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Julie Oustric, Yann Quilichini, Raphaël Morillon, Stéphane Herbette, Luro François, et al.. Tetraploid citrus seedlings subjected to long-term nutrient deficiency are less affected at the ultrastructural, physiological and biochemical levels than diploid ones. *Plant Physiology and Biochemistry*, 2019, 135, pp.372-384. 10.1016/j.plaphy.2018.12.020 . hal-02187423

HAL Id: hal-02187423

<https://hal.science/hal-02187423v1>

Submitted on 26 May 2020

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Tetraploid citrus seedlings subjected to long-term nutrient deficiency are less affected at the ultrastructural, physiological and biochemical levels than diploid ones

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Disclosures: The authors have no conflicts of interest to declare

1 **Abstract**

2 Nutrient deficiency has economic and ecological repercussions for citrus fruit crops worldwide.
3 Citrus crops rely on fertilization to maintain good fruit output and quality, whereas new crop
4 management policy aims to reduce fertilizers input. New rootstocks are needed to meet to this
5 constraint, and the use of new tetraploid rootstocks better adapted to lower nutrient intake could
6 offer a promising way forward. Here we compared physiological, biochemical and anatomic
7 traits of leaves in diploid (2x) and doubled-diploid (4x) Citrumelo 4475 (*Citrus paradisi* L.
8 Macf. \times *Poncirus trifoliata* L. Raf.) and Volkamer lemon (*Citrus limonia* Osb.) seedlings over
9 7 months of nutrient deficiency. Photosynthetic parameters (P_{net} , G_s and F_v/F_m) decreased, but
10 to a lesser extent in 4x genotypes than 2x. Degradation of the ultrastructural organelles
11 (chloroplasts and mitochondria) and compound cells (thylakoids and starches) was also lower
12 in 4x genotypes, suggesting that tetraploidy may enhance tolerance to nutrient deficiency.
13 However, leaf surface (stomata, stomatal density and epithelial cells) showed no nutrient
14 deficiency-induced change. In 4x Citrumelo 4475, the higher tolerance to nutrient deficiency
15 was associated with a lower MDA and H_2O_2 accumulation than in the 2x, suggesting a more
16 efficient antioxidant system in the 4x genotype. However, few differences in antioxidant system
17 and oxidative status were observed between 2x and 4x Volkamer lemons.
18

19 **Keywords** Citrus, Nutrient deficiency, Oxidative stress, Photosynthesis, Polyploidy,
20 Ultrastructure

21 **Abbreviations** 2x: diploid; 4x: doubled-diploid; APX: ascorbate peroxidase; CAT: catalase; CM2x: diploid
22 Citrumelo 4475; CM4x: doubled-diploid Citrumelo 4475; F_0 : minimal chlorophyll a fluorescence; F_m : maximal
23 fluorescence; F_v : variable fluorescence; F_v/F_m : maximum quantum efficiency of PSII; G_s : stomatal conductance;
24 MDA: malondialdehyde; P_{net} : Leaf net photosynthetic rate; ROS: reactive oxygen species; SOD: superoxide
25 dismutase; VK2x: diploid Volkameriana; VK4x: doubled-diploid Volkameriana.
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1. Introduction

4 Polyploidy is a common feature and a major facilitator of adaptive evolution in plants
5 (Wendel 2000; Chen, 2007). It arises spontaneously from unreduced gametes or duplicated
6 chromosomes. Two types of polyploidy are generally recognized: autopolyploidy, which is
7 considered extremely rare in nature (Stebbins 1950; Grant, 1981), and allopolyploidy.
8 Autopolyploid genomes are composed of identical sets of chromosomes, while allotetraploid
9 genomes are formed through interspecific hybridization (Soltis and Soltis, 2009). Genome
10 variations in polyploidy have induced changes in anatomical and morphological characteristics,
11 physiological functions and gene expression (Leitch and Leitch, 2008). Polyploid plants
12 frequently occur in adverse environmental conditions, like high altitude with high radiation,
13 more polar latitude, deserts, and nutrient-poor soil (Ehrendorfer et al., 1980; Thompson and
14 Lumaret, 1992; Soltis and Soltis 2000). Their potential to improve tolerance has prompted a
15 number of investigations into diploids (2x) and doubled-diploid (4x) genotypes under several
16 environmentally stressful conditions, and some studies have found a tolerance improvement in
17 4x under heat, drought and salt stress than in their related 2x (Saleh et al., 2008; Li et al., 2009;
18 Zhang et al., 2010).

19 A common consequence of environmental stresses is the overproduction of free radicals
20 and other reactive oxygen species (ROS) that can deteriorate the ultrastructure of plant
21 components (e.g. photosynthetic pigments) and cell membranes (Foyer et al. 1994, Taylor and
22 Craig 1971). The major sources of ROS in plants cells are chloroplasts, mitochondria and
23 peroxisomes. Plants possess two antioxidant systems—one enzymatic (mainly superoxide
24 dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and dehydroascorbate reductase
25 (DHAR)), the other non-enzymatic (mainly ascorbate, glutathione and proline). However,
26 stress response may change depending on genotype, intensity of stress, and ploidy level (Santini
27 et al., 2013; Liu et al., 2011).

28 Nutrient deficiency is one of the major environmental factors that severely limit crop
29 development and production worldwide (Foyer et al., 1997). Essential nutrients are mainly N,
30 P and K, which have a direct impact on plant growth. A deficiency in these elements would
31 disturb the vegetative or reproductive lifecycle of the plant. Mineral-deficient plants
32 consequently show various visual symptoms, such as necrosis, chlorosis, dark green foliage, or
33 stunted growth (Ericsson, 1995). These minerals have various functions: (i) as essential

1 metabolites (e.g. proteins, enzymes and coenzymes, cell walls and chlorophyll), (ii) as enzyme
2 activators or regulators of enzyme-associated processes, and (iii) as non-structural factors in
3 physiological processes (i.e. membrane integrity, photosynthesis, stomatal movement and
4 environmental signalling) (Lal, 2006). Deficiency in one or more mineral elements causes a
5 degradation of the chloroplasts and thus disruption of their photosynthetic capacity (Kalaji et
6 al., 2014).

7 The citrus industry has long used rootstocks to enhance tolerance to biotic and abiotic
8 stresses and to improve fruit output and quality. The majority of the rootstocks used are 2x,
9 with $2n = 2x = 18$ chromosomes (Krug, 1943). However, 4x resulting from incomplete mitosis
10 of somatic embryos frequently occur in seedlings of 2x apomictic genotypes (Cameron and
11 Frost, 1968). Tetraploid citrus are characterized by various phenotypic traits including greater
12 flower number, greener (high chlorophyll production), thicker and larger leaves and fruits, and
13 reduced growth compared to the related 2x (Stebbins, 1950; Ollitrault and Jacquemond, 1994;
14 Romero-Aranda et al., 1997). Leaves of 4x generally have larger cells, guard cells, stomata and
15 chlorophyll, and a higher number of chloroplasts (Beck et al., 2003; Jellings and Leech, 1984;
16 Zhang et al., 2010). The greater genetic variability of 4x could be used in citrus to improve
17 abiotic stress tolerance (Allario et al., 2013; Oustric et al., 2017; Balal et al., 2017).

18 Fertilization is a big economic and ecological issue in the citrus industry, making it
19 essential to better understand plant responses to nutrient deficiency and urgent to develop new
20 rootstocks that require less fertilizer. New tetraploid rootstocks offer a promising way forward
21 to lower use of fertilizers.

22 Here we compared the impact of nutrient deficiency in two rootstock genotypes that are
23 used worldwide, i.e. Citrumelo 4475 (*Citrus paradisi* L. Macf. \times *Poncirus trifoliata* L. Raf.
24 (CM2x)) and Volkamer lemon (*Citrus limonia* Osb. (VK2x)), in both 2x and their respective
25 4x types (CM4x and VK4x, respectively).

26 The objective of this study was to differentiate these 4 genotypes by investigating the
27 impact of nutrient deficiency on leaf structure and ultrastructure associated with physiological
28 and biochemical parameters. Increased tolerance to nutritional stress in 4x (lower structure and
29 ultrastructure damage associated with higher photosynthetic capacities and more efficient
30 antioxidant system) would confirm the value of using polyploids when exposed to
31 environmentally harsh conditions.

32

1

2 **2. Materials and Methods**3 *2.1. Plant material and experimental design*

4 Plants of 2x Citrumelo 4475 (CM2x) and Volkamer lemon (VK2x) genotypes and their
5 related doubled-diploids (CM4x and VK4x, respectively) were grown from seedling stages (8
6 weeks old). Seedlings of each genotype were selected after checking their ploidy status by flow
7 cytometry (Partec I, Germany) according to Froelicher et al. (2007). Clonal propagation by
8 nucellar embryogenesis was checked by genotyping with SSR markers as described in Vieira
9 et al. (2016).

10 The 6 seedlings of each genotype, giving a total of 24 plants, were grown under identical
11 conditions in vermiculite with ferti-irrigation and water (1L/h) during 4 years in a tunnel
12 greenhouse at the AREFLEC experimental station in San Giuliano, Corsica (41°47'27''N and
13 09°23'40''E). The ferti-irrigation solutions were prepared and applied with a metering pump.
14 The stock solution used for irrigation included: N (nitrogen), P (phosphorus) and K (potassium)
15 (20-5-10 fertilizer unit) + 2MgO (magnesium oxide) + trace elements. The fertilization rate
16 supplied was in accordance with the French department of agriculture recommendations.

17 After 4 years and before starting the experiment, the vermiculite was washed with
18 distilled water for 48 hours in order to eliminate any nutritional reserves in the pot. The 4-years-
19 old trees were then divided into 2 blocks: one with reference ferti-irrigation (control plants) and
20 the other with irrigation water (without nutrient inputs). In each block, a total of 3 trees of each
21 genotype was randomized, giving a total of 12 trees per block.

22 Experiments were carried out in December 2016 after 210 days of nutrient deficiency.
23 Measurements and sampling were performed on homogeneous plants composed of 4 branches
24 with fully-expanded leaves. For physiological measurements, 9 fully expanded leaves were
25 analyzed, i.e. 3 per tree (9 replicates), between 7 AM and 11 AM. For biochemical analyses, 3
26 samples were collected for each genotype, i.e. 1 per tree, and each sample is obtained by pooling
27 8 fully-expanded leaves. All leaves were immediately frozen in liquid nitrogen and stored at
28 -80°C, and each leaf sample was ground to a fine powder prior performing biochemical
29 analyses. For the mineral measurements, 3 samples were collected for each genotype, i.e. one
30 per tree, and each sample is obtained by pooling 8 fully-expanded leaves (3 replicates).

1 At the same time of measurements and sampling, five 1 cm² pieces for scanning electron
2 microscopy and five 1–2 mm² pieces for transmission electron microscopy were cut with a razor
3 blade from mid-laminar areas of both sets of leaves (5 replicates for each genotype).

4

5 *2.2. Measurements of gas exchange*

6 Leaf net photosynthetic rate (P_{net}) and stomatal conductance (G_s) were measured using
7 a LI-6400 portable photosynthesis system (Li-COR, Lincoln, NE, USA) with the LI6400-40
8 Leaf Chamber. This experiment was conducted at photosynthetic photon flux density (PPFD)
9 of 1400 $\mu\text{mol photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with a Li-COR red-blue light source (6400-02B no. SI-710, Li-
10 Cor, Lincoln, NE), a constant carbon dioxide concentration (CO_2) of 380 $\mu\text{mol}\cdot\text{mol}^{-1}$, air flow
11 rate of 500 $\mu\text{mol}\cdot\text{s}^{-1}$, and leaf temperature of 25°C.

12

13 *2.3. Measurements of chlorophyll a fluorescence*

14 Chlorophyll *a* fluorescence of dark-acclimated leaves was measured using a Handy-
15 PEA portable fluorometer (Hansatech Instruments, Ltd, Norfolk, UK). Leaves were firstly dark-
16 acclimated with a “leafclip” for 30 min. Then, leaves were illuminated with a light intensity of
17 3000 $\mu\text{mol photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, by an array of three light-emitting diodes (650 nm) for a duration of
18 1 s. Among the measured parameters, F_0 represents the minimal values of chlorophyll *a*
19 fluorescence, F_m represents the maximum fluorescence, and F_v is variable fluorescence, which
20 is obtained by subtracting F_0 from F_m . The F_v/F_m ratio corresponds to the maximum quantum
21 efficiency of photosystem II (PSII) (Maxwell and Johnson, 2000).

22

23 *2.4. Foliar mineral analysis*

24 All ground samples were dried at 65 ± 10° C in an oven overnight, transferred into a
25 desiccator until cooling and sent to a CIRAD laboratory (Montpellier, France) for analysis of
26 macro- and micro-nutrients.

27 Total P, K, Ca, Mg, Na, B, Cu, Fe, Zn and Mn in leaves were determined using an
28 Agilent 720 simultaneous ICP-OES after double calcination including silica removal by adding
29 hydrofluoric acid.

30 N content in leaves was measured by combustion using a Leco TruMac N determinator.

31

32 *2.5. Scanning electron microscopy (SEM)*

1 Leaf pieces (typically 1 cm²) were cut with a razor blade from mid-laminar areas of
2 leaves and fixed in cold (4°C) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH
3 7.2, rinsed in a 0.1 M cacodylate buffer at pH 7.2, dehydrated through a graded ethanol series
4 (30%, 50%, 75%, 90% and 100%), and dried with CO₂ in an Emitech K850 critical point dryer
5 (Quorum Technologies Ltd, Ashford, U.K.). Specimens were mounted on aluminum stubs with
6 carbon double-sided adhesive disks, coated with gold/palladium in a SC7640 sputter coater
7 (Quorum Technologies Ltd, Newhaven, U.K.), and examined under a S-3400N scanning
8 electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan) at an accelerating
9 voltage of 5 at the University of Corsica (Corte, France). SEM measurements were realized
10 using the software of the microscope. 30 independent measurements on 5 different leaves of
11 each variety and each genotype were realized in order to estimate length and width of stomata
12 and ostioles. 5 independent measurements on 5 different leaves of each variety and each
13 genotype were analyzed using Adobe Photoshop software in order to estimate the stomata
14 density. The number of stomata was counted on photographs and taken into account the stomata
15 cut only on two sides of the photograph.

16

17 2.6. *Transmission electron microscopy (TEM)*

18 Leaf pieces (typically 1 mm²) were cut with a razor blade from mid-laminar areas of
19 leaves and fixed in cold (4°C) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH
20 7.2, rinsed in a 0.1 M cacodylate buffer at pH 7.2, post-fixed in cold (4°C) 1% osmium tetroxide
21 in the same buffer for 1 h, dehydrated through a graded ethanol series (70% and 100%) and
22 propylene oxide, embedded in Spurr, and polymerized at 60°C for 24 h. Ultra-thin sections (60–
23 90 nm) were cut using an Power tome PC ultramicrotome (RMC Boeckeler, Tuscon, U.S.A.).
24 The sections were placed on 200- and 300-mesh copper grids and stained with UranyLess
25 (Delta Micoscopies, France) and lead citrate. Sections were examined on a Hitachi H-7650
26 (Hitachi High-Technologies Corporation, Tokyo, Japan) at an accelerating voltage of 80 kV at
27 the University of Corsica (Corte, France). TEM measurements were realized using the software
28 of the microscope. 10 cells of each variety, each genotype and each mesophyll type were
29 analysed in order to estimate length, width and thickness of cells and length, width and number
30 of chloroplasts, starches, plastoglobuli and mitochondria. 30 independent measurements of
31 each variety, each genotype and each mesophyll type were analysed in order to estimate length

1 and width of chloroplasts, starches, plastoglobuli, mitochondria, grana, number of grana per
2 cell section, and number of thylakoids per granum.

3

4 *2.7. Assay of oxidation markers*

5 Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents and antioxidant
6 enzyme activities (superoxide dismutase, catalase and ascorbate peroxidase) were determined
7 using the method described by Santini et al. (2013). A V-630 spectrophotometer (Jasco Inc.,
8 Tokyo, Japan) was used to measure the absorption spectra of the samples.

9

10 *2.8. Statistical analyses*

11 The experimental was set up as a split-plot design with genotype as main plot and ploidy
12 and treatment as subplot. R statistical software (<http://www.R-project.org>) was used to analyze
13 all the data. Multi-way ANOVA followed by least significant difference (LSD) test at $P < 0.05$
14 was used for evaluating the influence of genotypes, ploidy and treatments. Heat map was made
15 to determine the differences between genotypes and treatments for abaxial epidermis and
16 ultrastructural palisadic and spongy mesophyll characters.

17

18 **3. Results and Discussion**

19 *3.1. Anatomical properties of doubled-diploid versus diploid genotypes under control
20 conditions*

21 Regardless of seedling genotype and ploidy level, stomata were observed only on leaf
22 abaxial surface and were surrounded by ordinary epidermal cells (anomocytic organization).
23 The epidermal carpet architecture showed no appendages (trichome) (Fig. 1). Similar results
24 were found by Inyama et al. (2015) in other *Citrus* species. In agreement with other studies,
25 stomatal density was lower in 4x genotypes than in their related 2x, and correlated with an
26 increase in size (Fig. 1; Table 1; Sup. Table 1) (Aryavand et al., 2003; Li et al., 2009; Allario
27 et al., 2011; Meng et al., 2014). At ultrastructure level, whether in the palisade or spongy
28 mesophylls, cells were larger with a thicker wall in 4x genotypes than in their related 2x (Fig.
29 2 and 3; Tables 2 and 3; Sup. Tables 2 and 3), as reported in Romero-Aranda et al. (1997).

30 Doubled-diploid genotypes had more chloroplasts but their size remained broadly
31 similar to their 2x counterparts (Fig. 2 and 3; Tables 2 and 3; Sup. Tables 2 and 3), in line with
32 previous reports (Jellings and Leech, 1984; Mathura et al., 2006). Conversely, ploidy level did

1 not influence number and size of starches and plastoglobuli or number of grana per chloroplasts,
2 which were approximately identical between the different ploidy levels and whatever the
3 parenchyma (Fig. 2 and 3; Tables 2 and 3; Sup. Tables 2 and 3). This result was also observed
4 in 4x black locust (*Robinia pseudoacacia* L.) (Meng et al., 2014).

5

6 *3.2. Impact of tetraploidization on photosynthetic properties under nutrient deficiency*

7 Deficiency of one or more essential minerals is known to affect the photosynthetic
8 process (Lima et al., 1999; Huang et al., 2004; Boussadia et al., 2010). The decrease in P_{net} was
9 associated with a decrease in G_s in all genotypes (Fig. 4A and B; Sup. Table 4), suggesting a
10 stomatal limitation (Zhao et al., 2005) that would lead to down-regulation of the CO_2
11 accumulation (Flexas et al., 2013).

12 P_{net} was higher in 4x honeysuckle (*Lonicera japonica* Thunb.) (Li et al., 2009), spathe
13 flower (*Spathiphyllum wallisii*) (Van Laere et al., 2011) and in Carrizo citrange citrus seedlings
14 (*Citrus sinensis* (L.) Osbeck \times *Poncirus trifoliata* (L.) Raf) (Oliveira et al., 2017) than their 2x
15 counterparts under drought stress, due to higher maintained degree of stomatal opening.
16 However, nutrient deficiency caused less pronounced inhibition of photosynthesis in 4x
17 seedlings than in related 2x (Fig. 4C; Sup. Table 4) possibly due to an upregulation of non-
18 stomatal factors (biochemical) (Klamkowski and Treder, 2008; Shahid et al., 2011) or a
19 decrease in protective non-radiative energy dissipation, photodamage of the PSII centers, or
20 both (Osmond, 1994). Photoinhibitory impacts on PSII occurred proportionally more in 2x
21 honeysuckle (*Lonicera japonica* Thunb.) under salt stress (Yan et al., 2015) and heat stress (Li
22 et al., 2011) than in 4x counterparts. The lower decrease of photosynthetic rate in 4x genotypes
23 under nutrient deficiency could be due to ultrastructural changes in leaves (Parisod et al., 2010;
24 Xiong et al., 2006).

25

26 *3.3. The higher photosynthetic rates of doubled-diploid genotypes under nutrient stress*
27 *could be explained by their leaf and cell anatomy.*

28 In each genotype, nutrient deficiency decreased the total content of all minerals except
29 for K (Fig. 6) and was associated with leaf alteration (Fig. 5). Despite a similar decrease in
30 minerals content (Fig. 6), leaves in 2x genotypes had a greater proportion of chlorotic leaves
31 than in their related 4x (Fig. 5), suggesting a substantial loss of chlorophyll pigments (Zhang et
32 al., 2016). However, taking in account the long-term nutrient deficiency, there was no large

1 change in stomata size and density in either 2x or 4x genotypes (Fig. 1; Table 1; Sup. Table 1),
2 suggesting that stomata do not play a major role in response to nutritional stress.

3 Furthermore, all genotypes presented ultrastructural damages (chloroplasts, starches,
4 mitochondria and plastoglobuli) under nutrient deficiency. In spongy and palisadic mesophyll
5 cells, number of chloroplasts and mitochondria decreased and granal thylakoids were distorted
6 in all genotypes (Fig. 2 and 3; Tables 2 and 3; Sup. Tables 2 and 3). Doubled-diploid genotypes
7 were less impacted by these changes than their related 2x. Loss of chloroplast integrity and
8 functioning (photosynthesis, potential quantum yield of photosystem II) would be characteristic
9 of senescence. Chloroplast degradation plays an important role in leaf senescence for nutrient
10 recycling from reproductive organs or younger leaves (Makino and Osmond, 1991;
11 Hortensteiner and Feller, 2002). Moreover, thylakoid membranes and their associated PSII are
12 considered as targets of abiotic stress (Ding et al., 2005).

13 The lower deterioration of thylakoids in 4x genotypes could explain their better F_v/F_m
14 ratio and, in turn, the higher photosynthetic activity maintained in 4x genotypes than their
15 respective 2x (Fig. 4C; Sup. Table 4). Interestingly, lower chloroplast damage associated with
16 higher photosynthetic activity was also observed in 4x black locust (*Robinia pseudoacacia* L.)
17 under salt stress (Wang et al., 2013) than in their related 2x.

18 Plastoglobuli containing products of membrane degradation showed an abnormal
19 increase in size (spongy and palisadic mesophyll cells) in all genotypes except VK4x (Fig. 2
20 and 3; Tables 2 and 3; Sup. Tables 2 and 3), which may indicate a link between chloroplast
21 disorganization and plastoglobuli (Dou et al., 1999). In the chloroplast stroma, nutrient
22 deficiency led to a decrease in the number as well as the length and width of starch granules in
23 both spongy and palisadic mesophyll cells in all genotypes except VK4x (Fig. 2 and 3; Tables
24 2 and 3; Sup. Tables 2 and 3). VK4x showed an increase in starch length and width (Fig. 2 and
25 3; Tables 2 and 3; Sup. Tables 2 and 3), as reported in other studies under nutrient deficiency
26 (Bondada and Syvertsen 2003; Zhao et al, 2015). The swelling of starch granules could be
27 induced by an up-regulation of transcripts encoding key enzymes involved in starch
28 biosynthesis and a down-regulation of transcripts encoding enzymes involved in starch
29 consumption (Tao et al., 2013). In CM4x, VK2x and CM2x, the decline in length and width of
30 transitory starch (Fig. 2 and 3; Tables 2 and 3; Sup. Tables 2 and 3) would suggest a high
31 mobilization and translocation of starch leading, in turn, to a more advanced senescence process
32 than in VK4x (Lindroth et al., 2002). In leaves, stimulation of the degradation of starch granules

1 would supply the carbon and energy needed for the stress response, especially when
2 photosynthesis becomes too weak, and protect cellular structures by increasing soluble sugars
3 as osmoprotectants (Kaplan and Guy, 2004; Rizhsky et al., 2004; Naeem et al., 2012).

4

5 *3.4. The greater tolerance to nutrient stress of 4x genotypes is associated with better*
6 *redox status*

7 Mesenchymal cells, especially chloroplasts, endure ultrastructure damage from abiotic
8 stresses such as salinity (Hernandez et al., 1995; Fidalgo et al., 2004), drought and heat
9 (Grigorova et al., 2011), chilling stress (Kratsh and Wise, 2000), nutrient excess (Papadakis et
10 al., 2004) and nutrient deficiency (Bondada and Syvertsen, 2003; Papadakis et al., 2007).
11 Reduction of chloroplast numbers leads to a decrease in photosynthetic activity under nutrient
12 deficiency. This decrease is probably due to oxidative stress, i.e. an imbalance between ROS
13 accumulation and antioxidant system capacity (Djanaguiraman and Prasad, 2010). Previous
14 studies have reported that nutrient deficiency enhances oxidative metabolism (Cakmak, 1994;
15 Tewari et al., 2006). Here, SOD and APX activities differed between genotypes, but CAT
16 activity decreased with nutrient deficiency in all genotypes (Fig. 7A, B and C; Sup. Table 4).
17 CAT activity decreases in several species under numerous environmental stresses such as salt
18 stress (Tanaka et al., 1999), shading stress (Causin et al., 2015) and nutrient deficiency (Huang
19 et al., 2004; Tewari et al., 2004). The increase in APX only seemed to compensate for the lower
20 CAT activity in CM4x, which limited accumulation of H₂O₂ (Fig. 7D; Sup. Table 4). The
21 increase in MDA content in CM2x and CM4x (Fig. 7E; Sup. Table 4) could result directly from
22 the transformation of the H₂O₂ in OH° via Fenton or Haber-Weiss reactions, but also from a
23 direct conversion of O₂ to OH° from an addition of three electrons. The lower MDA and H₂O₂
24 accumulation in CM4x was associated with greater SOD and APX activities. This result could
25 explain that CM4x had less ultrastructural damage and decreased less in photosynthetic activity
26 than CM2x. Higher antioxidant capacity was associated to better ultrastructural cell integrity
27 for better protection under drought stress in a tolerant apple rootstock compared to a sensitive
28 one (Wang et al., 2012). In agreement with our results, MDA and H₂O₂ were also less
29 accumulated in 4x *Ignam* (*Dioscorea*) under heat stress (Zhang et al., 2010) and in black locust
30 (*Robinia pseudoacacia* L.) under salt stress (Meng et al., 2012) than in their related 2x.

31 In VK2x and VK4x, the increase in SOD activity and the lower or similar APX and
32 CAT activities could explain their higher accumulation of H₂O₂. These results imply that factors

1 other than redox status could explain the stronger ultrastructural degradations in VK2x than
2 VK4x, i.e. transcriptional regulations, phytohormones and nutrient signalling (Woo *et al.*, 2013;
3 Liu *et al.*, 2016).

4

5 **4. Conclusion**

6 Polyploidization lends citrus genotypes greater tolerance to a prolonged nutrient
7 deficiency, as indicated by better photosynthetic activity for doubled-diploid genotypes under
8 stress conditions. This greater tolerance and photosynthetic activity were associated with
9 limited ultrastructural changes of leaf cells, especially chloroplasts and starch granules, in
10 doubled-diploid genotypes. These limited structural changes argue for a long-lasting tolerance
11 of doubled-diploid genotypes under nutrient deficiency, suggesting that these genotypes may
12 be good candidates for citrus cultivation as part of the effort to reduce fertiliser use. However,
13 better protection against nutrient deficiency is not still related to a better antioxidant system for
14 citrus.

15 The next step of this study will be to test these genotypes as rootstocks and to graft them
16 with an economically productive variety.

17

18 **Contributions**

19 J. Oustric is a student and main author. She collected test data, performed statistical
20 analyses, interpreted the results and drafted the manuscript.

21 J. Santini was involved in the write-up, planning and discussion of results.

22 Y. Quilichini performed microscopic analysis.

23 L. Berti helped in paper discussion and was the leader of the team.

24 R. Morillon, S. Herbette, F. Luro and J. Giannettini helped in research trial and to draft
25 the manuscript.

26

27 **Acknowledgements**

28 Julie Oustric received grant support from the “Collectivité Territoriale de Corse” (CTC).
29 This work was funded by the “Collectivité Territoriale de Corse” as part of the research project
30 “Sélection de porte-greffes pour le développement d'une agrumiculture durable en Corse”. We
31 thank Jean-Claude Ribaut for hosting this study at the AREFLEC experimental station, and

1 Gilles Paolacci and Paul Martin for installing and monitoring the ferti-irrigation system in the
 2 greenhouse.

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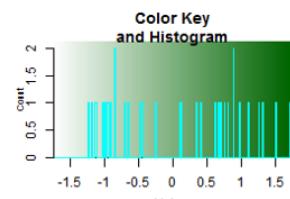
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Table 1. Abaxial epidermis study of diploid (CM2x) and doubled-diploid (CM4x) Citrumelo 4475 and diploid (VK2x) and doubled-diploid (VK4x) Volkamer lemon grown in nutrient reference solution (100%) and without nutrient solution (0%) for 210 days.



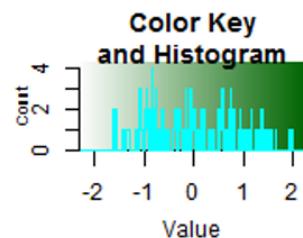
Characters	Citrumelo 2x		Citrumelo 4x		Volkamer lemon 2x		Volkamer lemon 4x	
	100%	0%	100%	0%	100%	0%	100%	0%
Position of stomata	Hypostomatic	Hypostomatic	Hypostomatic	Hypostomatic	Hypostomatic	Hypostomatic	Hypostomatic	Hypostomatic
Stomatal type	Anomocytic	Anomocytic	Anomocytic	Anomocytic	Anomocytic	Anomocytic	Anomocytic	Anomocytic
Stomatal density	60.44	54.10	26.60	35.80	26.96	27.16	19.44	21.00
Length of stomata (µm)	19.11	20.23	27.32	25.65	18.33	18.91	26.82	26.43
Width of stomata (µm)	16.45	16.76	22.70	21.43	16.24	16.57	21.14	21.65
Length of ostiole (µm)	5.51	7.98	10.60	8.95	5.49	6.22	8.46	9.04
Width of ostiole (µm)	2.68	4.14	7.19	5.05	2.78	3.43	5.17	5.70
Trichome type	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent

Values are means (\pm standard error) of 30 independent measurements on 5 different leaves ($n = 150$) for length and width of stomata and ostioles, and of 5 independent measurements on 5 different leaves ($n = 25$) for stomatal density. The heat map shows the differences between genotypes and treatments for each measured characteristic. Values are associated with color ranging from white (low) to dark green (high).

Comment citer ce document :

Oustric, J., Quilichini, Y., Morillon, R., Herbette, S., Luro, F., Giannettini, J., Berti, L., Santini, J. (2019). Tetraploid citrus seedlings subjected to long-term nutrient deficiency are less affected at the ultrastructural, physiological and biochemical levels than diploid ones. *Plant Physiology and Biochemistry*, 135, 372-384. DOI : 10.1016/j.plaphy.2018.12.020

Table 2. Ultrastructure of palisade mesophyll cells of diploid (CM2x) and doubled-diploid (CM4x) Citrumelo 4475 and diploid (VK2x) and doubled-diploid (VK4x) Volkamer lemon grown in nutrient reference solution (100%) and without nutrient solution (0%) for 210 days.



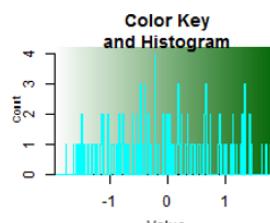
		Citrumelo 4475 2x		Citrumelo 4475 4x		Volkamer lemon 2x		Volkamer lemon 4x	
		100%	0%	100%	0%	100%	0%	100%	0%
Cells	Length (μm)	28.40	24.78	48.83	42.12	33.29	28.10	43.76	45.46
	Width (μm)	10.20	10.07	23.83	10.23	8.30	6.47	12.77	11.23
	Thickness (μm)	0.45	0.43	0.76	0.71	0.26	0.29	0.41	0.40
Chloroplasts	Number	8.88	3.00	15.20	7.25	9.40	4.60	13.60	9.60
	Length (μm)	5.41	2.87	6.34	3.91	5.03	3.45	5.75	5.44
	Width (μm)	2.40	1.16	2.80	1.28	2.81	2.47	2.60	3.41
Starches	Number	16.51	0.71	30.2	8.12	22.60	8.40	22.20	23.80
	Length (μm)	1.78	1.21	2.60	1.14	2.13	1.76	1.22	2.74
	Width (μm)	1.01	0.76	1.43	0.77	1.05	1.06	0.72	1.79
Plastoglobuli	Number	62.50	16.85	59.00	33.00	61.88	26.60	53.00	54.75
	Length (μm)	0.53	0.61	0.31	0.70	0.41	0.59	0.46	0.47
	Width (μm)	0.33	0.51	0.52	0.52	0.31	0.39	0.39	0.40
Mitochondria	Number	11.71	4.00	13.40	7.50	13.60	5.20	30.80	7.40
	Length (μm)	0.85	0.85	0.96	0.68	1.22	0.75	0.97	0.75
	Width (μm)	0.56	0.61	0.69	0.53	0.69	0.52	0.79	0.40
Grana	Number of grana per cell	22.93	Absent	13.93	Absent	20.60	Absent	26.26	Absent
	Length (μm)	0.52	Absent	0.34	Absent	0.40	Absent	0.38	Absent
	Width (μm)	0.60	Absent	0.56	Absent	0.42	Absent	0.42	Absent
	Number of thylakoids per grana	27.26	Absent	25.20	Absent	22.73	Absent	18.13	Absent

Values are means (\pm standard error) of independent measurements on five different cells section ($n = 5$) for length, width and thickness of cells and for number of chloroplasts, starches, plastoglobuli and mitochondria and of 30 independent measurements on different cells section ($n = 30$) for length and width of chloroplasts, starches, plastoglobuli, mitochondria, grana, number of grana per cells section and number of thylakoids per granum. The heat map shows the differences between genotypes and treatments for each measured characteristic. Values are associated with color ranging from white (low) to dark green (high).

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Table 3. Ultrastructure of spongy mesophyll cells of diploid (CM2x) and doubled-diploid (CM4x) Citrumelo 4475 and diploid (VK2x) and doubled-diploid (VK4x) Volkamer lemon grown in nutrient reference solution (100%) and without nutrient solution (0%) for 210 days.



		Citrumelo 4475 2x				Citrumelo 4475 4x		Volkamer lemon 2x		Volkamer lemon 4x	
		Spongy mesophyll cells									
		100%	0%	100%	0%	100%	0%	100%	0%	100%	0%
Cells	Length (μm)	20.15	22.64	42.53	28.54	24.51	24.15	31.63	33.56		
	Width (μm)	13.40	14.80	27.14	18.28	17.38	15.90	22.40	26.51		
	Thickness (μm)	0.78	0.82	0.82	0.89	0.76	0.63	1.08	0.86		
Chloroplasts	Number	7.00	2.42	10.00	5.50	7.40	3.66	10.20	6.00		
	Length (μm)	5.47	2.44	5.80	3.45	6.55	4.64	6.57	6.52		
	Width (μm)	2.83	0.79	2.15	1.04	3.68	3.12	3.40	3.97		
Starches	Number	16.60	0.57	16.80	4.87	21.10	6.80	31.80	18.00		
	Length (μm)	2.71	1.53	2.30	1.13	3.44	2.44	2.36	3.06		
	Width (μm)	1.75	0.81	1.28	0.68	2.13	1.73	1.31	1.68		
Plastoglobuli	Number	12.00	11.57	3.40	1.97	23.80	14.60	21.00	18.40		
	Length (μm)	0.30	0.54	0.35	0.70	0.38	0.55	0.39	0.41		
	Width (μm)	0.21	0.49	0.31	0.52	0.35	0.43	0.30	0.38		
Mitochondria	Number	10.80	2.71	8.00	5.50	14.00	9.80	20.80	7.40		
	Length (μm)	0.74	0.67	0.71	0.70	0.84	0.66	0.88	0.70		
	Width (μm)	0.49	0.45	0.44	0.57	0.70	0.50	0.65	0.58		
Grana	Number of grana per cell	11.00	Absent	11.23	Absent	14.06	Absent	16.66	Absent		
	Length (μm)	0.63	Absent	0.37	Absent	0.54	Absent	0.54	Absent		
	Width (μm)	0.39	Absent	0.51	Absent	0.61	Absent	0.52	Absent		
		Number of thylakoids per grana	26.66	Absent	24.69	Absent	21.26	Absent	23.13	Absent	

Values are mean (\pm standard error) of independent measurements on 10 different cells section ($n = 10$) for length, width and thickness of cells, and for number of chloroplasts, starches, plastoglobuli and mitochondria and of 30 independent measurements on different cells section ($n = 30$) for length and width of chloroplasts, starches, plastoglobuli, mitochondria, grana, number of grana per cell section, and number of thylakoids per granum. The heat map shows the differences between genotypes and treatments for each measured characteristic. Values are associated with color ranging from white (low) to dark green (high).

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Figure captions

Figure 1. Scanning electron micrographs of abaxial epidermis (1A, 1B, 1E, 1F, 1I, 1J, 1M, 1N; scale bar: 50 μ m) and stomata (1C, 1D, 1G, 1H, 1K, 1L, 1O, 1P; scale bar: 10 μ m) in diploid (CM2x) and doubled-diploid (CM4x) Citrumelo 4475 and diploid (VK2x) and doubled-diploid (VK4x) Volkamer lemon grown in nutrient reference solution (100%) and without nutrient solution (0%) for 210 days. ST: ostiole (stoma); EC: epithelial cells; GC: guard cells.

Figure 2. Transmission electron micrographs of palisade mesophyll cells (3A, 3B, 3C, 3D, 3I, 3J, 3K, 3L; scale bar: 5 μ m) and their respective chloroplasts (3E, 3F, 3G, 3H, 3M, 3N, 3O, 3P; scale bar: 1 μ m) in diploid (CM2x) and doubled-diploid (CM4x) Citrumelo 4475 and diploid (VK2x) and doubled-diploid (VK4x) Volkamer lemon grown in nutrient reference solution (100%) and without nutrient solution (0%) for 210 days. St: starch; Pg: plastoglobule; Mt: mitochondria; Gr: granum.

Figure 3. Transmission electron micrographs of spongy mesophyll cells (3A, 3B, 3C, 3D, 3I, 3J, 3K, 3L; scale bar: 5 μ m) and their respective chloroplasts (3E, 3F, 3G, 3H, 3M, 3N, 3O, 3P; scale bar: 1 μ m) in diploid (CM2x) and doubled-diploid (CM4x) Citrumelo 4475 and diploid (VK2x) and doubled-diploid (VK4x) Volkamer lemon grown in nutrient reference solution (100%) and without nutrient solution (0%) for 210 days. St: starch; Pg: plastoglobule; Mt: mitochondria; Gr: granum.

Figure 4. Determination of (A) net photosynthetic rate (P_{net}), (B) stomatal conductance (G_s), and (C) chlorophyll *a* fluorescence (F_v/F_m) after 210 days of nutrient deficiency in leaves of diploid (CM2x) and doubled-diploid (CM4x) Citrumelo 4475 and diploid (VK2x) and doubled-diploid (VK4x) Volkamer lemon. The white circles correspond to the control (100%) and the black circles to the plant without nutrition (0%). Values are mean (\pm standard error) of 9 independent measurements from 3 different trees for each genotype ($n = 9$). Ploidy and treatment effects were analyzed using ANOVA and Fisher LSD tests ($P < 0.05$). Distinct letters indicate significant differences between all genotypes and treatments.

Figure 5. Leaf damages after 210 days of total nutrient deficiency.

Figure 6. Total content of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), sodium (Na), iron (Fe), manganese (Mn), copper, zinc (Zn) and boron (B) in leaves of diploid (CM2x) and doubled-diploid (CM4x) Citrumelo 4475 and diploid (VK2x) and doubled-diploid (VK4x) Volkamer lemon grown in nutrient reference solution (100%) and without nutrient solution (0%) after 210 days. The white circles correspond to the control (100%) and the black circles to the plant without nutrition (0%). Values are mean (\pm standard error) of 3 independent measurements from 3 samples for each genotype, i.e. one per tree, obtained by pooling 8 fully-expanded leaves ($n = 3$). Data were analysed using ANOVA and Fisher LSD tests ($P < 0.05$). Different letters indicate significant differences between genotypes and treatments.

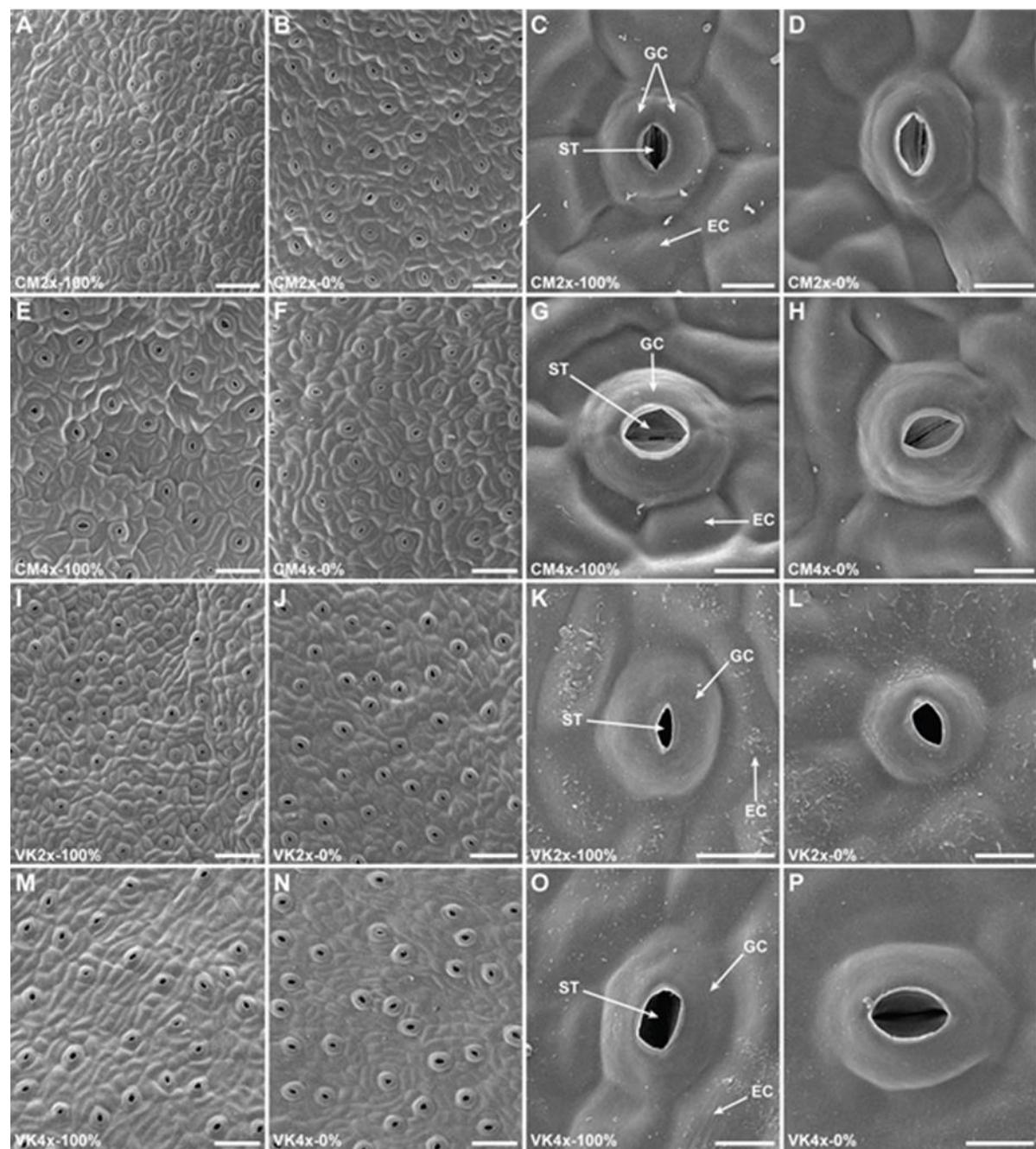
Figure 7. Determination of specific activities of (A) superoxide dismutase (SOD), (B) catalase (CAT) and (C) ascorbate peroxidase (APX) and (D) hydrogen peroxide (H_2O_2) and (E) malondialdehyde (MDA) after 210 days of nutrient deficiency in leaves of diploid (CM2x) and doubled-diploid (CM4x) Citrumelo 4475 and diploid (VK2x) and doubled-diploid (VK4x) Volkamer lemon. The white circles correspond to the control (100%) and the black circles to the plant without nutrition (0%). Values are mean (\pm standard error) of 3 independent measurements from 3 samples for each genotype, i.e. one per tree, obtained by pooling 8 fully-expanded leaves ($n = 3$). Ploidy and treatment effects were analyzed using ANOVA and Fisher LSD tests ($P < 0.05$). Distinct letters indicate significant differences between all genotypes and treatments.

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Figures

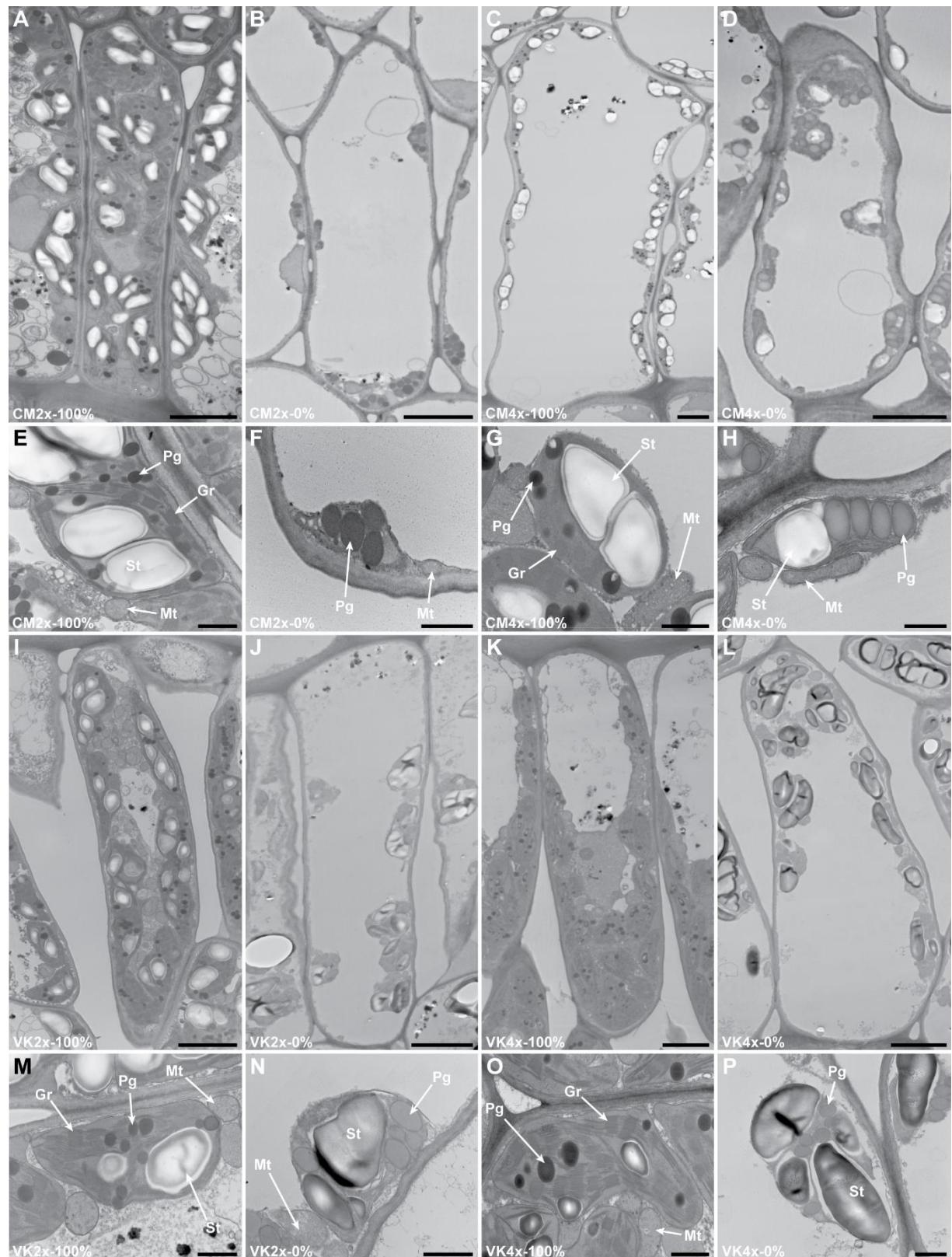
Fig. 1



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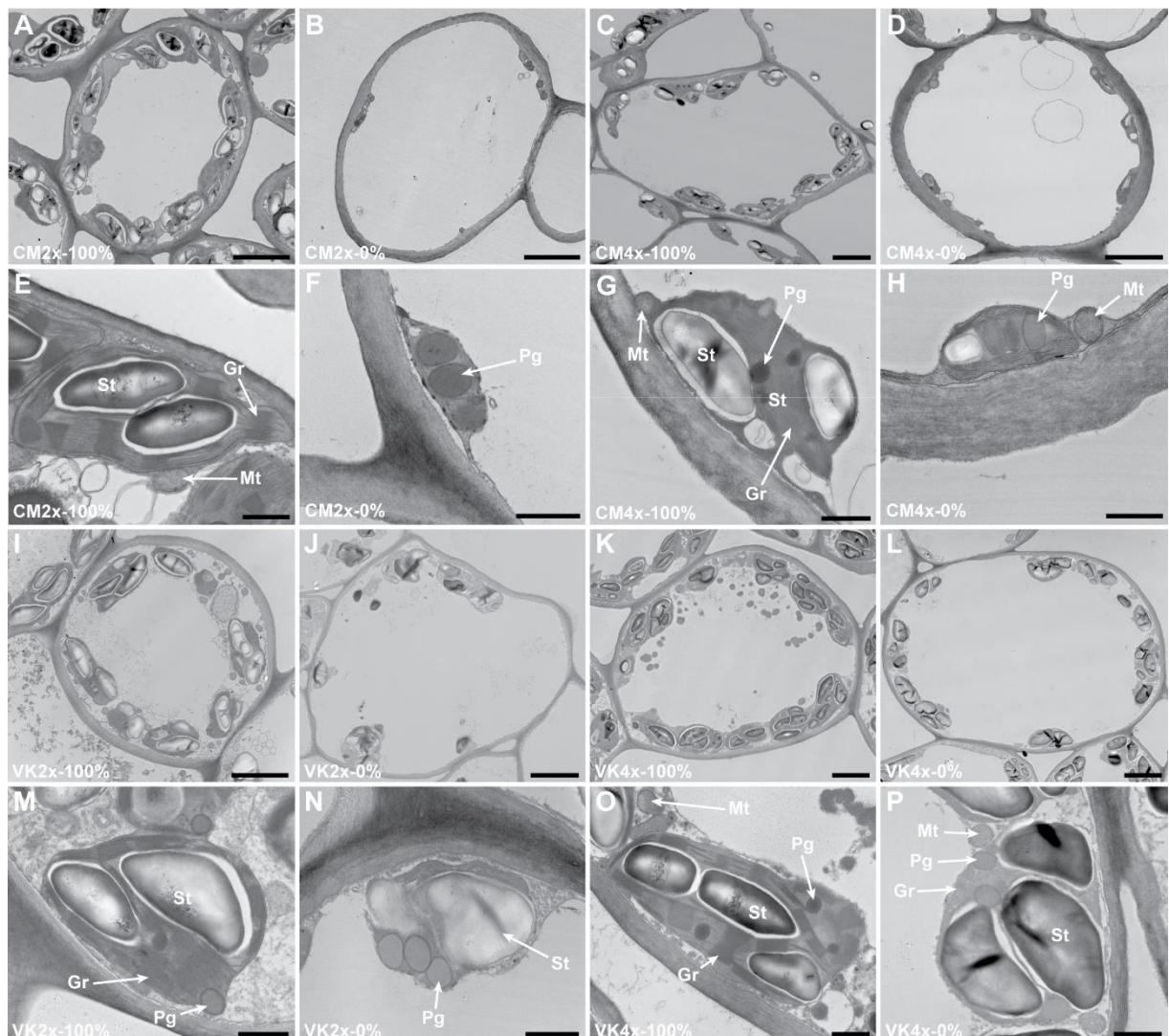
Fig. 2



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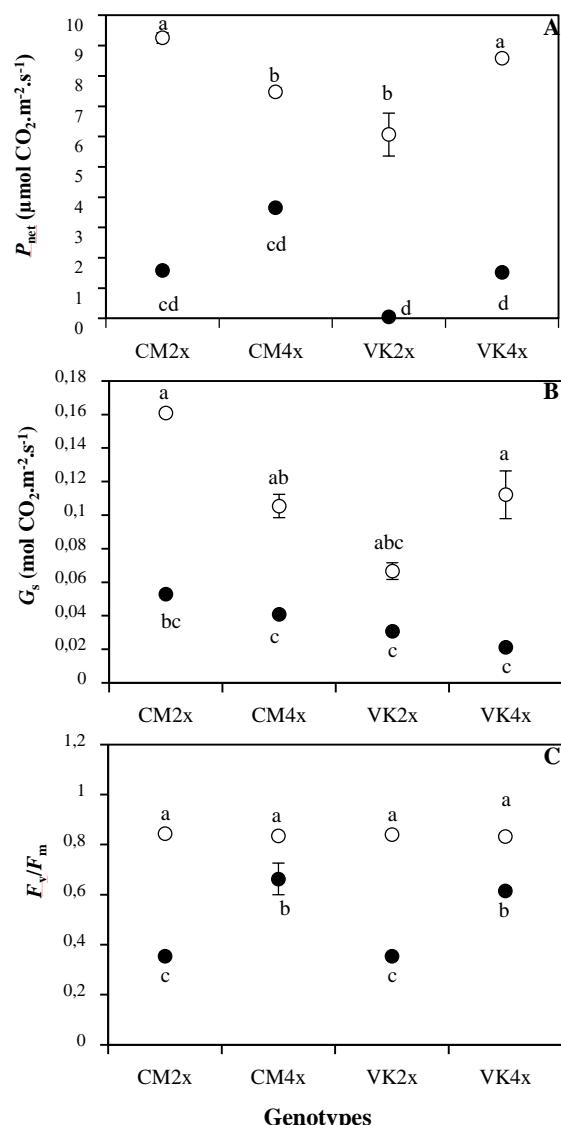
Fig. 3



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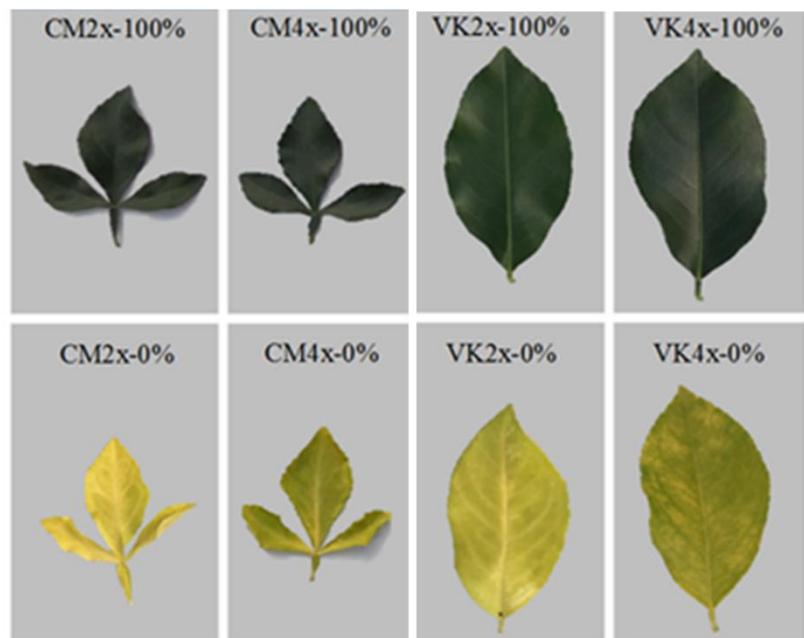
Fig. 4



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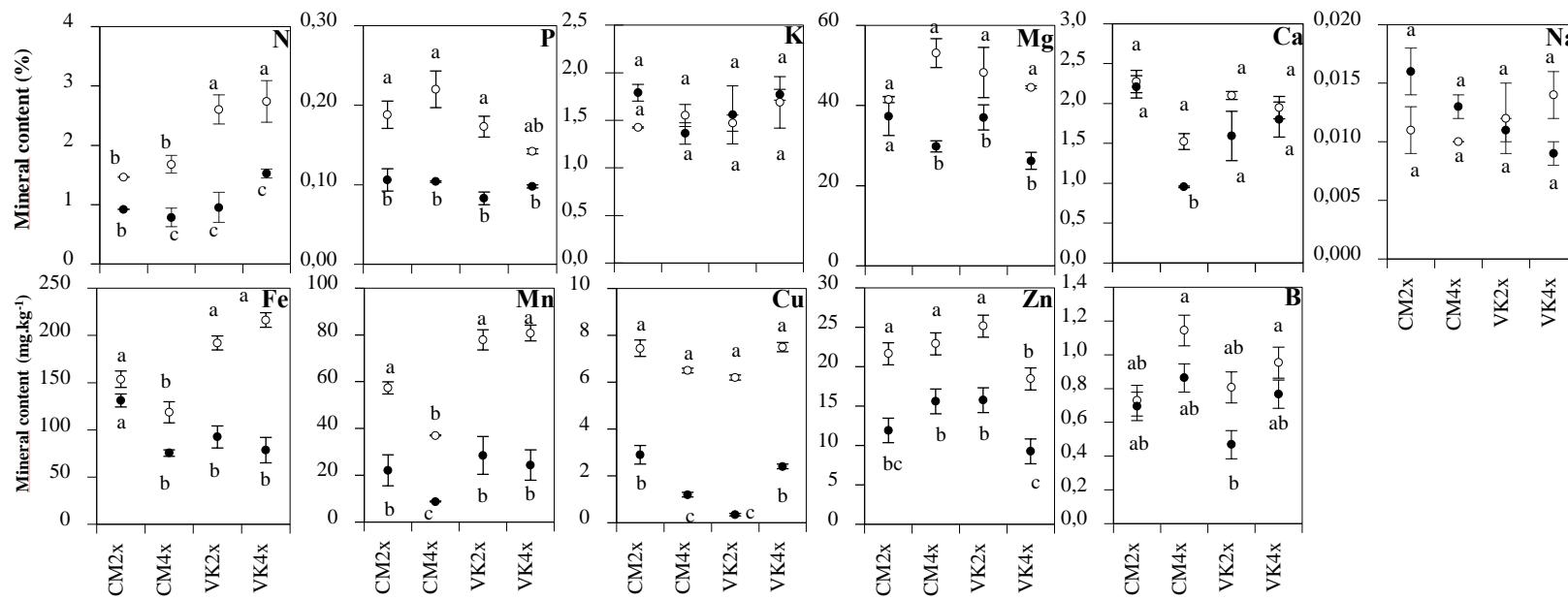
Fig. 5



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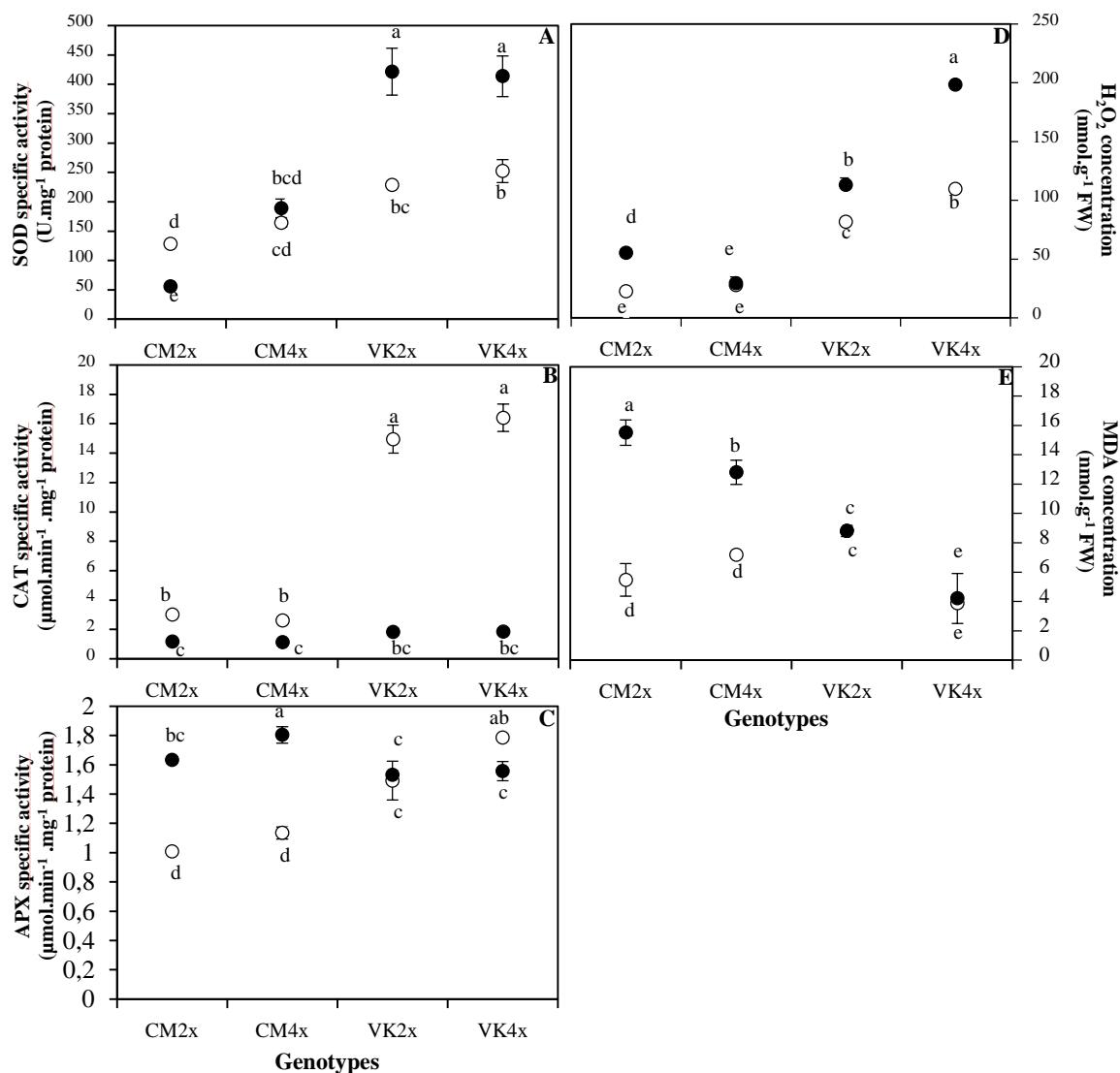
Fig 6.



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Fig. 7



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Supplementary data

Table 1. Probability values produced by analysis of variance (ANOVA) of abaxial epidermis datasets of experimentation conditions.

	Stomatal density	Length of stomata (µm)	Width of stomata (µm)	Length of ostiole (µm)	Width of ostiole (µm)
A (genotype)	10^{-15}	0.063	0.015	10^{-14}	10^{-5}
B (ploidy)	10^{-15}	10^{-15}	10^{-15}	10^{-15}	10^{-15}
C (treatment)	0.620	0.708	0.860	10^{-5}	0.225
D (A x B x C)	10^{-15}	10^{-15}	10^{-15}	10^{-15}	10^{-15}

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

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Table 2. Probability values produced by analysis of variance (ANOVA) on palisadic mesophyll cells ultrastructural datasets of experimentation conditions.

	Cells			Chloroplasts			Starches			Plastoglobuli			Mitochondria			Grana			
	Length (μm)	Width (μm)	Thickness (μm)	Number	Length (μm)	Width (μm)	Number	Length (μm)	Width (μm)	Number	Length (μm)	Width (μm)	Number	Length (μm)	Width (μm)	Number of grana per cell	Length (μm)	Width (μm)	Number of thylakoids per grana
A (genotype)	0.367	0.013	10^{-4}	0.149	10^{-12}	10^{-15}	10^{-3}	0.145	10^{-10}	10^{-4}	0.141	0.573	10^{-3}	0.032	0.800	10^{-4}	0.054	10^{-6}	0.010
B (ploidy)	10^{-8}	10^{-3}	10^{-5}	0.002	0.005	0.505	0.048	0.242	10^{-6}	0.91	0.876	0.849	0.573	0.535	0.330	10^{-3}	0.389	0.810	0.642
C (treatment)	0.093	0.008	0.048	10^{-8}	0.962	0.878	10-8	0.529	0.013	0.714	10^{-4}	10^{-8}	10^{-4}	0.088	0.211	NA	NA	NA	NA
D (A x B x C)	10^{-8}	10^{-14}	10^{-15}	10^{-10}	10^{-15}	10^{-15}	10^{-15}	0.333	10^{-15}	10^{-5}	10^{-15}	10^{-15}	10^{-8}	10^{-2}	0.509	10^{-4}	0.159	10^{-6}	0.023

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

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Table 3. Probability values produced by analysis of variance (ANOVA) on spongy mesophyll cells ultrastructural datasets of experimentation conditions.

	Cells			Chloroplasts			Starches			Plastoglobuli			Mitochondria			Grana			
	Length (μm)	Width (μm)	Thickness (μm)	Number	Length (μm)	Width (μm)	Number	Length (μm)	Width (μm)	Number	Length (μm)	Width (μm)	Number	Length (μm)	Width (μm)	Number of grana per cell	Length (μm)	Width (μm)	Number of thylakoids per grana
A (genotype)	0.727	0.113	0.455	0.149	10^{-12}	10^{-15}	10^{-3}	0.145	10^{-10}	10^{-4}	0.141	0.573	10^{-3}	0.032	0.8	10^{-4}	0.054	10^{-6}	0.01
B (ploidy)	10^{-4}	10^{-7}	10^{-7}	0.002	0.005	0.505	0.048	0.242	10^{-6}	0.91	0.876	0.849	0.573	0.535	0.33	10^{-3}	0.389	0.81	0.642
C (treatment)	0.446	0.743	0.743	10^{-8}	0.962	0.878	10^{-8}	0.529	0.013	0.714	10^{-4}	10^{-8}	10^{-4}	0.088	0.211	NA	NA	NA	NA
D (A x B x C)	10^{-4}	10^{-11}	10^{-11}	10^{-10}	10^{-15}	10^{-15}	10^{-15}	0.333	10^{-15}	10^{-5}	10^{-15}	10^{-15}	10^{-8}	10^{-3}	0.509	10^{-4}	0.159	10^{-6}	0.023

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

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Table 4. Probability values produced by analysis of variance (ANOVA) on physiological and biochemical datasets of experimentation conditions.

	P_{net}	G_s	F_v/F_m	SOD	CAT	APX	H_2O_2	MDA
A (genotype)	0.654	0.243	0.531	10^{-4}	0.005	0.07	0.000	0.022
B (ploidy)	0.288	0.992	0.256	0.305	0.845	0.13	0.195	0.148
C (treatment)	10^{-7}	10^{-4}	10^{-4}	0.082	0.002	0.04	0.078	0.002
D (A x B x C)	10^{-6}	0.008	10^{-4}	10^{-6}	10^{-5}	10^{-6}	0.000	10^{-5}

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

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Table 5. Probability values produced by analysis of variance (ANOVA) on minerals datasets of experimentation conditions.

	N%	P%	K%	Mg%	Ca%	Na%	Fe mg.kg ⁻¹	Mn mg.kg ⁻¹	Cu mg.kg ⁻¹	Zn mg.kg ⁻¹	B mg.kg ⁻¹
A (genotype)	0.174	0.066	0.259	0.635	0.235	0.448	0.818	0.976	0.930	0.766	0.144
B (ploidy)	0.739	0.261	0.898	0.079	0.795	0.234	0.463	0.205	0.944	0.063	0.261
C (treatment)	0.025	0.001	0.276	0.009	0.007	0.070	0.009	0.162	10 ⁻⁷	0.309	0.540
D (A x B x C)	10 ⁻⁴	10 ⁻⁴	0.386	0.028	10 ⁻⁴	0.251	0.089	0.318	10 ⁻⁴	0.042	0.441

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

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