis (Urban et al., 2017). The disturbance of

photosynthetic activity triggers oxidative stress and the accumulation of reactive oxygen species (ROS) in plant cells (Choudhury et al., 2017). ROS are ubiquitous in the cell and their production is localized in chloroplasts, mitochondria and peroxisomes. They contribute to extensive cell damage including lipid peroxidation and dysfunction of plant physiological and molecular processes which accelerate chlorophyll degradation and loss of cellular integrity. To cope with ROS accumulation, plants have developed complex antioxidant defence strategies. These include the up-regulation of enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) and molecules including ascorbate, proline, and glutathione (Mittler, 2002). Moreover, stress response in plants can be influenced by genotype (Zandalinas et al., 2017), the severity of stress conditions (Hong-Bo et al., 2006) and by ploidy level (Dutra de Souza et al., 2017). Given the above described physiologic and metabolic changes, it is not surprising that the ultrastructure of cell components, such as chloroplast, goes through dramatic changes during abiotic stress. Such modifications include changes in the number and size of chloroplasts, plastoglobules and starch reserves (Shao et al., 2016; Zellnig et al., 2010). Thus, analysing their structure and ultrastructure may be a reliable marker of abiotic stress in crop plants, associated with physiological and biochemical parameters related to stress tolerance.

Citrus is one of the main commercial fruit crops and the Mediterranean area is an extensive citrus producer, especially for mandarins. The harvest season of mandarin, clementine, and other hybrids is extended from September to February. The lack of citrus fruit production (especially small fruits like mandarin) beyond this period and the steady rise in both consumption and consumer expectations, such as the seedlessness, highlight the need to find suitable varieties to satisfy the market demand, mainly between February and May. The creation of triploid hybrids may be a promising breeding strategy to develop new seedless citrus varieties. Citrus trees are mostly diploid with a basic chromosome number in citrus fruits and related genera of x=9 (Krug, 1943). However, some exceptions can occur in natural triploid, especially 'Tahiti' lime, and in the tetraploid kumquat (Ollitrault et al., 2007). Triploid production has been already extended in the Mediterranean basin particularly in the mandarin breeding program (Ahmed et al., 2020; Aleza et al., 2010; Cuenca et al., 2010). Diploid x diploid hybridization is one of the main ways to produce triploid hybrids (Aleza et al., 2012; Ollitrault et al., 2008). Successful hybridization is mainly determined by the frequency of unreduced gametes by the female parent. In citrus triploid breeding programs, the 'Fortune' mandarin displayed the greatest frequency of triploid hybrids (Aleza et al., 2010; Navarro et al., 2015). Because of its polyploidy and fixed heterosis, triploid citrus can

have many benefits over their corresponding diploids including lower fertility, higher growth rate, larger leaf area and greater stress tolerance. These characteristics are very useful for economic and ecological purposes.

While the greater genetic variability of tetraploids has widely been used in citrus rootstocks to improve stress tolerance (Allario et al., 2013; Balal et al., 2017; Khalid et al., 2020; Oustric et al., 2019), few reports have focused on the improved stress tolerance of triploid citrus plants (Lourkisti et al., 2020). In addition, modifications in cell morphology induced by polyploidy could also affect the response to abiotic stresses, including water deficit (Khazaei et al., 2010; Leal-Bertioli et al., 2012; Xue et al., 2017). Thus, the hypothesis we want to verify in this study is that the better response of citrus triploids to water deficit could be partly explained by their cytological and anatomical characteristics. Therefore, the INRA-CIRAD research centre (San Giuliano, Corsica France) has developed a crossbreeding program between 'Fortune' mandarin and 'Ellendale' tangor to generate innovative seedless citrus hybrids (Ahmed et al., 2020). The hybridization resulted in diploid and triploid populations. While the 'Fortune' mandarin was chosen for its ability to produce triploid hybrids and its pomological features, the 'Ellendale' tangor was chosen for its later production (April) and its organoleptic quality.

This study sought to decipher the response of 3x compared to 2x under water deficit conditions taking account of the cytological traits changes induced by ploidy in the scion. To achieve this, we evaluate the drought tolerance mechanisms through the analysis of RWC and proline content. The impact of water deficit was evaluated through the analysis of photosynthetic capacity (leaf gas exchange, carboxylation efficiency and chlorophyll fluorescence) and oxidative stress (oxidative markers and antioxidant defences). Structural and ultrastructural traits were also studied to determine if the cellular properties of polyploids could explain the differences in water deficit responses between the varieties.

2. Materials and methods

2.1. Plant material and experimental design

Diploid (2x) and triploid (3x) citrus trees were generated from a hybridization between the 'Fortune' mandarin (*Citrus reticulata Blanco*) and the 'Ellendale' tangor [*Citrus reticulata Blanco x Citrus sinensis (L) Osb.*]. Diploid common clementine was also integrated in the study. Diploid and triploid scions were grafted on Citrange C-35 rootstock (*Citrus sinensis 'Ruby Blood' x Poncirus trifoliata*) and grown in a greenhouse during seven months at the

142 INRA-CIRAD experimental station in San Giuliano, Corsica, France, 50 m above sea level
143 (42°17'07.5" N, 9°31'21.9" E). C-35 rootstock was chosen for its tolerance to abiotic
144 (drought, cold) and biotic (Tristeza, Phytophthora) stresses. C-35 seedlings used for the
145 experiment were chosen carefully in the nursery to eliminate off-types. The 2x (D40-2x) and
146 3x varieties (T1-3x and T40-3x) were selected as described in a previous study (Lourkisti et
147 al., 2020). Six seedlings of each variety (a total of 24 plants) were transferred to 10 L pots
148 containing a substrate (topsoil, sand, and peat Klasmann TS1, 1:1:2) and grown under
149 identical conditions (photoperiod of 16h with day/night 27-30°C /20-25°C and the relative
150 humidity varying daily between 60% to 80%) in a greenhouse. The same weight of soil was
151 used so that the weight of each pot was 7 kg. During the acclimation period, plants were
152 watered near field capacity (FC) and fertilized with a nutrient solution with nitrogen,
153 phosphorous, potassium (N: 20, P: 5, K: 10, fertilizer unit), magnesium oxide and trace
154 elements.

155 One month before the experiment, plants were pruned to normalize tree size. To obtain
156 the maximum water holding capacity, the plants were watered until saturation and the excess
157 water was allowed to drain overnight. After draining, the pots were weighed to determine the
158 weight at FC. Each pot was then enclosed in a plastic bag to prevent water loss. The plants
159 were then divided into two randomized blocks: 12 plants (three independent biological
160 replicates per variety) were assigned as control plants and continuously irrigated near FC by
161 successive watering and 12 others plants (three independent biological replicates per variety)
162 were subjected to water stress so that the soil water content was only 35% to 45% of FC.
163 Watering was performed in each pot when the assigned minimal soil water content was
164 reached. To maintain this water condition, each pot was weighed daily before and after
165 irrigation. The water deficit lasted 6 days. At the end of the water deficit treatment period, the
166 physiological parameters were recorded. Three independent biological leaf sample replicates
167 were also harvested from each variety and treatment (control and water deficit), frozen
168 immediately, ground in liquid nitrogen and stored at −80°C for biochemical analysis.
169 Concomitantly with physiological measurements and samplings, five 1 cm^2 and 2 mm^2 pieces
170 were sampled from the mid-laminar areas of both sets of leaves to perform scanning and
171 transmission electron microscopy.

172 ### 2.2. Water potential and relative water content

173 Pre-dawn (Ψ_{PD}) water potential was determined in one fully expanded leaf per plant
174 (three independent biological replicates per variety) taken from the basal part of the primary

branch, using a Scholander-type chamber (PMS Instruments Co., Corvallis, OR, United States) as described by Scholander et al. (1965). Ψ_{PD} was recorded between 04:00 and 06:00 am solar time, at the beginning and the end of the water deficit period.

Leaf relative water content (RWC) was determined at midday as described by Barrs & Weatherley (1962). Three discs from pooled samples of each variety were cut out with a cork borer between 11:00 am and 12:00 pm, and immediately weighed to determine their fresh weight (FW). The leaf discs were then immersed in distilled water, stored at 4°C in the dark for 24 hours and weighed afterwards to assess their turgid weight (TW). Then, the leaf discs were dried in a forced air circulation oven at 80°C for 24 hours until their weight was constant; the dry weight (DW) was measured. RWC was calculated with the following equation (1):

$$RWC\ (\%) = \frac{Fresh\ weight - Dry\ weight}{Turgid\ weight - Dry\ weight} \times 100 \quad (1)$$

2.3. Leaf gas exchange and chlorophyll fluorescence

Before the beginning of the experiment, five mature leaves per plant were selected and marked for all physiological measurements (15 independent biological replicates per variety). For each treatment, the measurements were performed between 09:00 and 11:00 am.

Net photosynthesis (P_{net}), stomatal conductance (g_s) and transpiration (E) were monitored with an infrared gas analyser LCPRO-SD (ADC, BioScientific Ltd., UK) fitted with the broad-leaf chamber with the photosynthetic photon flux maintained at 1400 µmol.m^{-2}.s^{-1}. The CO_2 concentration of 380 µmol.mol^{-1}, air flow of 200 µmol.s^{-1} and leaf temperature were fixed according to the environmental values. Data were recorded when the parameters stabilized (3-6 min).

The chlorophyll fluorescence of dark and light-adapted leaves was determined with an OS1p chlorophyll fluorometer (Opti-Sciences, Inc. Hudson, United States). The maximum (F_m), and variable (F_v) fluorescence were measured under steady-state conditions in dark-adapted leaves (30 minutes) and were used to calculate the maximal quantum yield of PSII (F_v/F_m). Also, the same measurements were recorded under light-adapted conditions, being referred to as F'_o and F'_m (minimum and maximum fluorescence, respectively). These parameters were used to calculate the non-photochemical quenching (NPQ) as NPQ=[$(F_m-F'_m)/F'_m$]. NPQ referred to the efficiency of heat dissipation according to Baker et al. (2007).

2.4. Scanning electron microscopy (SEM)

206 At the end of the water deficit treatment period, leaf pieces (1 cm^2) were sampled from
207 mid-laminar areas of mature leaves and fixed immediately in cold 2.5% glutaraldehyde in 100
208 mM sodium cacodylate buffer (pH 7.2). Fixed tissue was then rinsed in 100 mM sodium
209 cacodylate buffer (pH 7.2) and dehydrated with a range of calibrated ethanol concentrations
210 (30%, 50%, 75%, 90%, and 100%). Tissues were dried with CO_2 in an Emitech K850 critical
211 point dryer (Quorum Technologies Ltd, Ashford, United Kingdom). The sample was then
212 mounted on aluminium beads with double-sided carbon adhesive discs, coated with
213 gold/palladium in a SC7640 sputter-coater (Quorum Technologies Ltd, Newhaven, United
214 Kingdom) and analysed with an S-3400N scanning electron microscope (Hitachi High-
215 Technologies Corporation, Tokyo, Japan) at an accelerating voltage of 5Kv at the University
216 of Corsica (Corte, France).
217 SEM measurements were performed using the microscope's software. Estimates of
218 length and width of stomata and ostioles were made using 30 independent measurements on
219 three different leaves for each variety and each condition (n=30). Stomatal density was
220 estimated in five independent measurements on three different leaves for each variety and
221 each condition (n=5) using Adobe Photoshop software. The number of stomata was counted
222 on the photographs and the stomata cut at the edge of the image was recorded on only two
223 adjacent sides.
224 2.5. Transmission electron microscopy (TEM)
225 At the end of the water deficit period, leaf pieces (1 mm^2) were sampled from mid-
226 laminar areas of mature leaves and fixed immediately in cold 2.5% glutaraldehyde in 100 mM
227 sodium cacodylate (pH 7.2). Fixed tissue was then rinsed in a 100 mM cacodylate buffer (pH
228 7.2), post-fixed in cold 1% osmium tetroxide in the same buffer for 1 hour, dehydrated
229 through a graded ethanol series (70% and 100%) and propylene oxide, embedded in Spurr,
230 and polymerized at 60°C for 24 hours. Ultra-thin sections (60-90 nm) were cut with Power
231 tome PC ultramicrotome (RMC Boeckeler, Tuscon, USA). The sections were placed on 200
232 copper grids and stained with UranyLess (Delta Microscopies, France) and lead citrate.
233 Sections were examined on a Hitachi H-7650 (Hitachi High-Technologies Corporation,
234 Tokyo, Japan) at an accelerating voltage of 80 Kv at the University of Corsica (Corte,
235 France).
236 TEM measurements were performed using the microscope's software. The length,
237 width, and thickness of cells and length, and the number of chloroplasts, starches,
238 plastoglobuli, and mitochondria were estimated on 10 cells of each variety, each treatment,

239 and each parenchyma type. The length and width of chloroplast, plastoglobuli, starches,
240 mitochondria, grana, the number of grana per cell section, and the number of thylakoids per
241 granum were estimated on 30 independent measurements of each variety, each treatment, and
242 each parenchyma type.

243 ### 2.6. Biochemical analysis

244 Three independent biological replicates of leaves samples (n=3) for each variety and
245 each treatment were harvested for biochemical analysis.
246 Lipid peroxidation was evaluated using the malondialdehyde concentration and was
247 determined in leaf samples as described by Santini et al. (2013). Frozen leaf powder (80 mg)
248 was homogenized in 2 mL 80% ethanol (v/v) and centrifuged at 3000 *x g* for 10 min at 4°C.
249 Absorbance was measured at 440, 535, and 600 nm against a blank.
250 The hydrogen peroxide concentration was determined using a PeroxiDetect Kit
251 (Sigma, Aldrich, St. Louis, MO, United States) as described by Lourkisti et al. (2020). Frozen
252 leaf powder (150 mg) was homogenized in 300 µL of distilled water and centrifuged at
253 21,000 *x g* for 15 min at 4°C. Absorbance was read at 560 nm with a microplate reader
254 (MULTISKAN FC^{TM}, Thermo Scientific, Waltham, MA, United States).
255 The concentration of proline was assayed as described by Carillo et al. (2011). Frozen
256 leaf powder (40 mg) was homogenized in 70% ethanol (v/v) and centrifuged at 15,000 *x g* for
257 15 min at 4°C. The absorbance was read at 520 nm with a microplate reader (MULTISKAN
258 FC^{TM}, Thermo Scientific, Waltham, MA, United States).
259 For measurements of antioxidant enzymatic activity, frozen powder (54 mg) was
260 homogenized in 2 mL of extraction buffer and centrifuged at 13,000 *x g* for 30 min at 4°C.
261 The supernatant was used to determine the activities of SOD (EC 1.15.1.1), CAT (EC
262 1.11.1.6), APX (EC 1.11.1.11), and DHAR (EC 1.8.5.1) as described by Santini et al. (2013).
263 Time-course measurements were done using a V-630 spectrophotometer (Jasco Inc., Tokyo,
264 Japan).

265 ### 2.7. Statistical analysis

266 Data were analysed with R statistical software (http://www.R-project.org). Multi-way
267 ANOVAs followed by the LSD test ($P<0.05$) were used to evaluate the influence of varieties
268 and treatments. A heat map was generated with RStudio software to determine the differences
269 between varieties and treatments for abaxial epidermis and ultrastructural spongic and
270 palisadic mesophyll features.

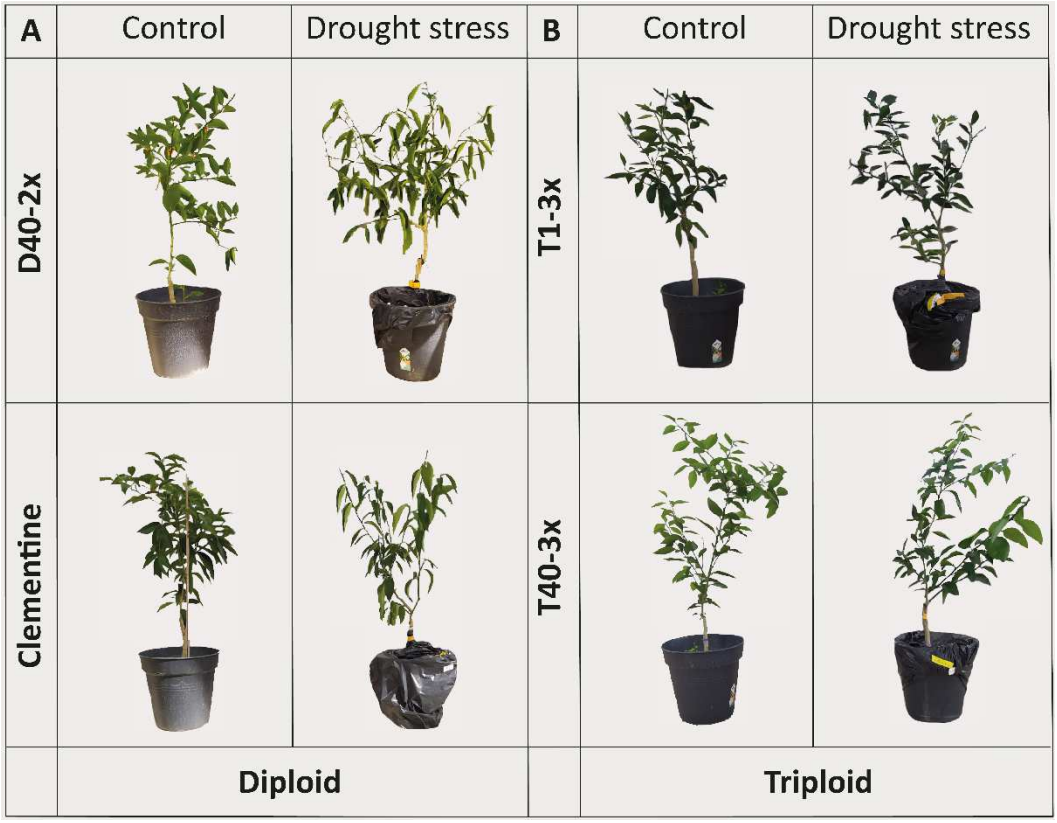


Figure 1: Morphological changes of control and drought-stressed plants in (A) diploid and (B) triploid varieties.

3. Results

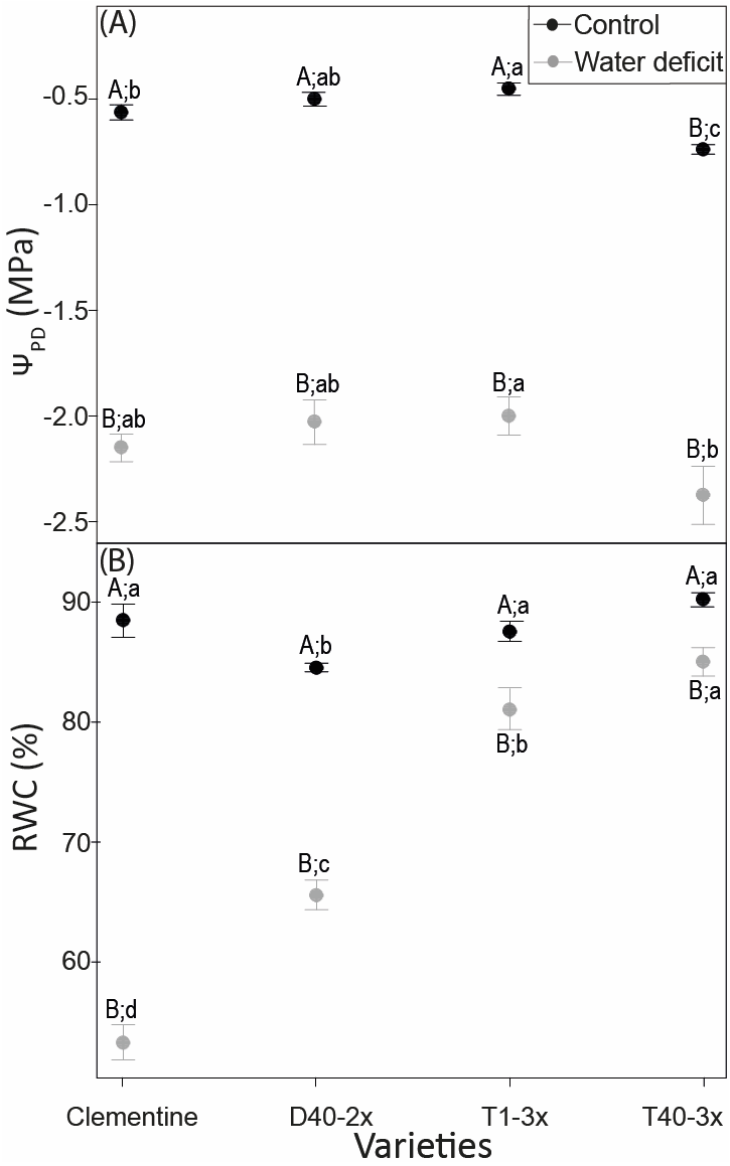
3.1. Leaf damages

Water deficit induced leaf wilting in 2x varieties (D40-2x and clementine) while no severe symptoms were observed in 3x varieties (Figure 1).

3.2. Plant water status

Under well-watered plants, leaf ψ_{PD} was similar through the studied varieties and reach -0.7 MPa (Figure 2A). Water deficit resulted in a decrease in ψ_{PD} in all varieties and T40-3x variety showed the lowest value (-2.4 MPa) under water deficit.

In control plants, leaf RWC was similar between varieties, except for D40-2x which exhibited the lowest value (84%) (Figure 2B). During water deficit, leaf RWC declined significantly in all varieties. In comparison with 2x varieties, 3x varieties showed highest values of RWC (between 81 and 85%).



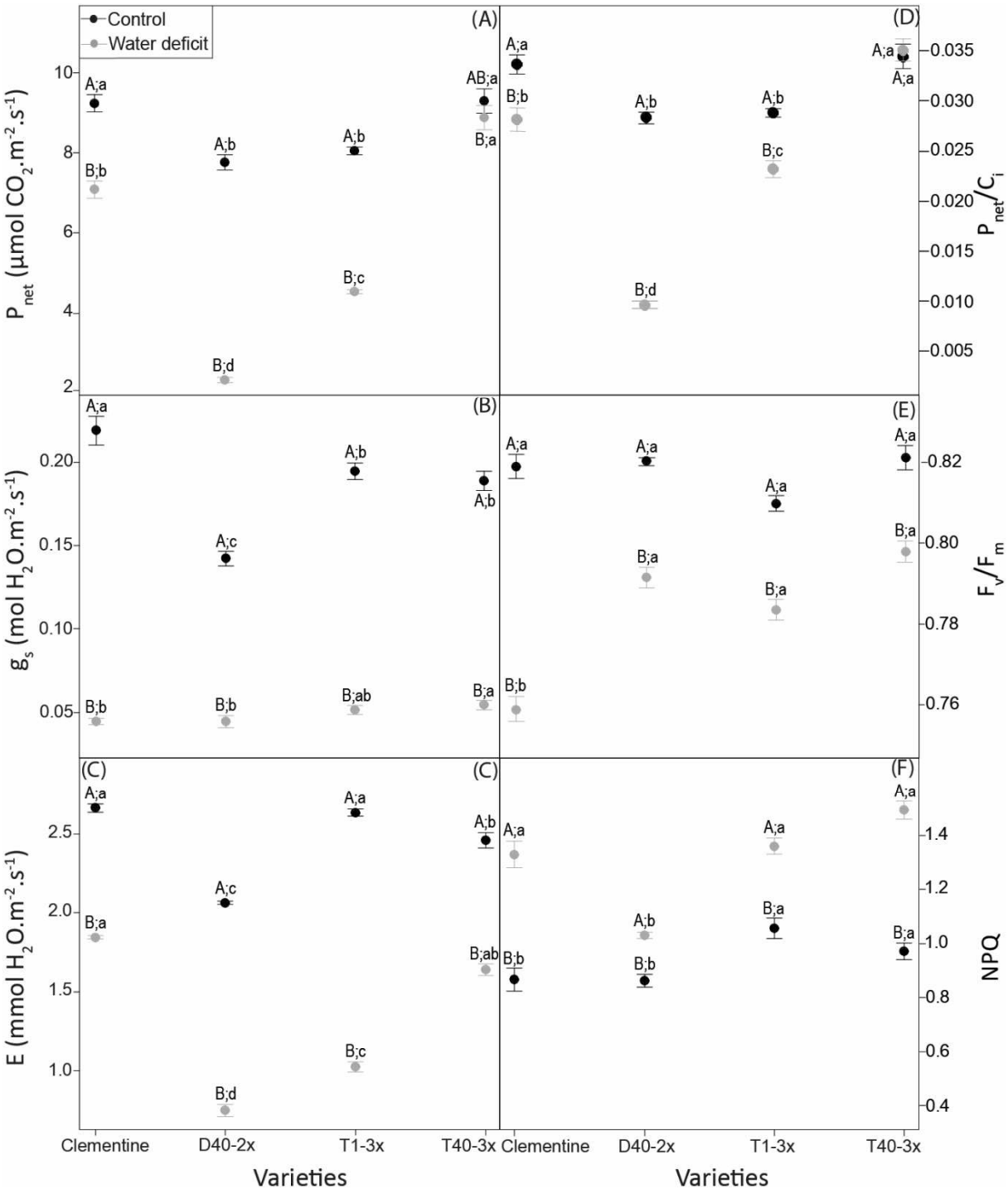
285

Figure 2: Changes in (A) pre-dawn leaf water potential (Ψ_{PD}) and (B) leaf relative water content (RWC) in varieties under control (black point) and water deficit (grey points) treatment. Data are mean values (± S.E.) of 3 independent measurements for Ψ_{PD} and RWC and 15 independent measurements (n=15). Data were analysed using ANOVA and Fisher's LSD test ($P<0.05$). Different uppercase letters show significant changes between the treatments (control and water deficit) for each variety and different lowercase letters show changes between the varieties for each treatment

3.3.Photosynthetic parameters

Photosynthesis rate (P_{net}) varied significantly among genotypes under well-watered plants where higher values were found in clementine and T40-3x variety (Figure 3A). Water deficit induced a sharp drop of P_{net} except in all varieties except for T40-3x which values were similar to control (Figure 3A). Similarly, significant differences in stomatal conductance (*gs*) were found among genotypes as well as among treatments (Figure 3B). Under water deficit, g_s declined significantly in all varieties where values were broadly similar among the varieties (Figure 3B). Transpiration rate (*E*) varied significantly among genotypes and among water regimes (Figure 3C). In control plants, the lowest value was found in D40-2x, while clementine and T1-3x exhibited the highest value. Water deficit resulted in decline in *E* in all varieties. Under water deficit, D40-2x variety showed the lowest value while the greatest value was found in clementine. In response to water deficit, carboxylation efficiency (*Pnet/Ci*) decreased in all varieties except for T40-3x (Figure 3D).

Similar values of the maximum quantum yield of efficiency (F_v/F_m) were found in well-watered plants (Figure 3E). In response to water deficit, F_v/F_m decreased in all varieties, although values of $\underline{F_v/F_m}$ was near to physiological value for T40-3x (Figure 3E). Non-photochemical quenching varied significantly among genotypes and treatments (Figure 3F). In control plants, 3x varieties showed the highest NPQ rate. Water deficit leads to an increase in NPQ rate in all varieties (Fig. 3F). While the NPQ rate was similar between clementine and 3x varieties, the latter had greater values than D40-2x variety.



30

Figure 3: Changes in (A) net photosynthesis (P_{net}), (B) stomatal conductance (g_s), (C) transpiration rate (E), (D) carboxylation efficiency (P_{net}/C_i), (E) maximal quantum yield of PSII (F_v/F_m) and (F) non-photochemical quenching (NPQ) rate in varieties under control (black points) and water deficit (grey points) treatments. Data are means values (± S.E.) of 15 independent measurements (n=15). Data were analysed using ANOVA and Fisher's LSD test (P<0.05). Different uppercase letters show significant changes between the treatments (control and water deficit) for each variety and different lowercase letters

3.4. Oxidative status

Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents varied significantly among genotypes and treatments (Figure 4). Water deficit induced increase in H_2O_2 content in D40-2x and T1-3x varieties while values of H_2O_2 content under water deficit were close to those obtain in control plants both in clementine and T40-3x (Figure 4A). A sharp increase in MDA content was also observed in clementine in response to water deficit (Figure 4B).

Antioxidant defences in terms of proline (Figure 4C) and antioxidant enzymes (SOD, CAT and APX) (Figures 4D, E, F) were also studied. Proline content rose in D40-2x and T40-3x in response to water deficit while a sharp decline was found in clementine (Figure 4C). The highest proline content was also observed in T40-3x under water deficit. Water deficit resulted in increase in SOD activity in clementine and T1-3x variety, while no significant change was observed in D40-2x and a decline was found in T40-3x (Figure 4D). Despite this decrease, this 3x variety still had higher activity than D40-2x. Except for clementine, a decline or a stability in CAT activity was observed in varieties in response to water deficit (Figure 4E). APX activity raised under water deficit in all varieties except for clementine where the same activity was reported in well-watered and drought-stressed plants (Figure 4F).

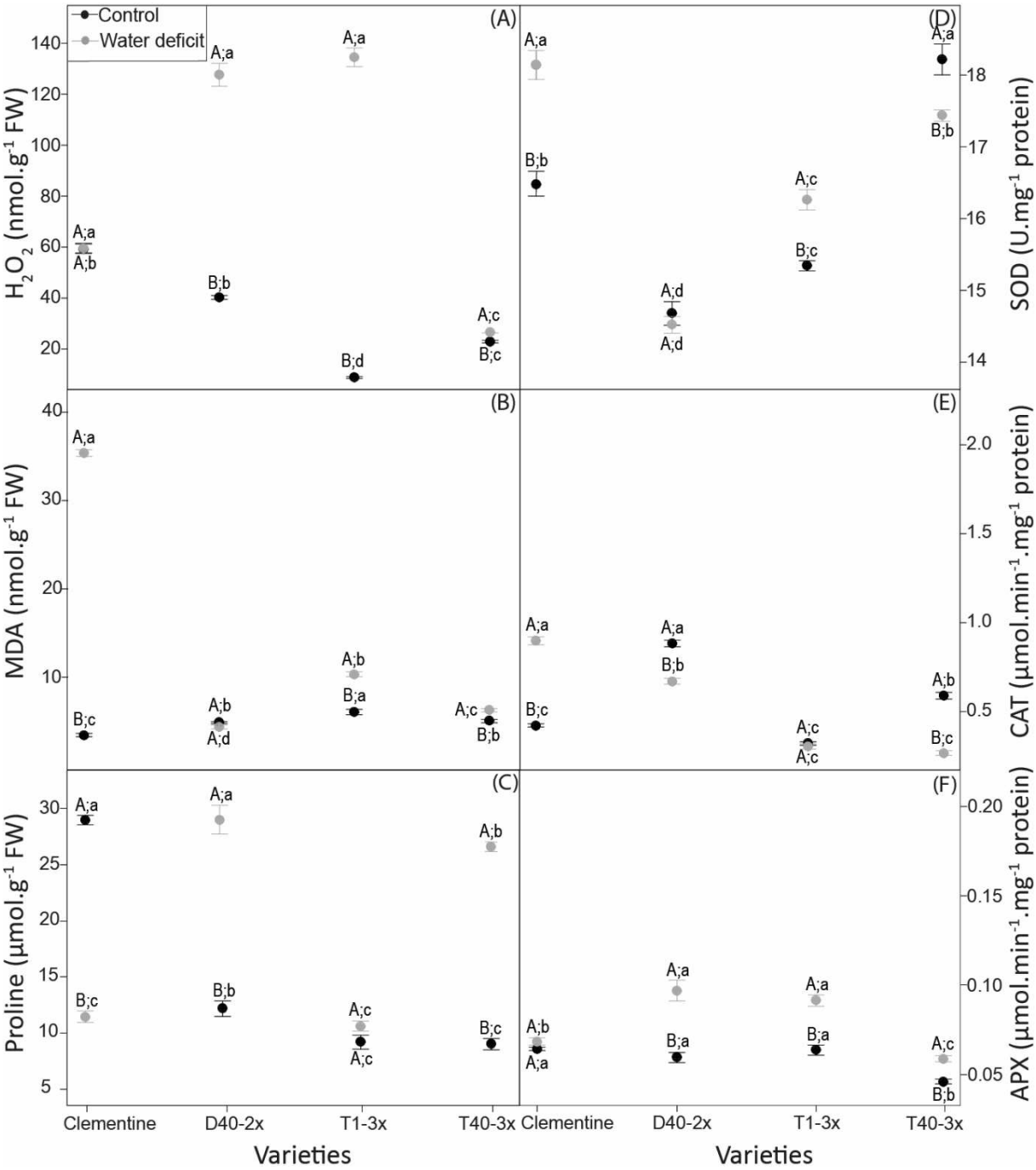


Figure 4: Changes in (A) malondialdehyde (MDA), (B) hydrogen peroxide (H_2O_2), (C) proline contents, (D) superoxide dismutase (SOD), (E) catalase (CAT) and (F) ascorbate peroxidase (APX) enzymatic activities in varieties under control (black points) and water deficit (grey points) treatments. Data are means values (± S.E.) of 15 independent measurements (*n*=15). Data were analysed using ANOVA and Fisher's LSD test (P<0.05). Different uppercase letters show significant changes between the treatments (control, water deficit, recovery) for each variety and different lowercase letters show changes between the varieties for each treatment.

323

3.5. Structural and ultrastructural changes in leaves

3.5.1. Stomatal characteristics

Stomata of all varieties were anomocytic (stomata surrounded by four subsidiary cells) and present on leaf abaxial surfaces. The epidermal carpet architecture was devoid of trichome (Figure 5). Stomata size and stomatal density varied among the ploidy level and treatment (Figs. 5, 6 and supplementary table 1). Triploidy was associated with increased stomata size and decreased stomatal density. Water deficit resulted in decline in ostiole size in D40-2x, T1-3x and T40-3x. This latter also showed a decrease in stomatal density under water deficit conditions (Figs. 5, 6).

3.5.2. Structural and ultrastructural changes in whole mesophyll cells and chloroplasts

Transmission electron micrographs of whole mesophyll cells of palisade and spongy parenchyma revealed several changes in the size of cells and chloroplasts among varieties and treatments (Figures 7;8;9;10 and supplementary tables 2, 3). Triploid varieties had a higher number of chloroplast, starches, mitochondria and grana in palisade parenchyma (Figs. 7; 8) while the number of these components differed greatly among the varieties in spongy parenchyma (Figs. 9; 10). A greater number of mitochondria was found in the spongy parenchyma of 3x varieties. Chloroplast of control plants were elongated with a typical disposition of thylakoid membranes with intact stacks of grana and stroma (Figs. 7, 9). Starch grains, plastoglobuli and mitochondria size were greater in 3x varieties both in palisade and spongy parenchyma (Figs. 8, 10).

Water deficit resulted in a decrease in components of mesophyll and spongy mesophyll cells (chloroplasts, starches and mitochondria) both in 2x and 3x varieties (Figs. 8, 10 and supplementary tables 2, 3). The number of chloroplasts found in both palisade and mesophyll parenchyma of 3x varieties was still higher than that of diploids under water deficit conditions. Water deficit resulted in damages in the ultrastructure of chloroplasts of mesophyll cells in 2x and 3x varieties (Figs. 7, 9). Less chloroplast damage was found in 3x varieties in comparison with 2x ones which had swollen thylakoids and dilated thylakoid membranes. Chloroplast of spongy mesophyll cells of D40-2x variety showed the most severe damage with undulated and disorganized thylakoid (Figure 9). Under water deficit, number and size of starch grain sharply declined in 2x and 3x varieties (Figs. 8, 10 and supplementary tables 2, 3). Starch grain number was more important in 3x varieties while D40-3x used all of its starch reserves in response to water deficit (Figs.7, 8, 9 and 10). Plastoglobuli decreased in

response to water deficit with some exceptions and a significant increase in length and width of PGs was observed in clementine and 3x varieties that had the highest values (Figs. 8, 10 and supplementary tables 2, 3).

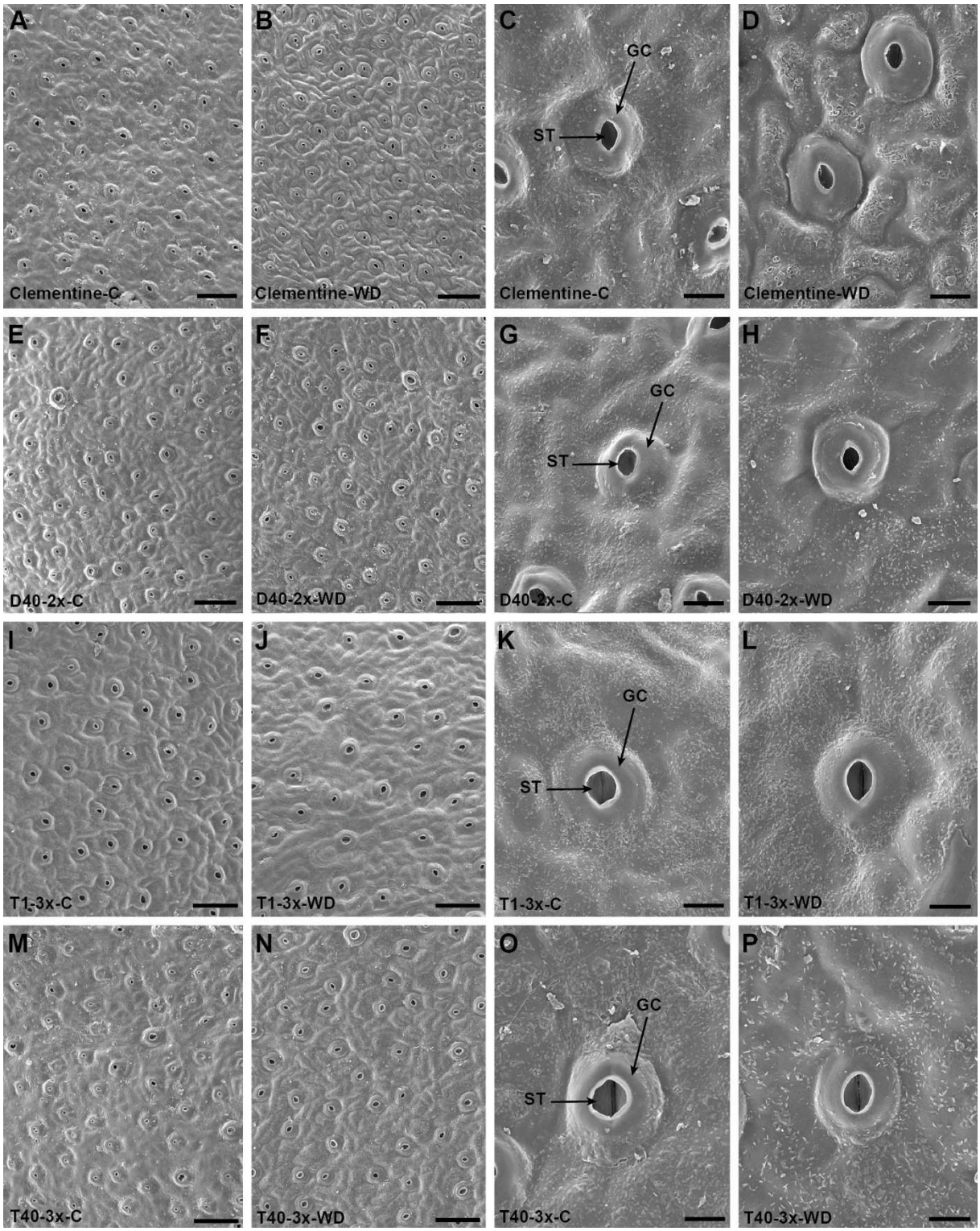


Figure 5: SEM micrographs of abaxial epidermis (1A, 1B, 1E, 1F, 1I,1J,1M, 1N; scale bar: 50 µm) and stomata (1C, 1D, 1G, 1H, 1K, 1L, 1O, 1P; scale bar: 10 µm) in diploid (clementine, D40-2x) and triploid (T1-3x and T40-3x) varieties under control (C) and water deficit (WD) conditions. GC: guard cells; ST: ostiole (Stoma).

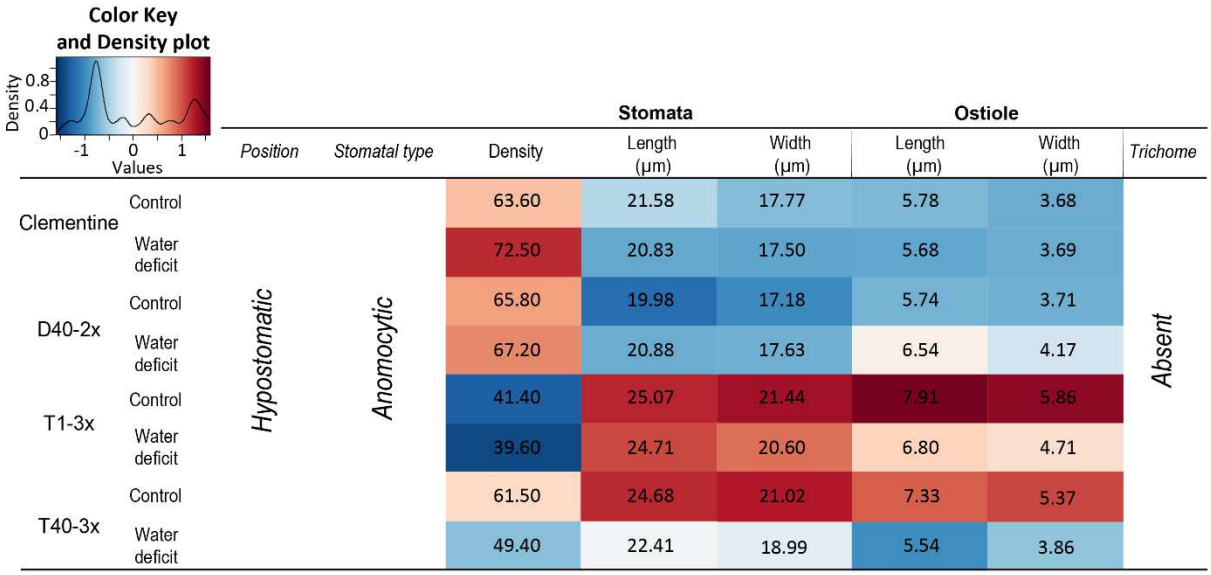


Figure 6: Heatmap showing changes between varieties and treatments (control and water deficit) for stomata and ostioles characteristics on leaf abaxial surface. Values are means of thirty independent measurements on five distinct leaves (n=150) for length and width of stomata and ostiole and of five independent measurements on five distinct leaves (n=25) for stomatal density. Values are associated with colour ranging from blue (low) to dark red (high).

361

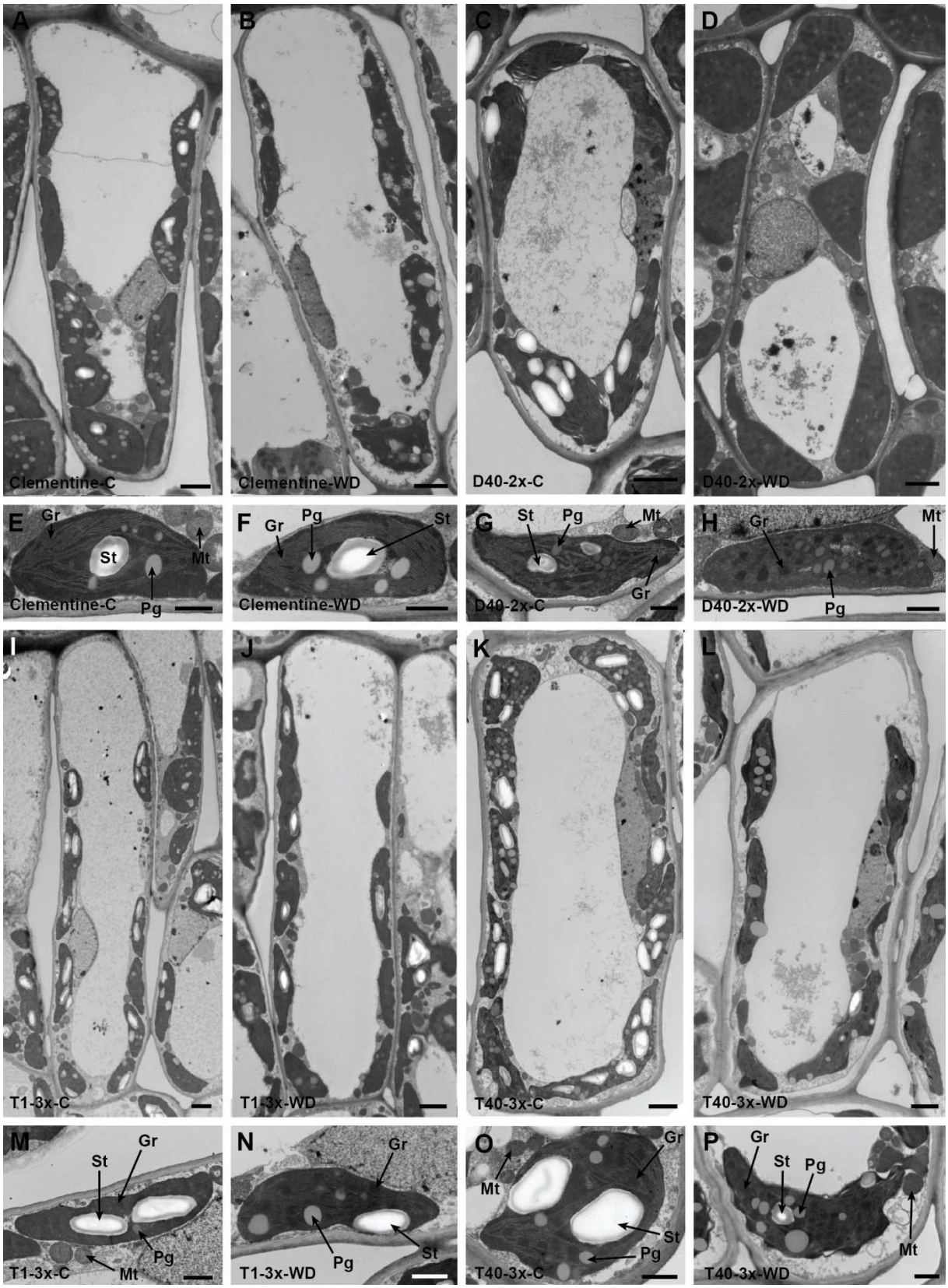
362

4. Discussion

4.1. Differences in water status in triploid and diploid varieties under water deficit

In the present study, the decrease in soil water potential showed that a water deficit occurred in all scions grafted to the same rootstock (Fig. 2A). However, we may not exclude that depending of the rootstock/scion association and the ploidy of the scion, the root development may have been changed and may have in turn modified the physiology of the tree. Water deficit induced significant changes in plant water relationships (Farooq et al., 2009) as evidenced by the decline in leaf relative water content (RWC) in all varieties in the present study (Figs. 2B). Triploid varieties were more capable of maintaining their water balance under severe water deficit as indicated by higher rates of RWC (Fig. 2B) in comparison with diploids which exhibited severe symptom as leaf wilting (Fig. 1A), at the same pre-dawn water potential (Fig. 2A). These results were in agreement with other studies on the effect that polyploidy has on water relations (Allario et al., 2013; Van Laere et al., 2011; Wang et al., 2012; Zandalinas et al., 2016). Further, osmotic adjustment referring to accumulation of several compounds such as soluble proline or sugars has been commonly observed in drought-stressed plants to help lower water potential and maintain turgor pressure (Kiani et al., 2007; Souza et al., 2004; Van Laere et al., 2011). In our study, proline accumulation was found in T40-3x variety (Fig. 4C). Related to high RWC, this could indicate a better osmotic adjustment under sever water deficit. However, high proline content was also observed in D40-2x which present low RWC and leaf wilting. Thus, the maintenance of water status in 3x varieties may be also due to accumulation of other osmolytes as soluble sugars (Al-Yasi et al., 2020; Blum, 2017). In the present study, the sharp decrease in starch grain size in chloroplasts of triploid and diploid varieties during water deficit suggested conversion of starch to soluble sugars to provide carbon and energy to the plant (Figs.7, 8,9, 10). Yang et al. (2004) reported that the decrease in starch reserves under drought conditions is mainly due to downregulation of enzymes involved in starch synthesis and upregulation of starch degradation. Hence, lower activity of starch synthesis enzymes (soluble starch synthase, sucrose synthase, and ADP Glucose pyrophosphorylase), resulting in an accumulation of sugars, were reported in plant cells' response to drought stress (Yang et al., 2019; Zhang et al., 2017). In the present study, 3x varieties had more starch grains even after its use (Figs. 8, 10). Associated with high RWC, this result suggested a better osmotic adjustment which probably resulted from soluble sugars in response to water deficit in triploids. Du et al. (2020) found that genes related to sucrose and starch metabolism were

upregulated in allotriploid *Populus* indicating the greater ability of triploids to metabolize sugar and sucrose and their high starch reserves and utilization. Since some studies suggested that carbon depletion was a mechanism underlying tree mortality during drought (McDowell et al., 2008), the increased carbon reserves induced by triploidy could play a significant role to withstand under severe water deficit conditions. Triploids showed a better water-holding capacity which seems to be associated with enhanced osmotic adjustment that may be crucial for better physiological and biochemical performance in water-limited conditions.



404 **Figure 7**: TEM micrographs of palisade mesophyll cells (3A; 3B; 3C; 3D; 3I; 3J; 3K; 3L; scale bar: 50 µm) and their respective chloroplasts (3E; 3F; 3G; 3H; 3M; 3N; 3O; 3P; scale bar: 1 µm) in diploid (clementine and D40-2x) and triploid (T1-3x and T40-3x) varieties control (C) and water deficit (WD) conditions. Gr: granum; Mt: mitochondria; Pg: plastoglobule; St: starch.

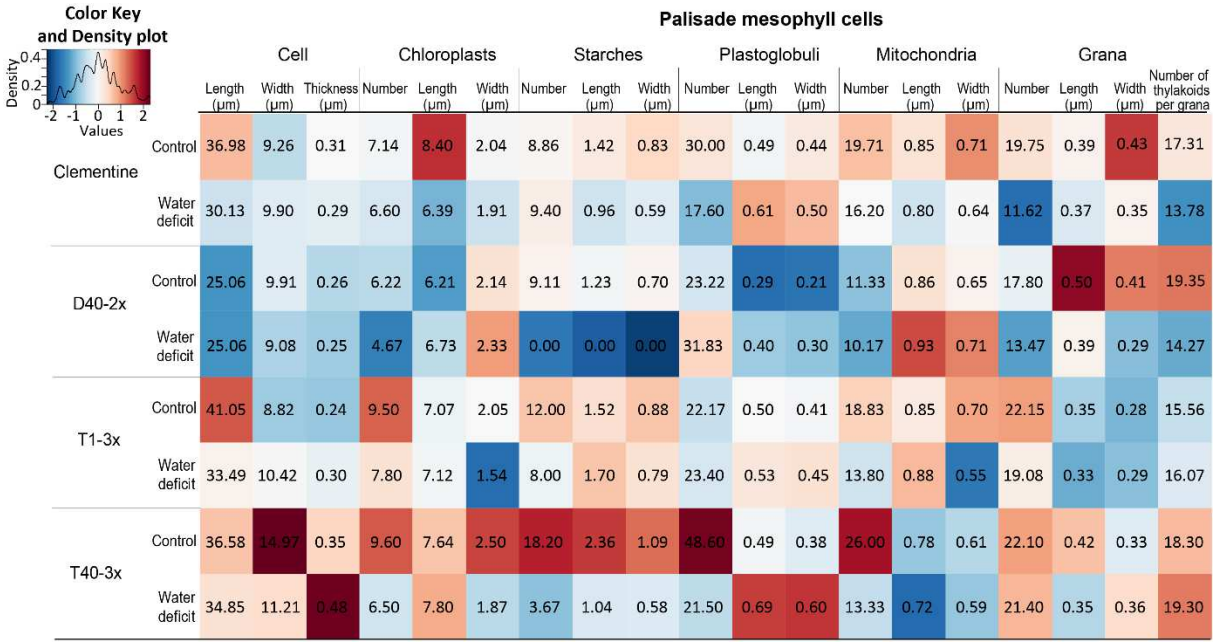


Figure 8: Heatmap showing changes between varieties and treatments (control and water deficit) for ultrastructure of palisade mesophyll cells. Values are means of independent measurements on five separate cell sections (n=5) for cell length, width and thickness and for cell constituents number (chloroplasts, starches, plastoglobuli, mitochondria) and of thirty independent measurements on separate cell sections (n=30) for cell constituents size, grana number per cell and thylakoid number per granum. Values are associated with colour ranging from blue (low) to dark

405

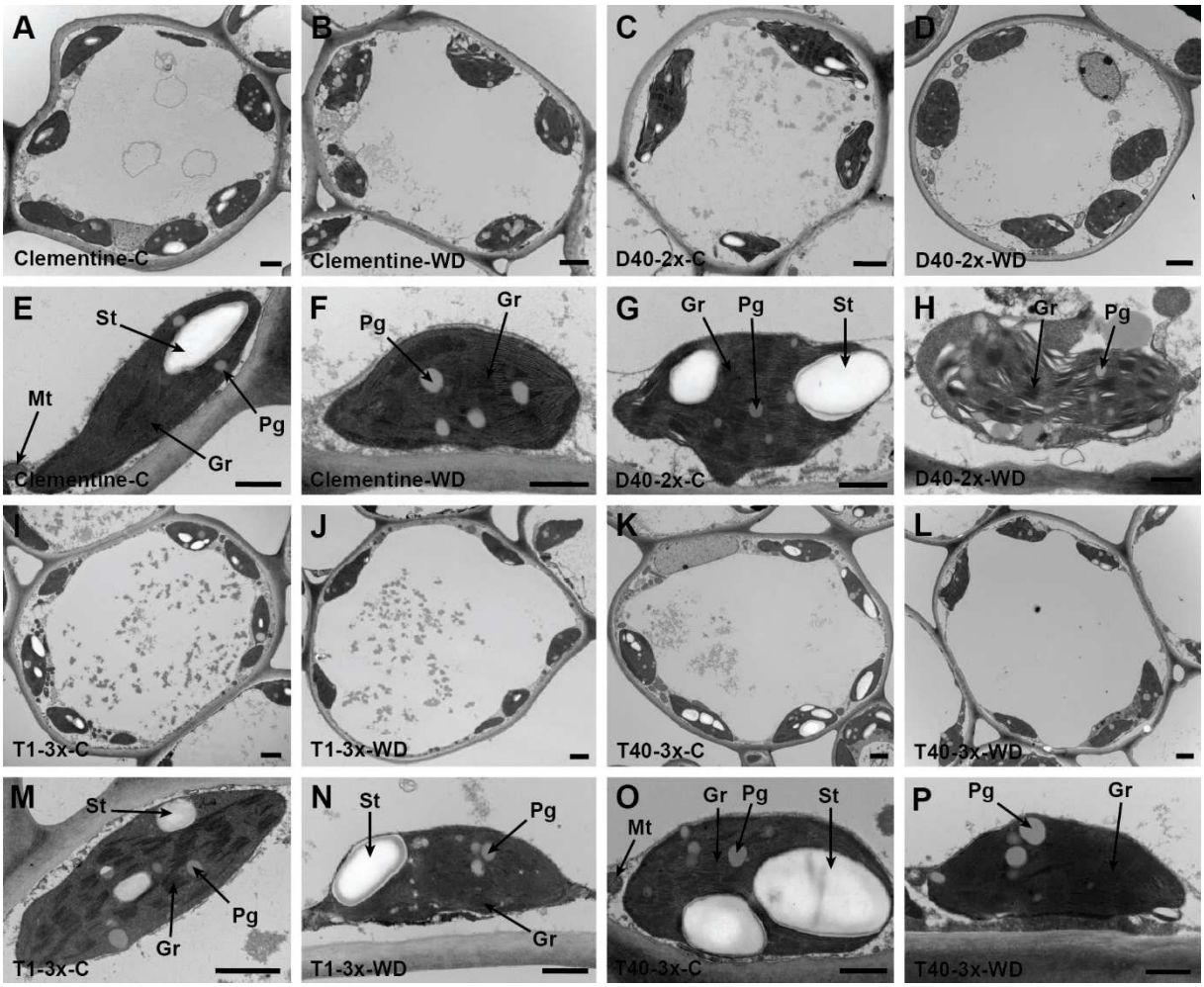
406 ### 4.2. Impact of water deficit on the photosynthetic activity of triploid and diploid
407 varieties

408 It is well known that stomatal closure appears to be the main physiological response to
409 water deficit and aims to minimize water loss (Chaves et al., 2003). In our study, water deficit
410 induced a sharp drop of g_s in all varieties where values were broadly similar among the
411 varieties indicating an effective stomatal response to water deficit (Fig. 3B). Stomatal traits
412 such as stomatal density and stomata size are vastly different across plant species (Caine et
413 al., 2019) and ploidy level (Chen, 2010) and thus are the main factors affecting leaf gas
414 exchange. Although Franks & Beerling (2009) reported that maximum stomatal conductance
415 to water vapor is negatively related to stomatal size in several species, some studies have
416 shown that higher stomatal conductance can be found in polyploid plants and resulted from
417 greater stomatal opening (Monda et al., 2016). In our study, stomatal changes induced by
418 triploidy, such as large stomata size and low stomatal density (Fig. 5; 6) could explain the
419 higher leaf gas exchange (Figs. 3) with greater RWC found in 3x varieties. In contrast, the
420 high transpiration rate in clementine could also be explained by high stomatal density found
421 under water deficit (Fig. 3C, 6). Muchow & Sinclair (1989) also suggested that water loss
422 above guard cell teichodes became a significant of water loss when stomata were nearly
423 closed. Our results suggested that maintenance of water balance in 3x varieties can protect
424 photosynthetic activity under water deficit conditions (Figs 2; 3). Similar relationships have
425 been found when comparing 4x and 2x citrus seedlings (Allario et al., 2013). Van Laere et al.
426 (2011) found that higher resistance of polyploid cultivars to drought stress was associated
427 with desirables changes in stomatal characteristics resulting in less disturbance of
428 photosynthetic capacity and better maintenance of water balance. Furthermore, studies
429 conducted on 3x *Populus* seedlings reported that both heterosis and polyploidy features
430 related to triploids were associated with better stomatal regulation in response to drought (Du
431 et al., 2020). Liqin et al. (2019) demonstrated that plant hormone-related genes involved in
432 the regulation of stomatal development were upregulated only in 3x *Populus* resulting in an
433 enhancement of auxin levels and an inhibition of stomatal development. These authors also
434 reported that the ABCG25 gene, involved in ABA signal stimulation, was upregulated in
435 triploids, compared to diploid and tetraploid *Populus* seedlings, leading to an enhancement of
436 ABA levels in guard cells and an inhibition in stomatal development.

437 Photosynthesis decline is one of the first consequence of stomatal closure (Flexas et
438 al., 1999) and can result from biochemical limitations especially under severe water deficit

(Manuela M. Chaves et al., 2003; Lawlor & Cornic, 2002). The P_{net}/C_i ratio is a useful indicator for estimation of Rubisco activity indicating its limitations under stressful conditions (Niinemets et al., 2009; Silva et al., 2013). In the present study, T40-3x maintained higher P_{net} rates than diploids, without decreasing P_{net}/C_i ratio (Figs. 3A, 3D) at the same water stress intensity (Fig. 2A). This result suggested that photosynthetic capacity was less affected and that the decrease in P_{net} was mainly induced by stomatal factor (Farquhar & Sharkey, 1982). In contrast, the decline of P_{net}/C_i ratio in D40-2x variety and to a lesser extent in clementine and T1-3x varieties indicated the decline in activity of CO_2 assimilation mechanisms at severe water deficit. Decrease in P_{net}/C_i ratio was also accompanied by a sharp drop in starch grain in D40-2x (Figs. 3D, 8, 10). This result suggested that production rather than utilization of photosynthates is reducing. Moreover, soluble sugars produced from starch conversion, particularly hexoses, may repress photosynthetic gene expression as gene encoding Rubisco nuclear sub-unit reducing Rubisco content and CO_2 assimilation (Sheen, 1994). Associated with low RWC, this result indicated that D40-2x variety was more affected by water deficit. Less disturbance of photosynthetic activity has already been reported in polyploid citrus genotypes under abiotic stresses (Khalid et al., 2020; Lourkisti et al., 2020; Oustric et al., 2019). For example, Li et al. (2009) showed that gas exchange and chlorophyll fluorescence were less affected for honeysuckle tetraploid cultivars than the diploids.

As a consequence, restricted CO_2 availability could lead to enhanced susceptibility to photo-damage and excess energy which must be dissipated. In our study, the maximum quantum yield of efficiency (F_v/F_m) decreased in all varieties in response to water deficit (Fig. 3E). Although low F_v/F_m (under the physiological value ~0.83) usually suggests PSII damage (Maxwell & Johnson, 2000), a decrease in F_v/F_m could also illustrate dynamic photoinhibition of the PSII and involvement of photoprotective mechanisms (Lambrev et al., 2012). Non-photochemical quenching (NPQ) is one of the major protective mechanisms that eliminates excess energy by heat dissipation and has been related to the xanthophyll cycle (Muller, 2001; Ruban, 2016). In the present study, water deficit leads to an increase in NPQ rate in all varieties (Fig. 3F). While the NPQ rate was similar between clementine and 3x varieties, the latter had greater values than D40-2x variety suggesting the effective removal of excess energy. Triploid varieties were less affected in terms of leaf gas exchange and chlorophyll fluorescence that may alleviate ROS accumulation in 3x varieties.



471

Figure 9: TEM micrographs of spongy mesophyll cells (3A; 3B; 3C; 3D; 3I; 3J; 3K; 3L; scale bar: 50 µm) and their respective chloroplasts (3E; 3F; 3G; 3H; 3M; 3N; 3O; 3P; scale bar: 1 µm) in diploid (Clementine and D40-2x) and triploid (T1-3x and T40-3x) varieties control (C) and water deficit (WD) conditions. Gr: granum; Mt: mitochondria; Pg: plastoglobule; St: starch.

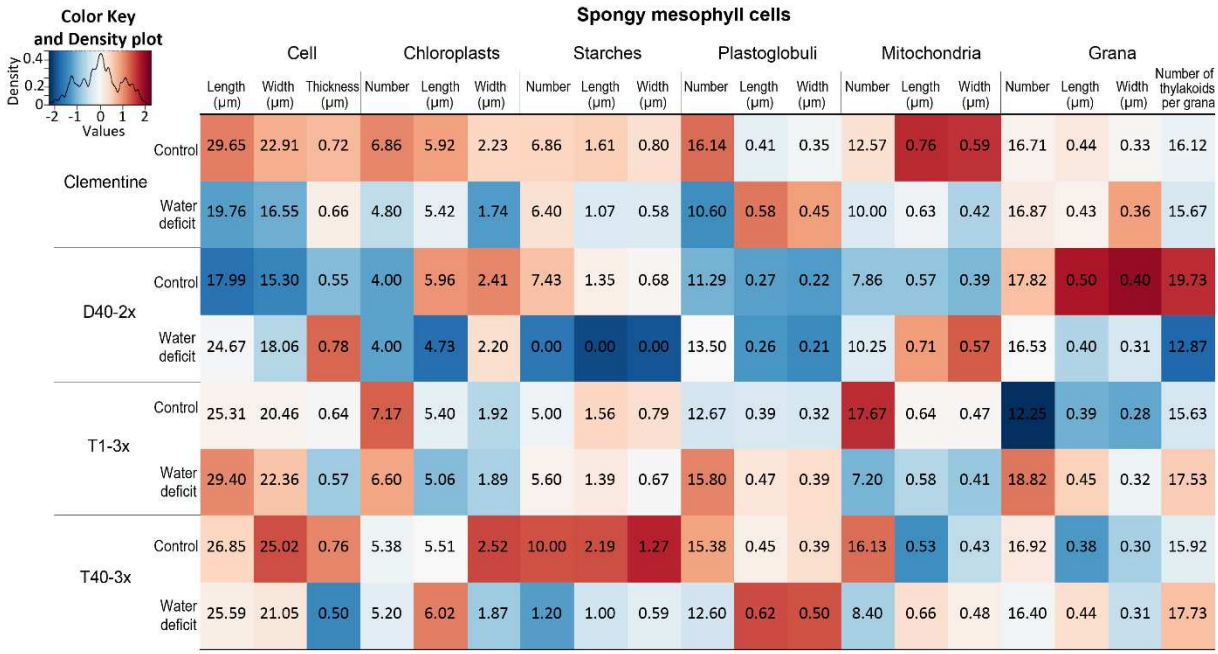


Figure 10: Heatmap showing changes between varieties and treatments (control and water deficit) for ultrastructure of spongy mesophyll cells. Values are means of independent measurements on five separate cell sections (*n*=5) for cell length, width and thickness and for cell constituents number (chloroplasts, starches, plastoglobuli, mitochondria) and of thirty independent measurements on separate cell sections (*n*=30) for cell constituents size, grana number per cell and thylakoid number per granum. Values are associated with colour ranging from blue (low) to dark red (high).

4.3. Role of the antioxidant system of 3x genotypes in water deficit tolerance

Exposure to adverse environmental conditions, including drought, can lead to oxidative stress when ROS production, such as hydrogen peroxide (H_2O_2), increases (Miller et al., 2010). Chloroplasts are a major source of ROS production and are consequently sensitive to oxidative damage when subjected to environmental stress including heat (Xu et al., 2006), drought (Wang et al., 2012) and nutrient deficiency (Oustric et al., 2019). Therefore, abiotic stresses were expected to induce damage due to ROS accumulation on chloroplast ultrastructure targeting thylakoid membranes and causing lipid peroxidation (Shao et al., 2016). In our study, T40-3x variety had the lowest oxidative markers levels (Fig. 4A, 4B) and both T1-3x and T40-3x exhibited less chloroplast damage (Figs.7, 9). While antioxidant enzyme activities did not discriminate 2x from 3x response (Figs. 4D, 4E, 4F), high proline content found in T40-3x (Fig. 4C) may explain less chloroplast damages since proline is involved in protection of membrane integrity and ROS scavenging (Dien et al., 2019; Szabados & Savouré, 2010). Triploid varieties had less cellular damage as a result of

water deficit (Figs.7 and 9) which was consistent with their water relationship and their gas exchange being less sensitive to water deficit (Figs. 2 and 3). Despite a sharp decrease, the number of chloroplasts found in both palisade and mesophyll parenchyma of 3x varieties was still higher than that of diploids (Figs. 8, 10) and could explain their better ability to maintain photosynthetic activity (Fig. 3). This result, associated with high RWC, indicated that triploids were less affected by water scarcity. In contrast, 2x varieties showed great content of H_2O_2 (D40-2x) and MDA (clementine) associated with more chloroplast damages (Figs. 3A, 3B, 9). Despite high activity of CAT and APX (Figs. 4E, 4F), 2x varieties showed severe damages as swollen thylakoids which were eventually destroyed (Fig. 9). Enhanced antioxidant activity, correlated with less oxidative damage, was also found in tetraploid citrus subjected to salinity stress (Khalid et al., 2020). Less chloroplast damage was also observed in polyploid citrus subjected to nutrient deficiency (Oustric et al., 2019) and polyploid apple rootstocks subjected to drought stress (Wang et al., 2012).

Plastoglobuli (PGs) containing lipids and antioxidants such as tocopherols, carotenes, and plastoquinones (Rottet et al., 2015) decreased in response to water deficit, with some exceptions (Figs. 7; 8; 9 and 10). However, a significant increase in length and width of PGs was observed in clementine and 3x varieties that had the highest values (Figs. 8, 10). This could illustrate an efficient antioxidant system, particularly in T40-3x. The involvement of PGs has already been proposed in response to drought stress (Mutava et al., 2015; Rey et al., 2000) although the mechanisms regulating this are still unclear. Moreover, an increase in PG size is suggestive of better metabolic exchange between the thylakoid membranes and PGs by storing destabilized lipids generated by oxidative stress and preserving thylakoid membranes (Figs. 4, 7, and 9).

5. Conclusion

The present study provides evidence that the better response of triploid varieties to water deficit can be attributed to changes in their morphological and cytological structure that provide more energy to adapt to adverse environments. The phenotypical advantages offered by triploidy results in greater water-holding capacity which seems to result from enhanced osmotic adjustment and protect the photosynthetic activity under drought conditions. The high carbohydrate reserve induced by triploidy results in better energy mobilization and may explain the better response to water deficit conditions in triploid varieties. It would be appropriate to dig deeper into the relationship between carbon storage and the tolerance to abiotic stress. This integrative approach also provides a deeper understanding of the basic mechanisms conferring water deficit tolerance. Combined with physiological and biochemical stress parameters, ultrastructural changes would be affected by various stresses and therefore appear to be suitable stress marker. Ploidy breeding to induce drought-tolerance-related morphological and physiological traits could be a useful tool leading to commercial success in fruit crops. The metabolic characterization of fruits of these new triploid varieties should be the next step before their introduction in the citrus market.

525 Author contributions

526 **Radia Lourkisti:** Acquisition of data, investigation, Formal analysis, Writing - original draft.
527 **Julie Oustric:** Acquisition of data, Investigation, Formal analysis, Writing – original draft.
528 **Yann Quilichini:** Formal analysis. **Yann Froelicher:** Writing – original draft. Supervision,
529 Validation. **Stéphane Herbette:** Supervision, Validation, Writing – original draft. **Raphael**
530 **Morillon:** Supervision, Validation, Writing – original draft. **Liliane Berti:** Supervision,
531 Validation. **Jérémie Santini:** Formal analysis, Investigation, Methodology, Supervision,
532 Validation, Visualization, writing – original draft, Writing – review & editing.

533 Funding

534 This work was supported by the “Collectivité de Corse” as part of the research project
535 “Innov’agrumes” (FEDER).

REFERENCES

Adams, K. L., & Wendel, J. F. (2005). Polyploidy and genome evolution in plants. *Current Opinion in Plant Biology*, *8*(2), 135–141. https://doi.org/10.1016/j.pbi.2005.01.001

Ahmed, D., Evrard, J.-C., Ollitrault, P., & Froelicher, Y. (2020). The effect of cross direction and ploidy level on phenotypic variation of reciprocal diploid and triploid mandarin hybrids. *Tree Genetics & Genomes*, *16*(1), 25. https://doi.org/10.1007/s11295-020-1417-7

Aleza, P., Juárez, J., Cuenca, J., Ollitrault, P., & Navarro, L. (2012). Extensive citrus triploid hybrid production by 2x × 4x sexual hybridizations and parent-effect on the length of the juvenile phase. *Plant Cell Reports*, *31*(9), 1723–1735. https://doi.org/10.1007/s00299-012-1286-0

Aleza, Pablo, Juárez, J., Ollitrault, P., & Navarro, L. (2010). Polyembryony in non-apomictic citrus genotypes. *Annals of Botany*, *106*(4), 533–545. https://doi.org/10.1093/aob/mcq148

Allario, T., Brumos, J., Colmenero-Flores, J. M., Iglesias, D. J., Pina, J. A., Navarro, L., Talon, M., Ollitrault, P., & Morillon, R. (2013). Tetraploid Rangpur lime rootstock increases drought tolerance via enhanced constitutive root abscisic acid production. *Plant, Cell & Environment*, *36*(4), 856–868. https://doi.org/10.1111/pce.12021

Al-Yasi, H., Attia, H., Alamer, K., Hassan, F., Ali, E., Elshazly, S., Siddique, K. H. M., & Hessini, K. (2020). Impact of drought on growth, photosynthesis, osmotic adjustment, and cell wall elasticity in Damask rose. *Plant Physiology and Biochemistry*, *150*, 133–139. https://doi.org/10.1016/j.plaphy.2020.02.038

Baker, N. R., Harbinson, J., & Kramer, D. M. (2007). Determining the limitations and regulation of photosynthetic energy transduction in leaves. *Plant, Cell & Environment*, *30*(9), 1107–1125. https://doi.org/10.1111/j.1365-3040.2007.01680.x

561 Balal, R. M., Shahid, M. A., Vincent, C., Zotarelli, L., Liu, G., Mattson, N. S.,
562 Rathinasabapathi, B., Martínez-Nicolas, J. J., & Garcia-Sanchez, F. (2017). Kinnow
563 mandarin plants grafted on tetraploid rootstocks are more tolerant to Cr-toxicity than
564 those grafted on its diploids one. *Environmental and Experimental Botany*, *140*, 8–18.
565 https://doi.org/10.1016/j.envexpbot.2017.05.011
566 Barrs, H., & Weatherley, P. (1962). A Re-Examination of the Relative Turgidity Technique
567 for Estimating Water Deficits in Leaves. *Australian Journal of Biological Sciences*,
568 *15*(3), 413. https://doi.org/10.1071/BI9620413
569 Birchler, J. A., Yao, H., Chudalayandi, S., Vaiman, D., & Veitia, R. A. (2010). Heterosis. *The*
570 *Plant Cell*, *22*(7), 2105–2112. https://doi.org/10.1105/tpc.110.076133
571 Blum, A. (2017). Osmotic adjustment is a prime drought stress adaptive engine in support of
572 plant production. *Plant, Cell & Environment*, *40*(1), 4–10.
573 https://doi.org/10.1111/pce.12800
574 Bondada, B. R., & Syvertsen, J. P. (2003). Leaf chlorophyll, net gas exchange and chloroplast
575 ultrastructure in citrus leaves of different nitrogen status. *Tree Physiology*, *23*(8), 553–
576 559. https://doi.org/10.1093/treephys/23.8.553
577 Caine, R. S., Yin, X., Sloan, J., Harrison, E. L., Mohammed, U., Fulton, T., Biswal, A. K.,
578 Dionora, J., Chater, C. C., & Coe, R. A. (2019). Rice with reduced stomatal density
579 conserves water and has improved drought tolerance under future climate conditions.
580 *New Phytologist*, *221*(1), 371–384.
581 Carillo, P., Gibon, Y., & contributors, P. (2011). PROTOCOL: Extraction and determination
582 of proline. *PrometheusWiki*.
583 Chaves, M. M., & Oliveira, M. M. (2004). Mechanisms underlying plant resilience to water
584 deficits: prospects for water-saving agriculture. *Journal of Experimental Botany*,
585 *55*(407), 2365–2384. https://doi.org/10.1093/jxb/erh269

586 Chaves, M. M., Maroco, J. P., & Pereira, J. S. (2003). Understanding plant responses to
587 drought — from genes to the whole plant. *Functional Plant Biology*, *30*(3), 239–264.
588 https://doi.org/10.1071/fp02076
589 Chen, Z. J. (2010). Molecular mechanisms of polyploidy and hybrid vigor. *Trends in Plant*
590 *Science*, *15*(2), 57–71. https://doi.org/10.1016/j.tplants.2009.12.003
591 Choudhury, F. K., Rivero, R. M., Blumwald, E., & Mittler, R. (2017). Reactive oxygen
592 species, abiotic stress and stress combination. *The Plant Journal: For Cell and*
593 *Molecular Biology*, *90*(5), 856–867. https://doi.org/10.1111/tpj.13299
594 Comai, L. (2005). The advantages and disadvantages of being polyploid. *Nature Reviews.*
595 *Genetics*, *6*(11), 836–846. https://doi.org/10.1038/nrg1711
596 Cuenca, J., Aleza, P., Juárez, J., Pina, J. A., & Navarro, L. (2010). 'Safor' Mandarin: A New
597 Citrus Mid-late Triploid Hybrid. *HortScience*, *45*(6), 977–980.
598 https://doi.org/10.21273/HORTSCI.45.6.977
599 Dien, D. C., Mochizuki, T., & Yamakawa, T. (2019). Effect of various drought stresses and
600 subsequent recovery on proline, total soluble sugar and starch metabolisms in Rice
601 (Oryza sativa L.) varieties. *Plant Production Science*, *22*(4), 530–545.
602 https://doi.org/10.1080/1343943X.2019.1647787
603 Du, K., Liao, T., Ren, Y., Geng, X., & Kang, X. (2020). Molecular Mechanism of Vegetative
604 Growth Advantage in Allotriploid Populus. *International Journal of Molecular*
605 *Sciences*, *21*(2). https://doi.org/10.3390/ijms21020441
606 Dutra de Souza, J., de Andrade Silva, E. M., Coelho Filho, M. A., Morillon, R., Bonatto, D.,
607 Micheli, F., & da Silva Gesteira, A. (2017). Different adaptation strategies of two
608 citrus scion/rootstock combinations in response to drought stress. *PLoS ONE*, *12*(5).
609 https://doi.org/10.1371/journal.pone.0177993

610 Farooq, M., Hussain, M., Wahid, A., & Siddique, K. H. M. (2012). Drought Stress in Plants:
611 An Overview. In R. Aroca (Ed.), *Plant Responses to Drought Stress: From*
612 *Morphological to Molecular Features* (pp. 1–33). Springer.
613 https://doi.org/10.1007/978-3-642-32653-0_1
614 Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., & Basra, S. M. A. (2009). Plant drought
615 stress: effects, mechanisms and management. *Agronomy for Sustainable Development*,
616 *29*(1), 185–212. https://doi.org/10.1051/agro:2008021
617 Farquhar, G.D., & Sharkey, T. D. (1982). Stomatal Conductance and Photosynthesis. *Annual*
618 *Review of Plant Physiology*, *33*(1), 317–345.
619 https://doi.org/10.1146/annurev.pp.33.060182.001533
620 Flexas, J., Bota, J., Loreto, F., Cornic, G., & Sharkey, T. D. (2004). Diffusive and metabolic
621 limitations to photosynthesis under drought and salinity in C(3) plants. *Plant Biology*
622 *(Stuttgart, Germany)*, *6*(3), 269–279. https://doi.org/10.1055/s-2004-820867
623 Flexas, J., Badger, M., Chow, W. S., Medrano, H., & Osmond, C. B. (1999). Analysis of the
624 Relative Increase in Photosynthetic O2 Uptake When Photosynthesis in Grapevine
625 Leaves Is Inhibited following Low Night Temperatures and/or Water Stress. *Plant*
626 *Physiology*, *121*(2), 675–684. https://doi.org/10.1104/pp.121.2.675
627 Franks, P. J., & Beerling, D. J. (2009). Maximum leaf conductance driven by CO2 effects on
628 stomatal size and density over geologic time. *Proceedings of the National Academy of*
629 *Sciences*, *106*(25), 10343–10347. https://doi.org/10.1073/pnas.0904209106
630 Gao, X., & Giorgi, F. (2008). Increased aridity in the Mediterranean region under greenhouse
631 gas forcing estimated from high resolution simulations with a regional climate model.
632 *Global and Planetary Change*, *62*(3–4), 195–209.
633 Hong-Bo, S., Xiao-Yan, C., Li-Ye, C., Xi-Ning, Z., Gang, W., Yong-Bing, Y., Chang-Xing,
634 Z., & Zan-Min, H. (2006). Investigation on the relationship of proline with wheat anti-

drought under soil water deficits. *Colloids and Surfaces. B, Biointerfaces*, *53*(1), 113–119. https://doi.org/10.1016/j.colsurfb.2006.08.008

IPCC (2014). Climate change 2014: synthesis report. Contribution of working groups I, II and III to the fifth assessment report of the intergovernmental panel on climate change. eds. Core Writing Team, R. K. Pachauri and L. A. Meyer (Geneva: IPCC), 151.

Khalid, M. F., Hussain, S., Anjum, M. A., Ahmad, S., Ali, M. A., Ejaz, S., & Morillon, R. (2020). Better salinity tolerance in tetraploid vs diploid volkamer lemon seedlings is associated with robust antioxidant and osmotic adjustment mechanisms. *Journal of Plant Physiology*, *244*, 153071. https://doi.org/10.1016/j.jplph.2019.153071

Khazaei, H., Monneveux, P., Hongbo, S., & Mohammady, S. (2010). Variation for stomatal characteristics and water use efficiency among diploid, tetraploid and hexaploid Iranian wheat landraces. *Genetic Resources and Crop Evolution*, *57*(2), 307–314.

Kiani, S. P., Talia, P., Maury, P., Grieu, P., Heinz, R., Perrault, A., Nishinakamasu, V., Hopp, E., Gentzbittel, L., Paniego, N., & Sarrafi, A. (2007). Genetic analysis of plant water status and osmotic adjustment in recombinant inbred lines of sunflower under two water treatments. *Plant Science*, *172*(4), 773–787. https://doi.org/10.1016/j.plantsci.2006.12.007

Krug, C. A. (1943). Chromosome Numbers in the Subfamily Aurantioideae with Special Reference to the Genus Citrus. *Botanical Gazette*, *104*(4), 602–611. https://doi.org/10.1086/335173

Lambrev, P. H., Miloslavina, Y., Jahns, P., & Holzwarth, A. R. (2012). On the relationship between non-photochemical quenching and photoprotection of Photosystem II. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1817*(5), 760–769. https://doi.org/10.1016/j.bbabio.2012.02.002

Lawlor, D. W. (2002). Limitation to Photosynthesis in Water-stressed Leaves: Stomata vs. Metabolism and the Role of ATP. *Annals of Botany*, *89*(7), 871–885. https://doi.org/10.1093/aob/mcf110

Lawlor, D. W., & Cornic, G. (2002). Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. *Plant, Cell & Environment*, *25*(2), 275–294. https://doi.org/10.1046/j.0016-8025.2001.00814.x

Leal-Bertioli, S. C. M., Bertioli, D. J., Guimarães, P. M., Pereira, T. D., Galhardo, I., Silva, J. P., Brasileiro, A. C. M., Oliveira, R. S., Silva, P. Í. T., Vadez, V., & Araujo, A. C. G. (2012). The effect of tetraploidization of wild Arachis on leaf morphology and other drought-related traits. *Environmental and Experimental Botany*, *84*, 17–24. https://doi.org/10.1016/j.envexpbot.2012.04.005

Li, W.-D., Biswas, D. K., Xu, H., Xu, C.-Q., Wang, X.-Z., Liu, J.-K., & Jiang, G.-M. (2009). Photosynthetic responses to chromosome doubling in relation to leaf anatomy in Lonicera japonica subjected to water stress. *Functional Plant Biology*, *36*(9), 783–792. https://doi.org/10.1071/FP09022

Liqin, G., Jianguo, Z., Xiaoxia, L., & Guodong, R. (2019). Polyploidy-related differential gene expression between diploid and synthesized allotriploid and allotetraploid hybrids of Populus. *Molecular Breeding*, *39*(5), 69. https://doi.org/10.1007/s11032-019-0975-6

Lourkisti, R., Froelicher, Y., Herbette, S., Morillon, R., Tomi, F., Gibernau, M., Giannettini, J., Berti, L., & Santini, J. (2020). Triploid Citrus Genotypes Have a Better Tolerance to Natural Chilling Conditions of Photosynthetic Capacities and Specific Leaf Volatile Organic Compounds. *Frontiers in Plant Science*, *11*. https://doi.org/10.3389/fpls.2020.00330

683 Maxwell, K., & Johnson, G. N. (2000). Chlorophyll fluorescence--a practical guide. *Journal*
684 *of Experimental Botany*, *51*(345), 659–668.
685 McDowell, N., Pockman, W. T., Allen, C. D., Breshears, D. D., Cobb, N., Kolb, T., Plaut, J.,
686 Sperry, J., West, A., Williams, D. G., & Yepez, E. A. (2008). Mechanisms of plant
687 survival and mortality during drought: why do some plants survive while others
688 succumb to drought? *New Phytologist*, *178*(4), 719–739.
689 https://doi.org/10.1111/j.1469-8137.2008.02436.x
690 Miller, G., Suzuki, N., Ciftci-Yilmaz, S., & Mittler, R. (2010). Reactive oxygen species
691 homeostasis and signalling during drought and salinity stresses. *Plant, Cell &*
692 *Environment*, *33*(4), 453–467. https://doi.org/10.1111/j.1365-3040.2009.02041.x
693 Mittler, R. (2002). *Oxidative stress, antioxidants and stress tolerance*. 6.
694 Monda, K., Araki, H., Kuhara, S., Ishigaki, G., Akashi, R., Negi, J., Kojima, M., Sakakibara,
695 H., Takahashi, S., & Hashimoto-Sugimoto, M. (2016). Enhanced stomatal
696 conductance by a spontaneous Arabidopsis tetraploid, Me-0, results from increased
697 stomatal size and greater stomatal aperture. *Plant Physiology*, *170*(3), 1435–1444.
698 Muchow, R., & Sinclair, T. (1989). Epidermal conductance, stomatal density and stomatal
699 size among genotypes of Sorghum bicolor (L.) Moench. *Plant, Cell & Environment*,
700 *12*(4), 425–431.
701 Muller, P. (2001). Non-Photochemical Quenching. A Response to Excess Light Energy.
702 *PLANT PHYSIOLOGY*, *125*(4), 1558–1566. https://doi.org/10.1104/pp.125.4.1558
703 Mutava, R. N., Prince, S. J. K., Syed, N. H., Song, L., Valliyodan, B., Chen, W., & Nguyen,
704 H. T. (2015). Understanding abiotic stress tolerance mechanisms in soybean: A
705 comparative evaluation of soybean response to drought and flooding stress. *Plant*
706 *Physiology and Biochemistry*, *86*, 109–120.
707 https://doi.org/10.1016/j.plaphy.2014.11.010

Navarro L., Aleza P., Cuenca J., Juárez J., José A. Pina, Carmen Ortega, Antonio Navarro, & Violeta Ortega. (2015). THE MANDARIN TRIPLOID BREEDING PROGRAM IN SPAIN. *Acta Horticulturae*, *1065*, 389–395. https://doi.org/10.17660/ActaHortic.2015.1065.48

Niinemets, U., Díaz-Espejo, A., Flexas, J., Galmés, J., & Warren, C. R. (2009). Importance of mesophyll diffusion conductance in estimation of plant photosynthesis in the field. *Journal of Experimental Botany*, *60*(8), 2271–2282. https://doi.org/10.1093/jxb/erp063

Ollitrault, P., Guo, W., & Grosser, J. W. (2007). Somatic hybridization. In I. A. Khan (Ed.), *Citrus genetics, breeding and biotechnology* (pp. 235–260). CABI. https://doi.org/10.1079/9780851990194.0235

Ollitrault, Patrick, Dambier, D., Luro, F., & Froelicher, Y. (2008). Ploidy Manipulation for Breeding Seedless Triploid Citrus. In *Plant Breeding Reviews* (pp. 323–352). John Wiley & Sons, Ltd. https://doi.org/10.1002/9780470380130.ch7

Oustric, J., Morillon, R., Luro, F., Herbette, S., Martin, P., Giannettini, J., Berti, L., & Santini, J. (2019). Nutrient Deficiency Tolerance in Citrus Is Dependent on Genotype or Ploidy Level. *Frontiers in Plant Science*, *10*. https://doi.org/10.3389/fpls.2019.00127

Rey, P., Gillet, B., Römer, S., Eymery, F., Massimino, J., Peltier, G., & Kuntz, M. (2000). Over-expression of a pepper plastid lipid-associated protein in tobacco leads to changes in plastid ultrastructure and plant development upon stress. *The Plant Journal: For Cell and Molecular Biology*, *21*(5), 483–494. https://doi.org/10.1046/j.1365-313x.2000.00699.x

Rottet, S., Besagni, C., & Kessler, F. (2015). The role of plastoglobules in thylakoid lipid remodeling during plant development. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1847*(9), 889–899. https://doi.org/10.1016/j.bbabio.2015.02.002

Ruban, A. V. (2016). Nonphotochemical Chlorophyll Fluorescence Quenching: Mechanism and Effectiveness in Protecting Plants from Photodamage1. *Plant Physiology*, *170*(4), 1903–1916. https://doi.org/10.1104/pp.15.01935

Santana-Vieira, D. D. S., Freschi, L., Almeida, L. A. da H., Moraes, D. H. S. de, Neves, D. M., Santos, L. M. dos, Bertolde, F. Z., Soares Filho, W. dos S., Coelho Filho, M. A., & Gesteira, A. da S. (2016). Survival strategies of citrus rootstocks subjected to drought. *Scientific Reports*, *6*(1), 38775. https://doi.org/10.1038/srep38775

Santini, J., Giannettini, J., Pailly, O., Herbette, S., Ollitrault, P., Berti, L., & Luro, F. (2013). Comparison of photosynthesis and antioxidant performance of several Citrus and Fortunella species (Rutaceae) under natural chilling stress. *Trees*, *27*(1), 71–83. https://doi.org/10.1007/s00468-012-0769-5

Scholander, P. F., Bradstreet, E. D., Hemmingsen, E. A., & Hammel, H. T. (1965). Sap Pressure in Vascular Plants: Negative hydrostatic pressure can be measured in plants. *Science*, *148*(3668), 339–346. https://doi.org/10.1126/science.148.3668.339

Shao, R. X., Xin, L. F., Zheng, H. F., Li, L. L., Ran, W. L., Mao, J., & Yang, Q. H. (2016). Changes in chloroplast ultrastructure in leaves of drought-stressed maize inbred lines. *Photosynthetica*, *54*(1), 74–80. https://doi.org/10.1007/s11099-015-0158-6

Sheen, J. (1994). Feedback control of gene expression. *Photosynthesis Research*, *39*(3), 427–438.

Silva, M. de A., Jifon, J. L., Santos, C. M. dos, Jadoski, C. J., & Silva, J. A. G. da. (2013). Photosynthetic capacity and water use efficiency in sugarcane genotypes subject to water deficit during early growth phase. *Brazilian Archives of Biology and Technology*, *56*(5), 735–748. https://doi.org/10.1590/S1516-89132013000500004

Souza, R. P., Machado, E. C., Silva, J. A. B., Lagôa, A. M. M. A., & Silveira, J. A. G. (2004). Photosynthetic gas exchange, chlorophyll fluorescence and some associated metabolic

changes in cowpea (Vigna unguiculata) during water stress and recovery. *Environmental and Experimental Botany*, *51*(1), 45–56. https://doi.org/10.1016/S0098-8472(03)00059-5

Szabados, L., & Savouré, A. (2010). Proline: a multifunctional amino acid. *Trends in Plant Science*, *15*(2), 89–97. https://doi.org/10.1016/j.tplants.2009.11.009

Tan, F.-Q., Tu, H., Liang, W.-J., Long, J.-M., Wu, X.-M., Zhang, H.-Y., & Guo, W.-W. (2015). Comparative metabolic and transcriptional analysis of a doubled diploid and its diploid citrus rootstock (C. junos cv. Ziyang xiangcheng) suggests its potential value for stress resistance improvement. *BMC Plant Biology*, *15*. https://doi.org/10.1186/s12870-015-0450-4

Urban, L., Aarrouf, J., & Bidel, L. P. R. (2017). Assessing the Effects of Water Deficit on Photosynthesis Using Parameters Derived from Measurements of Leaf Gas Exchange and of Chlorophyll a Fluorescence. *Frontiers in Plant Science*, *8*. https://doi.org/10.3389/fpls.2017.02068

Van Laere, K., França, S. C., Vansteenkiste, H., Van Huylenbroeck, J., Steppe, K., & Van Labeke, M.-C. (2011). Influence of ploidy level on morphology, growth and drought susceptibility in Spathiphyllum wallisii. *Acta Physiologiae Plantarum*, *33*(4), 1149–1156.

Wang, S., Liang, D., Li, C., Hao, Y., Ma, F., & Shu, H. (2012). Influence of drought stress on the cellular ultrastructure and antioxidant system in leaves of drought-tolerant and drought-sensitive apple rootstocks. *Plant Physiology and Biochemistry: PPB*, *51*, 81–89. https://doi.org/10.1016/j.plaphy.2011.10.014

Xu, S., Li, J., Zhang, X., Wei, H., & Cui, L. (2006). Effects of heat acclimation pretreatment on changes of membrane lipid peroxidation, antioxidant metabolites, and ultrastructure of chloroplasts in two cool-season turfgrass species under heat stress. *Environmental*

783 *and Experimental Botany*, *56*(3), 274–285.
784 https://doi.org/10.1016/j.envexpbot.2005.03.002
785 Xue, H., Zhang, B., Tian, J.-R., Chen, M.-M., Zhang, Y.-Y., Zhang, Z.-H., & Ma, Y. (2017).
786 Comparison of the morphology, growth and development of diploid and autotetraploid
787 'Hanfu' apple trees. *Scientia Horticulturae*, *225*, 277–285.
788 https://doi.org/10.1016/j.scienta.2017.06.059
789 Yang, H., Gu, X., Ding, M., Lu, W., & Lu, D. (2019). Activities of starch synthetic enzymes
790 and contents of endogenous hormones in waxy maize grains subjected to post-silking
791 water deficit. *Scientific Reports*, *9*(1), 7059. https://doi.org/10.1038/s41598-019-
792 43484-0
793 Yang, J., Zhang, J., Wang, Z., Xu, G., & Zhu, Q. (2004). Activities of Key Enzymes in
794 Sucrose-to-Starch Conversion in Wheat Grains Subjected to Water Deficit during
795 Grain Filling. *Plant Physiology*, *135*(3), 1621–1629.
796 https://doi.org/10.1104/pp.104.041038
797 Zandalinas, S. I., Balfagón, D., Arbona, V., & Gómez-Cadenas, A. (2017). Modulation of
798 Antioxidant Defense System Is Associated with Combined Drought and Heat Stress
799 Tolerance in Citrus. *Frontiers in Plant Science*, *8*.
800 https://doi.org/10.3389/fpls.2017.00953
801 Zandalinas, S. I., Rivero, R. M., Martínez, V., Gómez-Cadenas, A., & Arbona, V. (2016).
802 Tolerance of citrus plants to the combination of high temperatures and drought is
803 associated to the increase in transpiration modulated by a reduction in abscisic acid
804 levels. *BMC Plant Biology*, *16*(1), 105. https://doi.org/10.1186/s12870-016-0791-7
805 Zellnig, G., Perktold, A., & Zechmann, B. (2010). Fine structural quantification of drought-
806 stressed Picea abies (L.) organelles based on 3D reconstructions. *Protoplasma*, *243*(1–
807 4), 129–136.

Zhang, W., Gu, J., Wang, Z., Wei, C., Yang, J., & Zhang, J. (2017). Comparison of Structural and Functional Properties of Wheat Starch Under Different Soil Drought Conditions. *Scientific Reports*, *7*(1), 12312. https://doi.org/10.1038/s41598-017-10802-3