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

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

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17  
18

19 **Abstract**

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

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

## 42        **1. Introduction**

43            Polyploidy plays a major role in plant evolution and is considered an effective tool for  
44 crop improvement (Adams & Wendel, 2005; Comai, 2005). Polyploidy can occur  
45 spontaneously from unreduced gametes of somatic hybridization. Allopolyploids can thus  
46 combine polyploid advantages and heterosis that refers to the phenomenon that hybrid  
47 offspring have greater biomass and faster growth than the average of both parents (Birchler et  
48 al., 2010). Polyploid plants are widely associated with desirable traits such as large leaves and  
49 flowers, bigger leaf thickness, darker green coloration of leaves and changes in stomatal cell  
50 size and density (Allario et al., 2013; Santana-Vieira et al., 2016; Xue et al., 2017). Such  
51 anatomical, morphological, physiological and gene expression changes confer superior  
52 adaptation to a wider range of environmental stresses (Tan et al., 2015). Thus, examining  
53 cytological properties related to physiological and biochemical parameters may be an  
54 effective tool for explaining the better tolerance to abiotic stresses including drought. Some  
55 studies have shown the relationship between morphological variations and physiological  
56 changes with better tolerance of polyploid plants, especially tetraploids, under stressful  
57 conditions relative to their diploid counterparts (Allario et al., 2013; Bondada & Syvertsen,  
58 2003; Oustric et al., 2019). However, the tolerance of seedlings achieved by other  
59 economically relevant ploidy levels, such as triploidy, has not been studied.

60            Drought is the main environmental factor impacting crop yield and fruit quality  
61 (Chaves & Oliveira, 2004; LAWLOR, 2002). As climate prediction models show increased  
62 occurrences of drought episodes (IPCC 2014), global crop production will continue to be  
63 challenged. This trend is particularly worrying in the Mediterranean region which will  
64 experience more frequent periods of intensive drought, resulting in the extension of arid areas  
65 (Gao & Giorgi, 2008). Drought-sensitive plants exhibit various anatomical features including  
66 leaf wilting and leaf shedding that disrupt plant growth. Stomatal closure is among the first  
67 response of plants upon exposure to water-deficit and aims to limit water loss through  
68 transpiration (Farooq et al., 2012). However, it also limits CO<sub>2</sub> influx resulting in a lower  
69 photosynthesis rate. Although stomatal limitations may be the key factor responsible for the  
70 lower photosynthesis rate, non-stomatal limitations can occur and may be more important  
71 during severe water deficit (Farooq et al., 2009; Flexas et al., 2004). These limitations include  
72 the decline of major components of photosynthetic capacity, as the maximum carboxylation  
73 rate related to Rubisco activity, the light-saturated rate of electron transport and triose-  
74 phosphate used for starch and sucrose synthesis (Urban et al., 2017). The disturbance of

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

92 *Citrus* is one of the main commercial fruit crops and the Mediterranean area is an  
93 extensive citrus producer, especially for mandarins. The harvest season of mandarin,  
94 clementine, and other hybrids is extended from September to February. The lack of citrus fruit  
95 production (especially small fruits like mandarin) beyond this period and the steady rise in  
96 both consumption and consumer expectations, such as the seedlessness, highlight the need to  
97 find suitable varieties to satisfy the market demand, mainly between February and May. The  
98 creation of triploid hybrids may be a promising breeding strategy to develop new seedless  
99 citrus varieties. Citrus trees are mostly diploid with a basic chromosome number in citrus  
100 fruits and related genera of  $x=9$  (Krug, 1943). However, some exceptions can occur in natural  
101 triploid, especially ‘Tahiti’ lime, and in the tetraploid kumquat (Ollitrault et al., 2007).  
102 Triploid production has been already extended in the Mediterranean basin particularly in the  
103 mandarin breeding program (Ahmed et al., 2020; Aleza et al., 2010; Cuenca et al., 2010).  
104 Diploid x diploid hybridization is one of the main ways to produce triploid hybrids (Aleza et  
105 al., 2012; Ollitrault et al., 2008). Successful hybridization is mainly determined by the  
106 frequency of unreduced gametes by the female parent. In citrus triploid breeding programs,  
107 the ‘Fortune’ mandarin displayed the greatest frequency of triploid hybrids (Aleza et al.,  
108 2010; Navarro et al., 2015). Because of its polyploidy and fixed heterosis, triploid citrus can

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

112 While the greater genetic variability of tetraploids has widely been used in citrus  
113 rootstocks to improve stress tolerance (Allario et al., 2013; Balal et al., 2017; Khalid et al.,  
114 2020; Oustric et al., 2019), few reports have focused on the improved stress tolerance of  
115 triploid citrus plants (Lourkisti et al., 2020). In addition, modifications in cell morphology  
116 induced by polyploidy could also affect the response to abiotic stresses, including water  
117 deficit (Khazaei et al., 2010; Leal-Bertioli et al., 2012; Xue et al., 2017). Thus, the hypothesis  
118 we want to verify in this study is that the better response of citrus triploids to water deficit  
119 could be partly explained by their cytological and anatomical characteristics. Therefore, the  
120 INRA-CIRAD research centre (San Giuliano, Corsica France) has developed a crossbreeding  
121 program between ‘Fortune’ mandarin and ‘Ellendale’ tangor to generate innovative seedless  
122 citrus hybrids (Ahmed et al., 2020). The hybridization resulted in diploid and triploid  
123 populations. While the ‘Fortune’ mandarin was chosen for its ability to produce triploid  
124 hybrids and its pomological features, the ‘Ellendale’ tangor was chosen for its later  
125 production (April) and its organoleptic quality.

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

134

## 135 **2. Materials and methods**

### 136 **2.1. Plant material and experimental design**

137 Diploid (2x) and triploid (3x) citrus trees were generated from a hybridization between  
138 the ‘Fortune’ mandarin (*Citrus reticulata* Blanco) and the ‘Ellendale’ tangor [*Citrus reticulata*  
139 Blanco x *Citrus sinensis* (L) Osb.]. Diploid common clementine was also integrated in the  
140 study. Diploid and triploid scions were grafted on Citrange C-35 rootstock (*Citrus sinensis*  
141 ‘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<sup>2</sup> and 2 mm<sup>2</sup> 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 ( $\Psi_{PD}$ ) 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

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

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

186 
$$RWC (\%) = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100 \quad (1)$$

### 187 2.3. Leaf gas exchange and chlorophyll fluorescence

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

191 Net photosynthesis ( $P_{net}$ ), stomatal conductance ( $g_s$ ) and transpiration (E) were  
192 monitored with an infrared gas analyser LCPRO-SD (ADC, BioScientific Ltd., UK) fitted  
193 with the broad-leaf chamber with the photosynthetic photon flux maintained at 1400  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .  
194 The  $\text{CO}_2$  concentration of 380  $\mu\text{mol}\cdot\text{mol}^{-1}$ , air flow of 200  $\mu\text{mol}\cdot\text{s}^{-1}$  and leaf temperature  
195 were fixed according to the environmental values. Data were recorded when the parameters  
196 stabilized (3-6 min).

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

### 205 2.4. Scanning electron microscopy (SEM)



206 At the end of the water deficit treatment period, leaf pieces (1 cm<sup>2</sup>) 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<sub>2</sub> 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<sup>2</sup>) 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 UranylLess (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  $\times$  g for 10 min at 4°C.  
249 Absorbance was measured at 440, 535, and 600 nm against a blank.

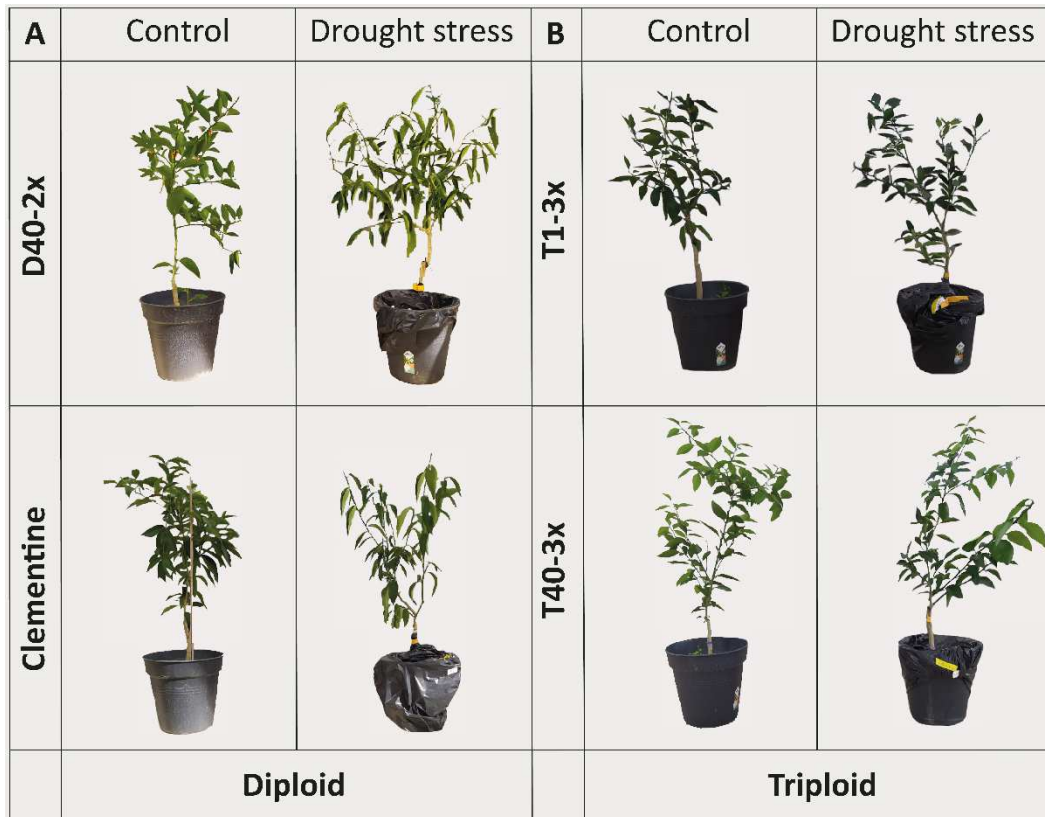
250 The hydrogen peroxide concentration was determined using a PeroxiDetect Kit  
251 (Sigma, Aldrich, St. Louis, MO, United States) as described by Lourkisti et al. (2020). Frozen  
252 leaf powder (150 mg) was homogenized in 300  $\mu$ L of distilled water and centrifuged at  
253 21,000  $\times$  g for 15 min at 4°C. Absorbance was read at 560 nm with a microplate reader  
254 (MULTISKAN FC<sup>TM</sup>, 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  $\times$  g for  
257 15 min at 4°C. The absorbance was read at 520 nm with a microplate reader (MULTISKAN  
258 FC<sup>TM</sup>, 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  $\times$  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 spongy and  
270 palisadic mesophyll features.



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

272 **3. Results**

273 **3.1. Leaf damages**

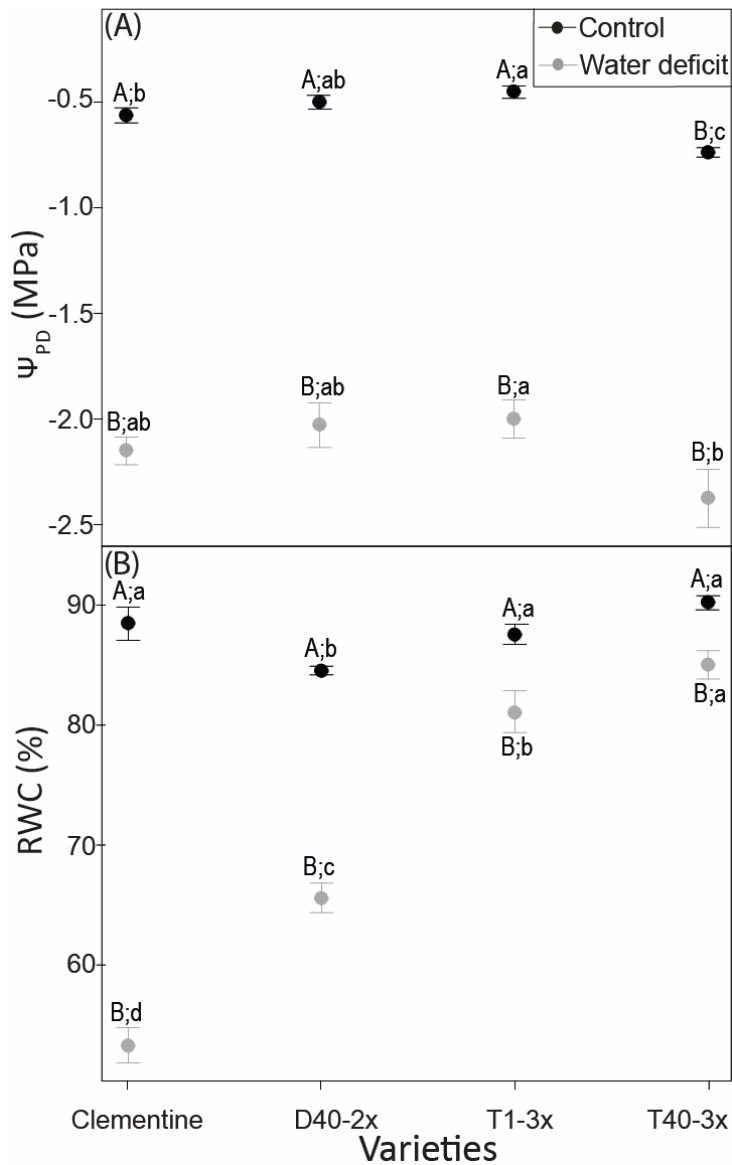
274 Water deficit induced leaf wilting in 2x varieties (D40-2x and clementine) while no  
275 severe symptoms were observed in 3x varieties (Figure 1).

276

277 **3.2. Plant water status**

278 Under well-watered plants, leaf  $\psi_{PD}$  was similar through the studied varieties and reach  
279 -0.7 MPa (Figure 2A). Water deficit resulted in a decrease in  $\psi_{PD}$  in all varieties and T40-3x  
280 variety showed the lowest value (-2.4 MPa) under water deficit.

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



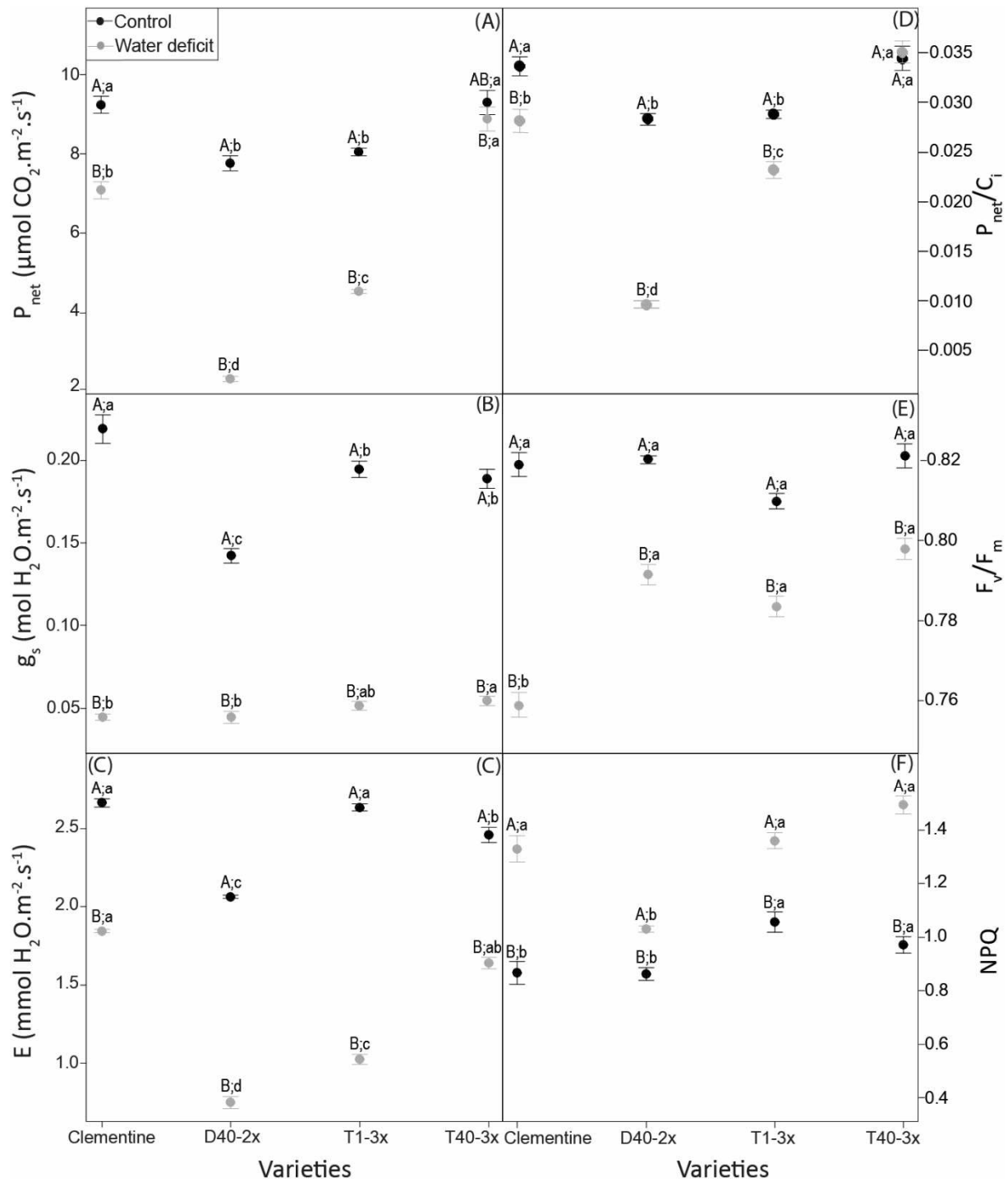
285

**Figure 2:** Changes in (A) pre-dawn leaf water potential ( $\Psi_{PD}$ ) and (B) leaf relative water content (RWC) in varieties under control (black point) and water deficit (grey points) treatment. Data are mean values ( $\pm$  S.E.) of 3 independent measurements for  $\Psi_{PD}$  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

286 **3.3. Photosynthetic parameters**

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

299 Similar values of the maximum quantum yield of efficiency ( $F_v/F_m$ ) were found in  
300 well-watered plants (Figure 3E). In response to water deficit,  $F_v/F_m$  decreased in all varieties,  
301 although values of  $F_v/F_m$  was near to physiological value for T40-3x (Figure 3E). Non-  
302 photochemical quenching varied significantly among genotypes and treatments (Figure 3F).  
303 In control plants, 3x varieties showed the highest NPQ rate. Water deficit leads to an increase  
304 in NPQ rate in all varieties (Fig. 3F). While the NPQ rate was similar between clementine and  
305 3x varieties, the latter had greater values than D40-2x variety.



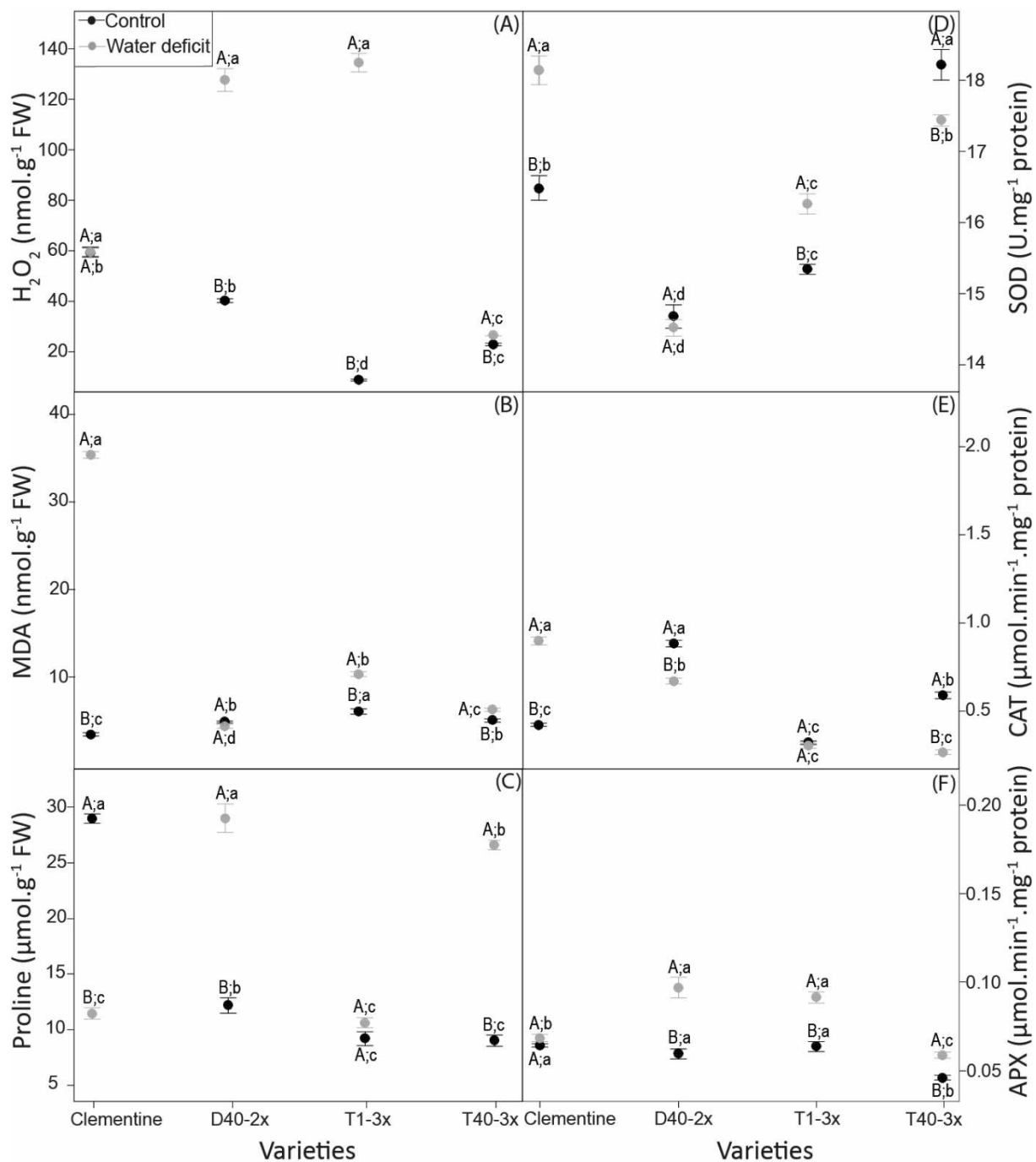
30<sup>~</sup> **Figure 3:** Changes in (A) net photosynthesis ( $P_{net}$ ), (B) stomatal conductance ( $g_s$ ), (C) transpiration rate ( $E$ ), (D) carboxylation efficiency ( $P_{net}/C_i$ ), (E) maximal quantum yield of PSII ( $F_v/F_m$ ) and (F) non-photochemical quenching (NPQ) rate in varieties under control (black points) and water deficit (grey points) treatments. Data are means values ( $\pm$  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

307 **3.4.Oxidative status**

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

313 Antioxidant defences in terms of proline (Figure 4C) and antioxidant enzymes (SOD,  
314 CAT and APX) (Figures 4D, E, F) were also studied. Proline content rose in D40-2x and T40-  
315 3x in response to water deficit while a sharp decline was found in clementine (Figure 4C).  
316 The highest proline content was also observed in T40-3x under water deficit. Water deficit  
317 resulted in increase in SOD activity in clementine and T1-3x variety, while no significant  
318 change was observed in D40-2x and a decline was found in T40-3x (Figure 4D). Despite this  
319 decrease, this 3x variety still had higher activity than D40-2x. Except for clementine, a  
320 decline or a stability in CAT activity was observed in varieties in response to water deficit  
321 (Figure 4E). APX activity raised under water deficit in all varieties except for clementine  
322 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<sub>2</sub>O<sub>2</sub>), (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.

324 **3.5. Structural and ultrastructural changes in leaves**

325 **3.5.1. Stomatal characteristics**

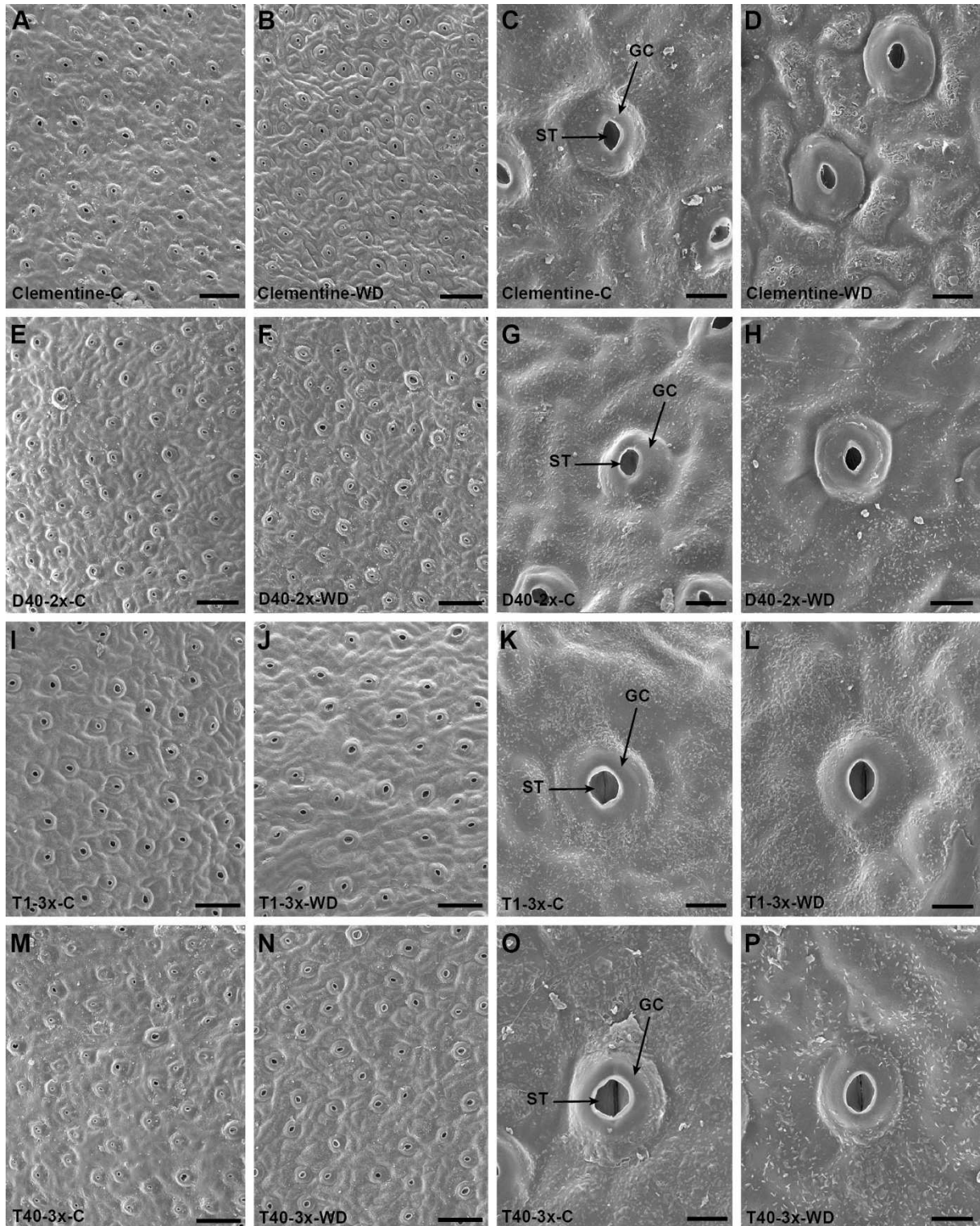
326 Stomata of all varieties were anomocytic (stomata surrounded by four subsidiary cells)  
327 and present on leaf abaxial surfaces. The epidermal carpet architecture was devoid of  
328 trichome (Figure 5). Stomata size and stomatal density varied among the ploidy level and  
329 treatment (Figs.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

333 **3.5.2. Structural and ultrastructural changes in whole mesophyll cells and**  
334 **chloroplasts**

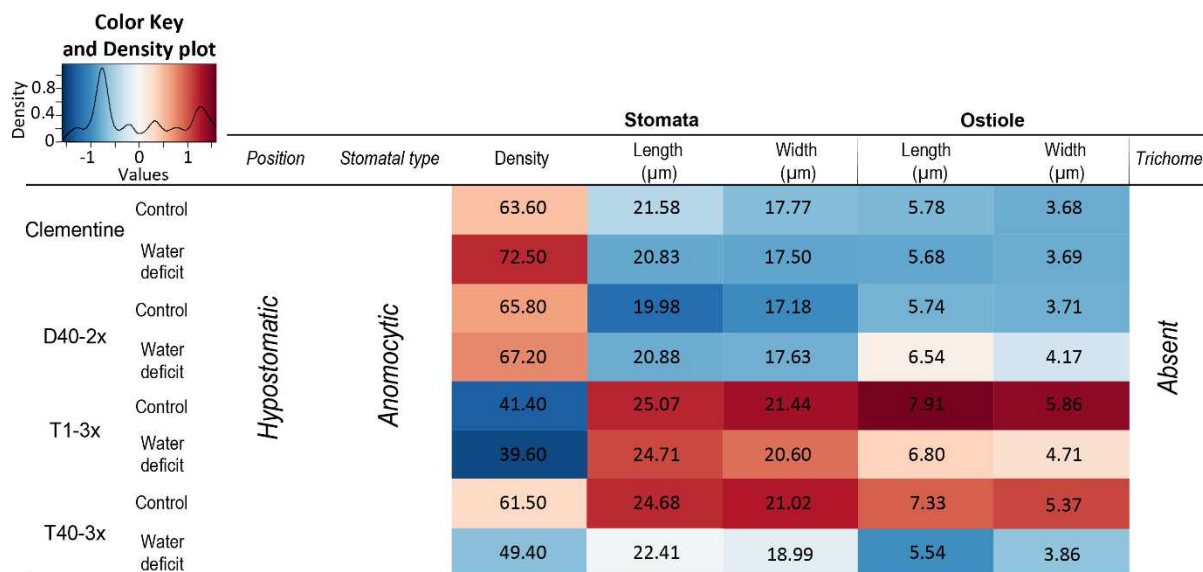
335 Transmission electron micrographs of whole mesophyll cells of palisade and spongy  
336 parenchyma revealed several changes in the size of cells and chloroplasts among varieties and  
337 treatments (Figures 7;8;9;10 and supplementary tables 2, 3). Triploid varieties had a higher  
338 number of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

345 Water deficit resulted in a decrease in components of mesophyll and spongy  
346 mesophyll cells (chloroplasts, starches and mitochondria) both in 2x and 3x varieties (Figs. 8,  
347 10 and supplementary tables 2, 3). The number of chloroplasts found in both palisade and  
348 mesophyll parenchyma of 3x varieties was still higher than that of diploids under water deficit  
349 conditions. Water deficit resulted in damages in the ultrastructure of chloroplasts of  
350 mesophyll cells in 2x and 3x varieties (Figs. 7, 9). Less chloroplast damage was found in 3x  
351 varieties in comparison with 2x ones which had swollen thylakoids and dilated thylakoid  
352 membranes. Chloroplast of spongy mesophyll cells of D40-2x variety showed the most severe  
353 damage with undulated and disorganized thylakoid (Figure 9). Under water deficit, number  
354 and size of starch grain sharply declined in 2x and 3x varieties (Figs. 8, 10 and supplementary  
355 tables 2, 3). Starch grain number was more important in 3x varieties while D40-3x used all of  
356 its starch reserves in response to water deficit (Figs.7, 8, 9 and 10). Plastoglobuli decreased in

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



36  
**Figure 5:** SEM micrographs of abaxial epidermis (1A, 1B, 1E, 1F, 1I,1J,1M, 1N; scale bar: 50  $\mu$ m) and stomata (1C, 1D, 1G, 1H, 1K, 1L, 1O, 1P; scale bar: 10  $\mu$ m) 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

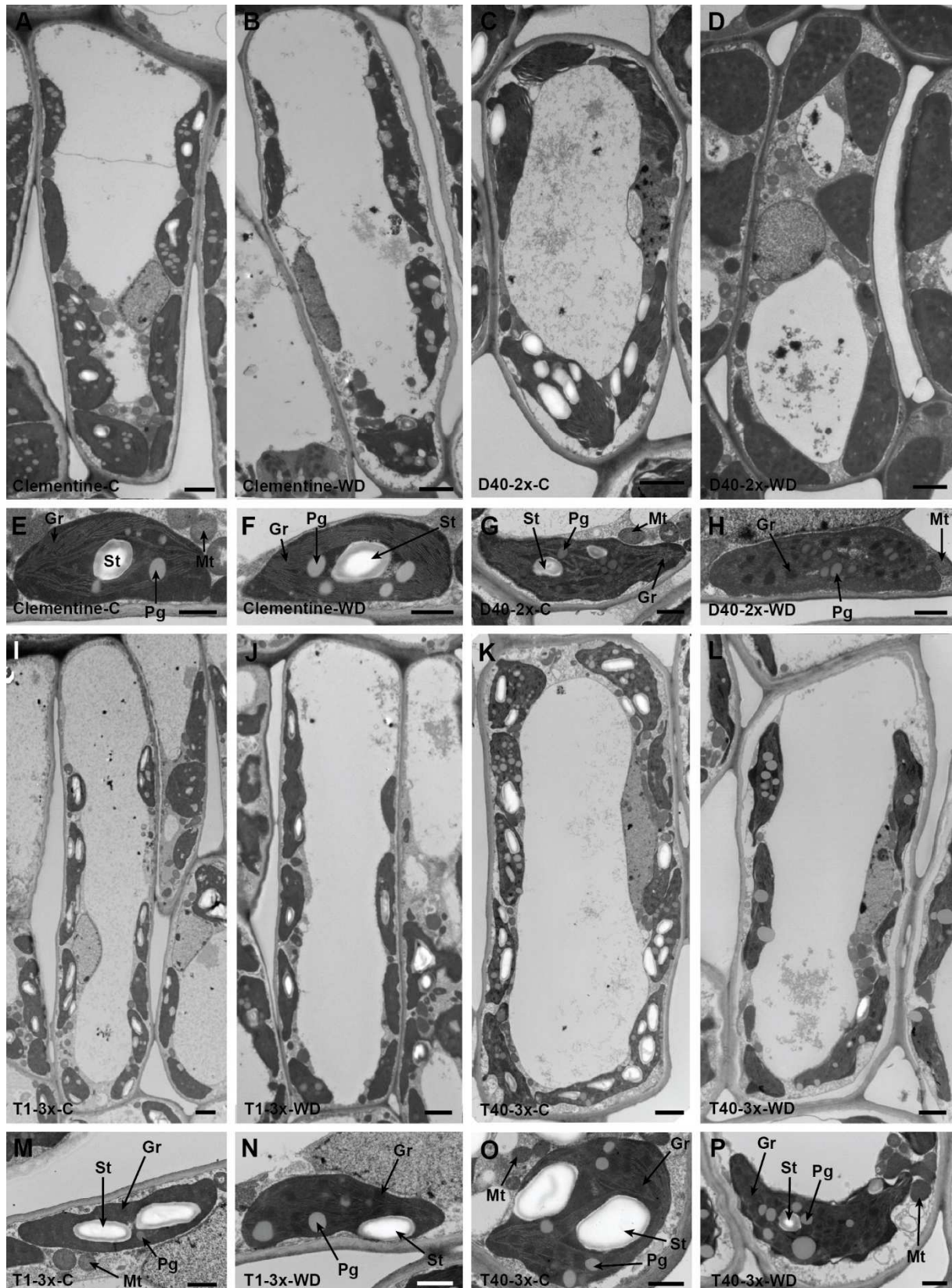


## 4. Discussion

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

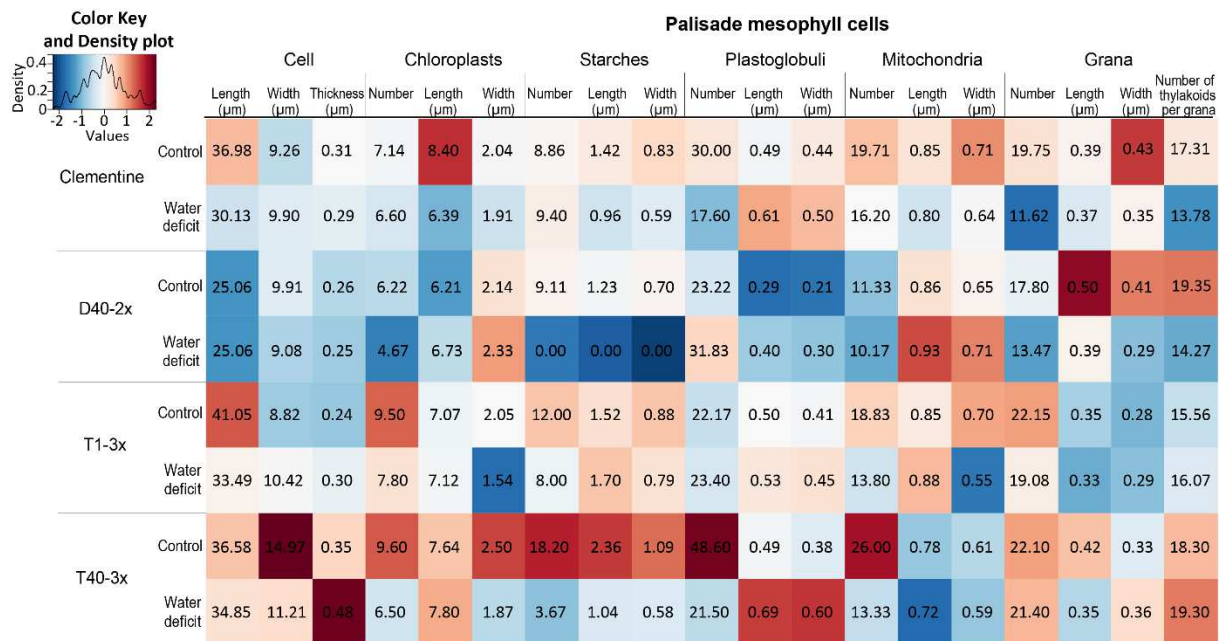
In the present study, the decrease in soil water potential showed that a water deficit occurred in all scions grafted to the same rootstock (Fig. 2A). However, we may not exclude that depending of the rootstock/scion association and the ploidy of the scion, the root development may have been changed 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

397 upregulated in allotriploid *Populus* indicating the greater ability of triploids to metabolize  
398 sugar and sucrose and their high starch reserves and utilization. Since some studies suggested  
399 that carbon depletion was a mechanism underlying tree mortality during drought (McDowell  
400 et al., 2008), the increased carbon reserves induced by triploidy could play a significant role  
401 to withstand under severe water deficit conditions. Triploids showed a better water-holding  
402 capacity which seems to be associated with enhanced osmotic adjustment that may be crucial  
403 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  $\mu\text{m}$ ) and their respective chloroplasts (3E; 3F; 3G; 3H; 3M; 3N; 3O; 3P; scale bar: 1  $\mu\text{m}$ ) 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

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

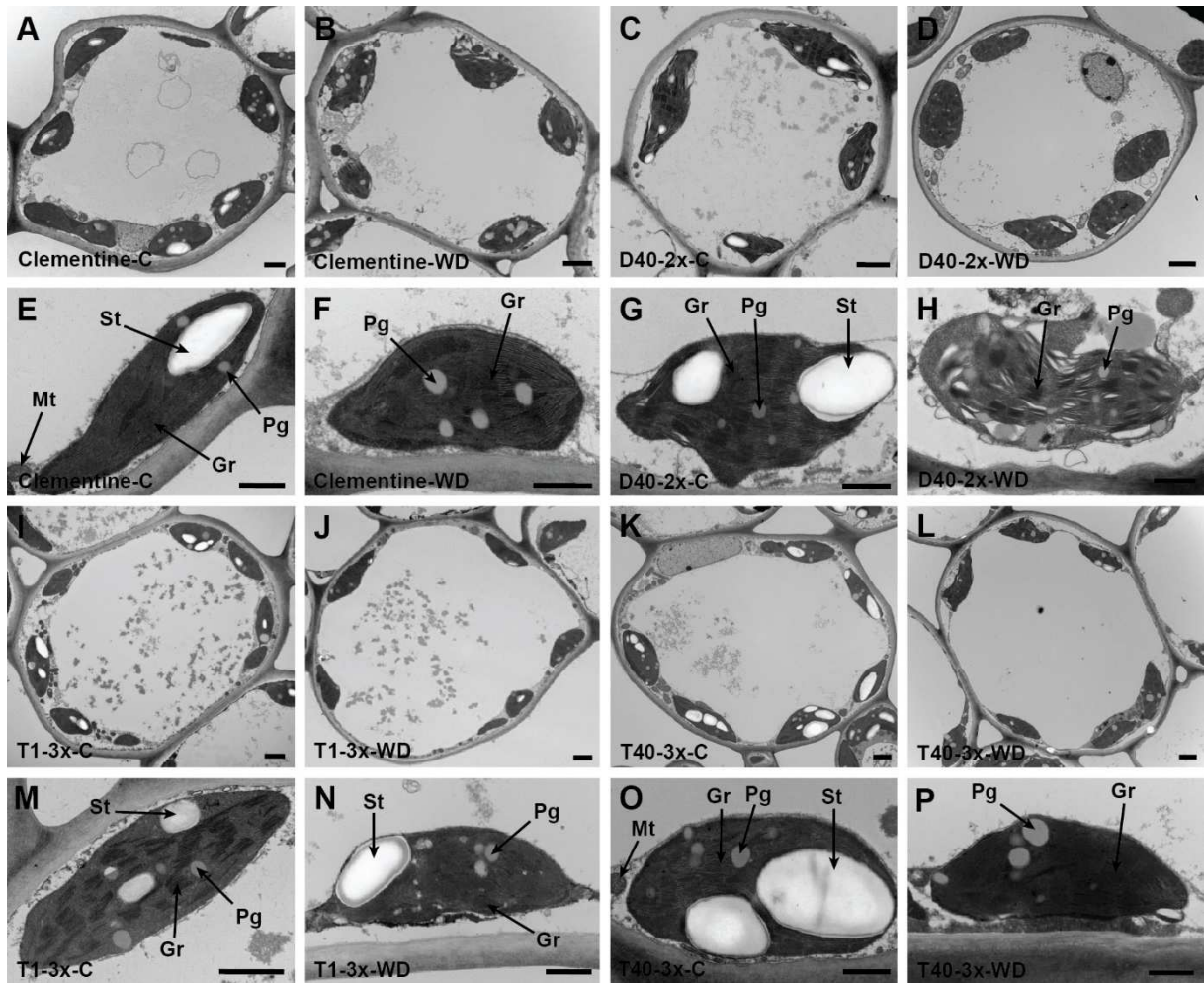
408 It is well known that stomatal closure appears to be the main physiological response to  
409 water deficit and aims to minimize water loss (Chaves et al., 2003). In our study, water deficit  
410 induced a sharp drop of  $g_s$  in all varieties where values were broadly similar among the  
411 varieties 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

439 (Manuela M. Chaves et al., 2003; Lawlor & Cornic, 2002). The  $P_{net}/C_i$  ratio is a useful  
440 indicator for estimation of Rubisco activity indicating its limitations under stressful conditions  
441 (Niinemets et al., 2009; Silva et al., 2013). In the present study, T40-3x maintained higher  $P_{net}$   
442 rates than diploids, without decreasing  $P_{net}/C_i$  ratio (Figs. 3A, 3D) at the same water stress  
443 intensity (Fig. 2A). This result suggested that photosynthetic capacity was less affected and  
444 that the decrease in  $P_{net}$  was mainly induced by stomatal factor (Farquhar & Sharkey, 1982).  
445 In contrast, the decline of  $P_{net}/C_i$  ratio in D40-2x variety and to a lesser extent in clementine  
446 and T1-3x varieties indicated the decline in activity of CO<sub>2</sub> assimilation mechanisms at severe  
447 water deficit. Decrease in  $P_{net}/C_i$  ratio was also accompanied by a sharp drop in starch grain in  
448 D40-2x (Figs. 3D, 8, 10). This result suggested that production rather than utilization of  
449 photosynthates is reducing. Moreover, soluble sugars produced from starch conversion,  
450 particularly hexoses, may repress photosynthetic gene expression as gene encoding Rubisco  
451 nuclear sub-unit reducing Rubisco content and CO<sub>2</sub> assimilation (Sheen, 1994). Associated  
452 with low RWC, this result indicated that D40-2x variety was more affected by water deficit.  
453 Less disturbance of photosynthetic activity has already been reported in polyploid citrus  
454 genotypes under abiotic stresses (Khalid et al., 2020; Lourkisti et al., 2020; Oustric et al.,  
455 2019). For example, Li et al. (2009) showed that gas exchange and chlorophyll fluorescence  
456 were less affected for honeysuckle tetraploid cultivars than the diploids.

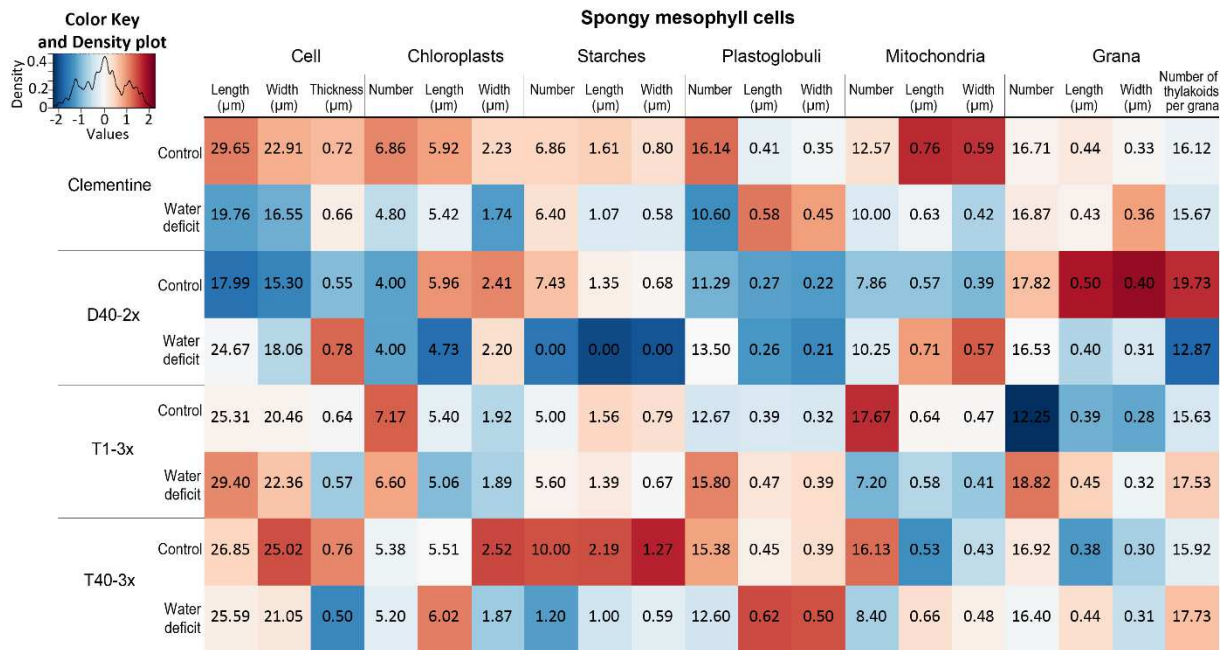
457 As a consequence, restricted CO<sub>2</sub> availability could lead to enhanced susceptibility to  
458 photo-damage and excess energy which must be dissipated. In our study, the maximum  
459 quantum yield of efficiency ( $F_v/F_m$ ) decreased in all varieties in response to water deficit (Fig.  
460 3E). Although low  $F_v/F_m$  (under the physiological value ~0.83) usually suggests PSII damage  
461 (Maxwell & Johnson, 2000), a decrease in  $F_v/F_m$  could also illustrate dynamic photoinhibition  
462 of the PSII and involvement of photoprotective mechanisms (Lambrev et al., 2012). Non-  
463 photochemical quenching (NPQ) is one of the major protective mechanisms that eliminates  
464 excess energy by heat dissipation and has been related to the xanthophyll cycle (Muller, 2001;  
465 Ruban, 2016). In the present study, water deficit leads to an increase in NPQ rate in all  
466 varieties (Fig. 3F). While the NPQ rate was similar between clementine and 3x varieties, the  
467 latter had greater values than D40-2x variety suggesting the effective removal of excess  
468 energy. Triploid varieties were less affected in terms of leaf gas exchange and chlorophyll  
469 fluorescence that may alleviate ROS accumulation in 3x varieties.

470



471

**Figure 9:** TEM micrographs of spongy mesophyll cells (3A; 3B; 3C; 3D; 3I; 3J; 3K; 3L; scale bar: 50  $\mu$ m) and their respective chloroplasts (3E; 3F; 3G; 3H; 3M; 3N; 3O; 3P; scale bar: 1  $\mu$ m) 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).

### 472 **4.3. Role of the antioxidant system of 3x genotypes in water deficit tolerance**

473 Exposure to adverse environmental conditions, including drought, can lead to  
 474 oxidative stress when ROS production, such as hydrogen peroxide ( $H_2O_2$ ), increases (Miller  
 475 et al., 2010). Chloroplasts are a major source of ROS production and are consequently  
 476 sensitive to oxidative damage when subjected to environmental stress including heat (Xu et  
 477 al., 2006), drought (Wang et al., 2012) and nutrient deficiency (Oustric et al., 2019).  
 478 Therefore, abiotic stresses were expected to induce damage due to ROS accumulation on  
 479 chloroplast ultrastructure targeting thylakoid membranes and causing lipid peroxidation (Shao  
 480 et al., 2016). In our study, T40-3x variety had the lowest oxidative markers levels (Fig. 4A,  
 481 4B) and both T1-3x and T40-3x exhibited less chloroplast damage (Figs.7, 9). While  
 482 antioxidant enzyme activities did not discriminate 2x from 3x response (Figs. 4D, 4E, 4F),  
 483 high proline content found in T40-3x (Fig. 4C) may explain less chloroplast damages since  
 484 proline is involved in protection of membrane integrity and ROS scavenging (Dien et al.,  
 485 2019; Szabados & Saviouré, 2010). Triploid varieties had less cellular damage as a result of

486 water deficit (Figs.7 and 9) which was consistent with their water relationship and their gas  
487 exchange being less sensitive to water deficit (Figs. 2 and 3). Despite a sharp decrease, the  
488 number of chloroplasts found in both palisade and mesophyll parenchyma of 3x varieties was  
489 still higher than that of diploids (Figs. 8, 10) and could explain their better ability to maintain  
490 photosynthetic activity (Fig. 3). This result, associated with high RWC, indicated that  
491 triploids were less affected by water scarcity. In contrast, 2x varieties showed great content of  
492 H<sub>2</sub>O<sub>2</sub> (D40-2x) and MDA (clementine) associated with more chloroplast damages (Figs. 3A,  
493 3B, 9). Despite high activity of CAT and APX (Figs. 4E, 4F), 2x varieties showed severe  
494 damages as swollen thylakoids which were eventually destroyed (Fig. 9). Enhanced  
495 antioxidant activity, correlated with less oxidative damage, was also found in tetraploid citrus  
496 subjected to salinity stress (Khalid et al., 2020). Less chloroplast damage was also observed in  
497 polyploid citrus subjected to nutrient deficiency (Oustric et al., 2019) and polyploid apple  
498 rootstocks subjected to drought stress (Wang et al., 2012).

499 Plastoglobuli (PGs) containing lipids and antioxidants such as tocopherols, carotenes,  
500 and plastoquinones (Rottet et al., 2015) decreased in response to water deficit, with some  
501 exceptions (Figs. 7; 8; 9 and 10). However, a significant increase in length and width of PGs  
502 was observed in clementine and 3x varieties that had the highest values (Figs. 8, 10). This  
503 could illustrate an efficient antioxidant system, particularly in T40-3x. The involvement of  
504 PGs has already been proposed in response to drought stress (Mutava et al., 2015; Rey et al.,  
505 2000) although the mechanisms regulating this are still unclear. Moreover, an increase in PG  
506 size is suggestive of better metabolic exchange between the thylakoid membranes and PGs by  
507 storing destabilized lipids generated by oxidative stress and preserving thylakoid membranes  
508 (Figs. 4, 7, and 9).

509        **5. Conclusion**

510        The present study provides evidence that the better response of triploid varieties to water  
511 deficit can be attributed to changes in their morphological and cytological structure that  
512 provide more energy to adapt to adverse environments. The phenotypical advantages offered  
513 by triploidy results in greater water-holding capacity which seems to result from enhanced  
514 osmotic adjustment and protect the photosynthetic activity under drought conditions. The high  
515 carbohydrate reserve induced by triploidy results in better energy mobilization and may  
516 explain the better response to water deficit conditions in triploid varieties. It would be  
517 appropriate to dig deeper into the relationship between carbon storage and the tolerance to  
518 abiotic stress. This integrative approach also provides a deeper understanding of the basic  
519 mechanisms conferring water deficit tolerance. Combined with physiological and biochemical  
520 stress parameters, ultrastructural changes would be affected by various stresses and therefore  
521 appear to be suitable stress marker. Ploidy breeding to induce drought-tolerance-related  
522 morphological and physiological traits could be a useful tool leading to commercial success in  
523 fruit crops. The metabolic characterization of fruits of these new triploid varieties should be  
524 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.

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