



## Research article

# 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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## ARTICLE INFO

## Keywords:

Citrus  
Nutrient deficiency  
Oxidative stress  
Photosynthesis  
Polyploidy  
Ultrastructure

## ABSTRACT

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

## 1. Introduction

Polyploidy is a common feature and a major facilitator of adaptive evolution in plants (Wendel, 2000; Chen, 2007). It arises spontaneously from unreduced gametes or duplicated chromosomes. Two types of polyploidy are generally recognized: autopolyploidy, which is considered extremely rare in nature (Stebbins, 1950; Grant, 1981), and allopolyploidy. Autopolyploid genomes are composed of identical sets of chromosomes, while allotetraploid genomes are formed through interspecific hybridization (Soltis and Soltis, 2009). Genome variations in

polyploidy have induced changes in anatomical and morphological characteristics, physiological functions and gene expression (Leitch and Leitch, 2008). Polyploid plants frequently occur in adverse environmental conditions, like high altitude with high radiation, more polar latitude, deserts, and nutrient-poor soil (Ehrendorfer, 1980; Thompson and Lumaret, 1992; Soltis and Soltis, 2000). Their potential to improve tolerance has prompted a number of investigations into diploids (2x) and doubled-diploid (4x) genotypes under several environmentally stressful conditions, and some studies have found a tolerance improvement in 4x under heat, drought and salt stress than in their related

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

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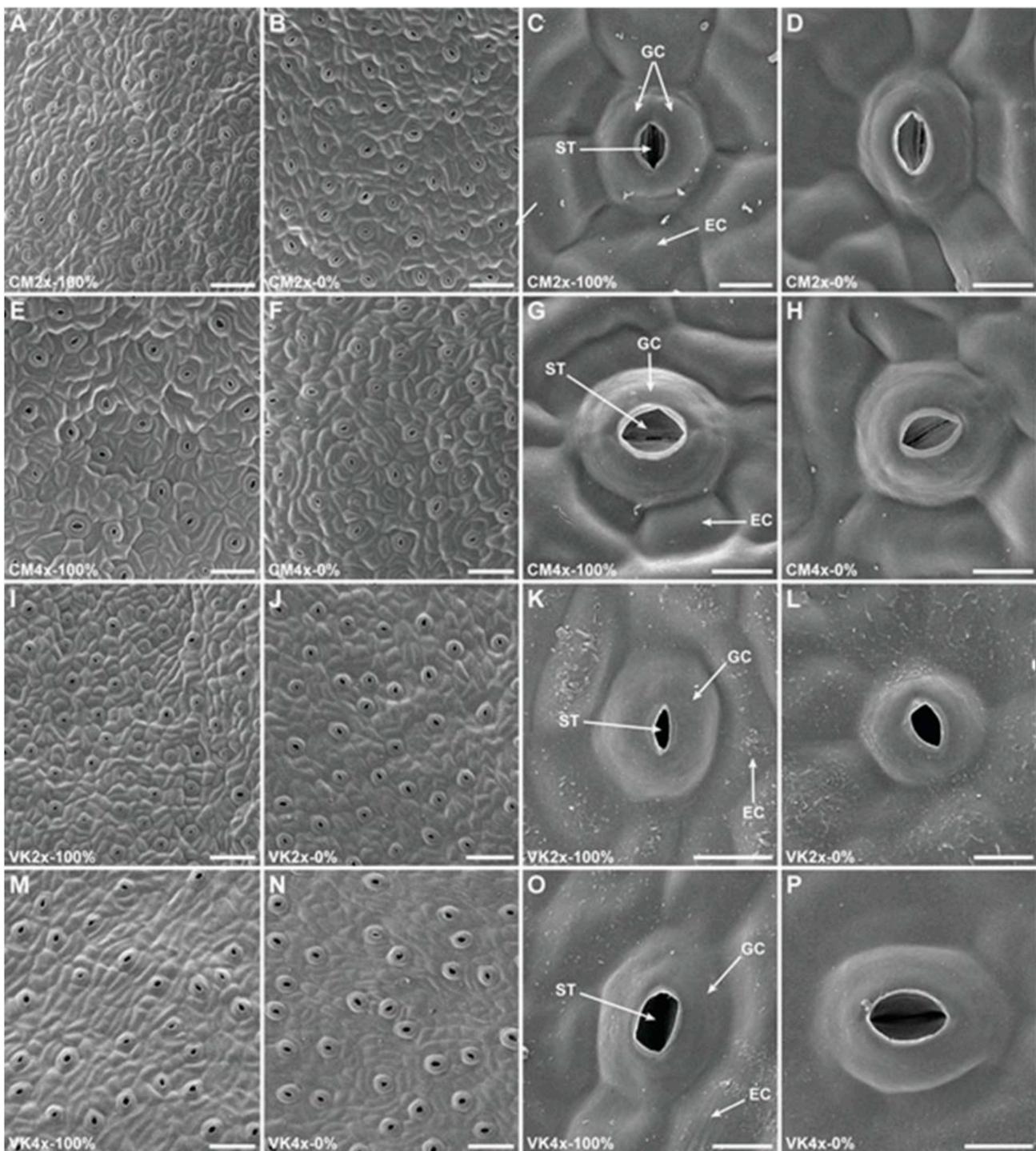
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<https://doi.org/10.1016/j.plaphy.2018.12.020>

Received 13 September 2018; Received in revised form 19 December 2018; Accepted 19 December 2018

Available online 27 December 2018

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**Fig. 1.** Scanning electron 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 (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.

2x (Saleh et al., 2008; Li et al., 2009; Zhang et al., 2010).

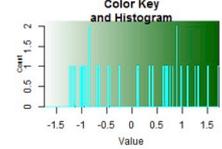
A common consequence of environmental stresses is the overproduction of free radicals and other reactive oxygen species (ROS) that can deteriorate the ultrastructure of plant components (e.g. photosynthetic pigments) and cell membranes (Foyer et al., 1994; Taylor and Craig, 1971). The major sources of ROS in plants cells are chloroplasts, mitochondria and peroxisomes. Plants possess two antioxidant systems—one enzymatic (mainly superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and dehydroascorbate reductase (DHAR)), the other non-enzymatic (mainly ascorbate, glutathione and

proline). However, stress response may change depending on genotype, intensity of stress, and ploidy level (Santini et al., 2013; Liu et al., 2011).

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

**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).

1995). These minerals have various functions: (i) as essential metabolites (e.g. proteins, enzymes and coenzymes, cell walls and chlorophyll), (ii) as enzyme activators or regulators of enzyme-associated processes, and (iii) as non-structural factors in physiological processes (i.e. membrane integrity, photosynthesis, stomatal movement and environmental signalling) (Lal, 2006). Deficiency in one or more mineral elements causes a degradation of the chloroplasts and thus disruption of their photosynthetic capacity (Kalaji et al., 2014).

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

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

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

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

## 2. Materials and methods

### 2.1. Plant material and experimental design

Plants of 2x Citrumelo 4475 (CM2x) and Volkamer lemon (VK2x) genotypes and their related doubled-diploids (CM4x and VK4x,

respectively) were grown from seedling stages (8 weeks old). Seedlings of each genotype were selected after checking their ploidy status by flow cytometry (Partec I, Germany) according to Froelicher et al. (2007). Clonal propagation by nucellar embryogenesis was checked by genotyping with SSR markers as described in Vieira et al. (2016).

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

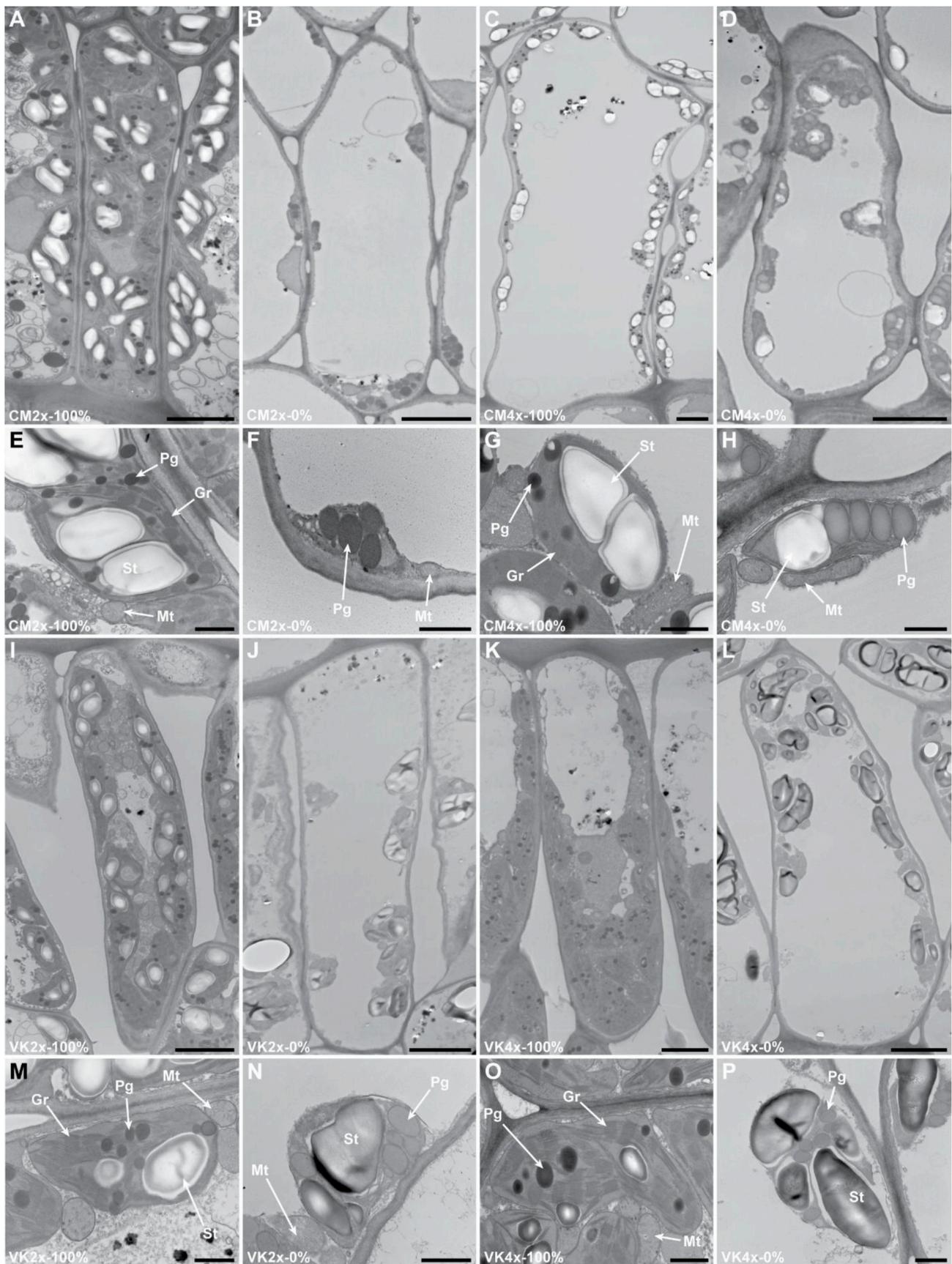
After 4 years and before starting the experiment, the vermiculite was washed with distilled water for 48 h in order to eliminate any nutritional reserves in the pot. The 4-years-old trees were then divided into 2 blocks: one with reference ferti-irrigation (control plants) and the other with irrigation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

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

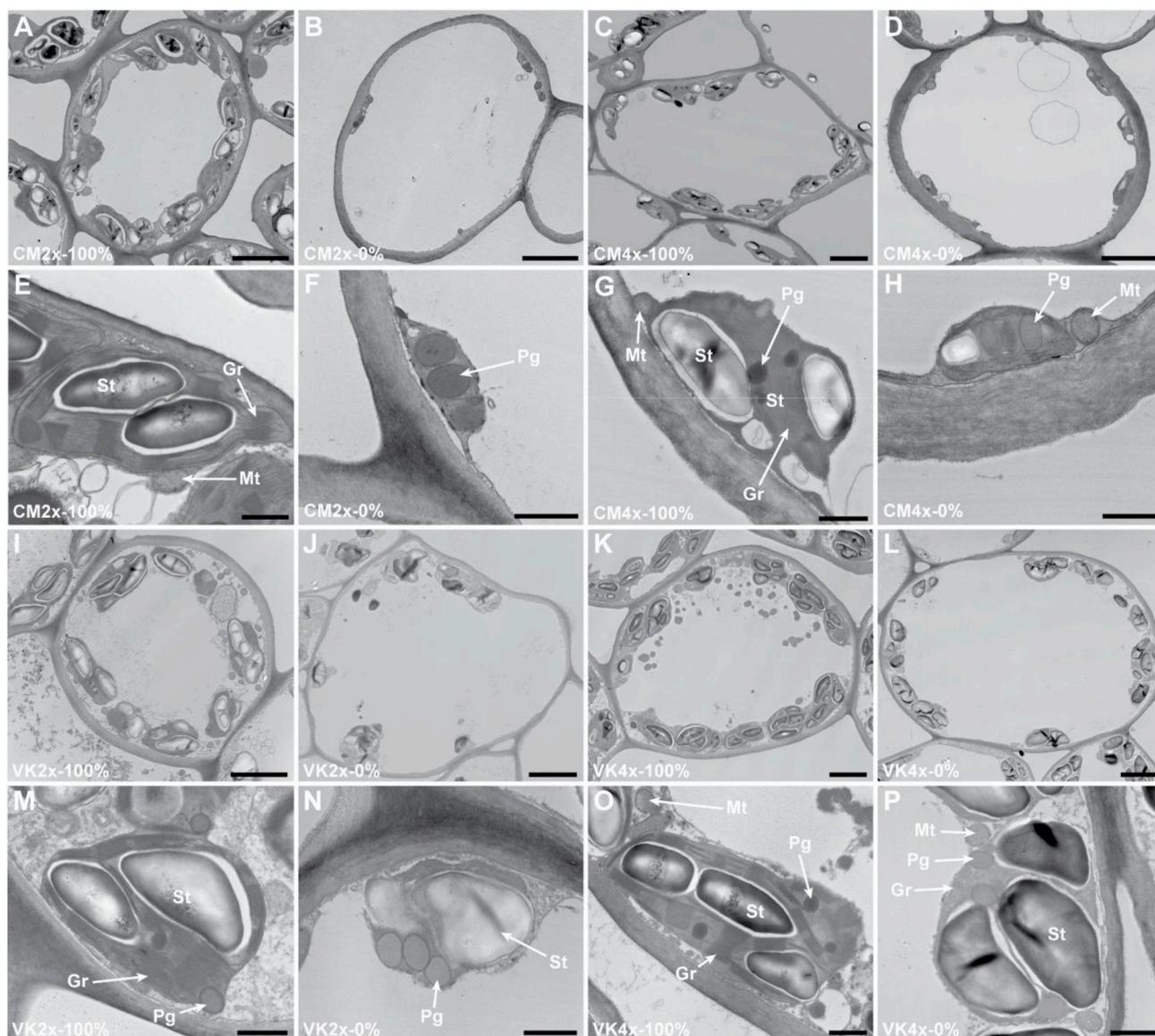
At the same time of measurements and sampling, five 1 cm<sup>2</sup> pieces for scanning electron microscopy and five 1–2 mm<sup>2</sup> pieces for transmission electron microscopy were cut with a razor blade from mid-laminar areas of both sets of leaves (5 replicates for each genotype).

### 2.2. Measurements of gas exchange

Leaf net photosynthetic rate ( $P_{\text{net}}$ ) and stomatal conductance ( $G_s$ ) were measured using a LI-6400 portable photosynthesis system (LI-COR, Lincoln, NE, USA) with the LI6400-40 Leaf Chamber. This experiment was conducted at photosynthetic photon flux density (PPFD) 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-Cor, Lincoln, NE), a constant carbon dioxide



**Fig. 2.** Transmission electron micrographs of palisade mesophyll cells (3A, 3B, 3C, 3D, 3I, 3J, 3K, 3L; scale bar: 5 μm) and their respective chloroplasts (3E, 3F, 3G, 3H, 3M, 3N, 3O, 3P; scale bar: 1 μm) 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.



**Fig. 3.** Transmission electron micrographs of spongy mesophyll cells (3A, 3B, 3C, 3D, 3I, 3J, 3K, 3L; scale bar: 5  $\mu\text{m}$ ) and their respective chloroplasts (3E, 3F, 3G, 3H, 3M, 3N, 3O, 3P; scale bar: 1  $\mu\text{m}$ ) 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.

concentration ( $\text{CO}_2$ ) of  $380 \mu\text{mol mol}^{-1}$ , air flow rate of  $500 \mu\text{mol s}^{-1}$ , and leaf temperature of  $25^\circ\text{C}$ .

### 2.3. Measurements of chlorophyll *a* fluorescence

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

### 2.4. Foliar mineral analysis

All ground samples were dried at  $65 \pm 10^\circ\text{C}$  in an oven overnight,

transferred into a desiccator until cooling and sent to a CIRAD laboratory (Montpellier, France) for analysis of macro- and micro-nutrients.

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

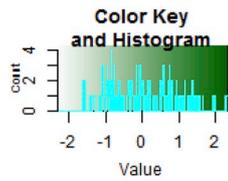
N content in leaves was measured by combustion using a Leco TruMac N determinator.

### 2.5. Scanning electron microscopy (SEM)

Leaf pieces (typically  $1 \text{ cm}^2$ ) were cut with a razor blade from mid-laminar areas of leaves and fixed in cold ( $4^\circ\text{C}$ ) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2, rinsed in a 0.1 M cacodylate buffer at pH 7.2, dehydrated through a graded ethanol series (30%, 50%, 75%, 90% and 100%), and dried with  $\text{CO}_2$  in an Emitech K850 critical point dryer (Quorum Technologies Ltd, Ashford, U.K.). Specimens were mounted on aluminum stubs with carbon double-sided adhesive disks, coated with gold/palladium in a SC7640 sputter coater (Quorum Technologies Ltd, Newhaven, U.K.), and examined under a S-

**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%
Palisade mesophyll cells									
Cells	Length (µm)	28.40	<b>24.78</b>	48.83	<b>42.12</b>	33.29	<b>28.10</b>	43.76	<b>45.46</b>
	Width (µm)	10.20	<b>10.07</b>	23.83	<b>10.23</b>	8.30	<b>6.47</b>	12.77	<b>11.23</b>
	Thickness (µm)	0.45	<b>0.43</b>	0.76	<b>0.71</b>	0.26	<b>0.29</b>	0.41	<b>0.40</b>
Chloroplasts	Number	8.88	<b>3.00</b>	15.20	<b>7.25</b>	9.40	<b>4.60</b>	13.60	<b>9.60</b>
	Length (µm)	5.41	<b>2.87</b>	6.34	<b>3.91</b>	5.03	<b>3.45</b>	5.75	<b>5.44</b>
Starches	Width (µm)	2.40	<b>1.16</b>	2.80	<b>1.28</b>	2.81	<b>2.47</b>	2.60	<b>3.41</b>
	Number	16.51	<b>0.71</b>	30.2	<b>8.12</b>	22.60	<b>8.40</b>	22.20	<b>23.80</b>
Plastoglobuli	Length (µm)	1.78	<b>1.21</b>	2.60	<b>1.14</b>	2.13	<b>1.76</b>	1.22	<b>2.74</b>
	Width (µm)	1.01	<b>0.76</b>	1.43	<b>0.77</b>	1.05	<b>1.06</b>	0.72	<b>1.79</b>
Mitochondria	Number	62.50	<b>16.85</b>	59.00	<b>33.00</b>	61.88	<b>26.60</b>	53.00	<b>54.75</b>
	Length (µm)	0.53	<b>0.61</b>	0.31	<b>0.70</b>	0.41	<b>0.59</b>	0.46	<b>0.47</b>
Grana	Width (µm)	0.33	<b>0.51</b>	0.52	<b>0.52</b>	0.31	<b>0.39</b>	0.39	<b>0.40</b>
	Number	11.71	<b>4.00</b>	13.40	<b>7.50</b>	13.60	<b>5.20</b>	20.80	<b>7.40</b>
Grana	Length (µm)	0.85	<b>0.85</b>	0.96	<b>0.68</b>	1.22	<b>0.75</b>	0.97	<b>0.75</b>
	Width (µm)	0.56	<b>0.61</b>	0.69	<b>0.53</b>	0.69	<b>0.52</b>	0.79	<b>0.40</b>
	Number of grana per cell	22.93	<b>Absent</b>	13.93	<b>Absent</b>	20.60	<b>Absent</b>	26.26	<b>Absent</b>
	Length (µm)	0.52	<b>Absent</b>	0.34	<b>Absent</b>	0.40	<b>Absent</b>	0.38	<b>Absent</b>
Grana	Width (µm)	0.60	<b>Absent</b>	0.56	<b>Absent</b>	0.42	<b>Absent</b>	0.42	<b>Absent</b>
	Number of thylakoids per grana	27.26	<b>Absent</b>	25.20	<b>Absent</b>	22.73	<b>Absent</b>	18.13	<b>Absent</b>

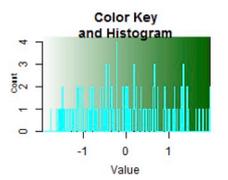
Values are means ( ± standard error) of independent measurements on five different cells section (n = 5) for length, width and thickness of cells and for number of chloroplasts, starches, plastoglobuli and mitochondria and of 30 independent measurements on different cells section (n = 30) for length 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).

3400N scanning electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan) at an accelerating voltage of 5 at the University of Corsica (Corte, France). SEM measurements were realized

using the software of the microscope. 30 independent measurements on 5 different leaves of each variety and each genotype were realized in order to estimate length and width of stomata and ostioles. 5

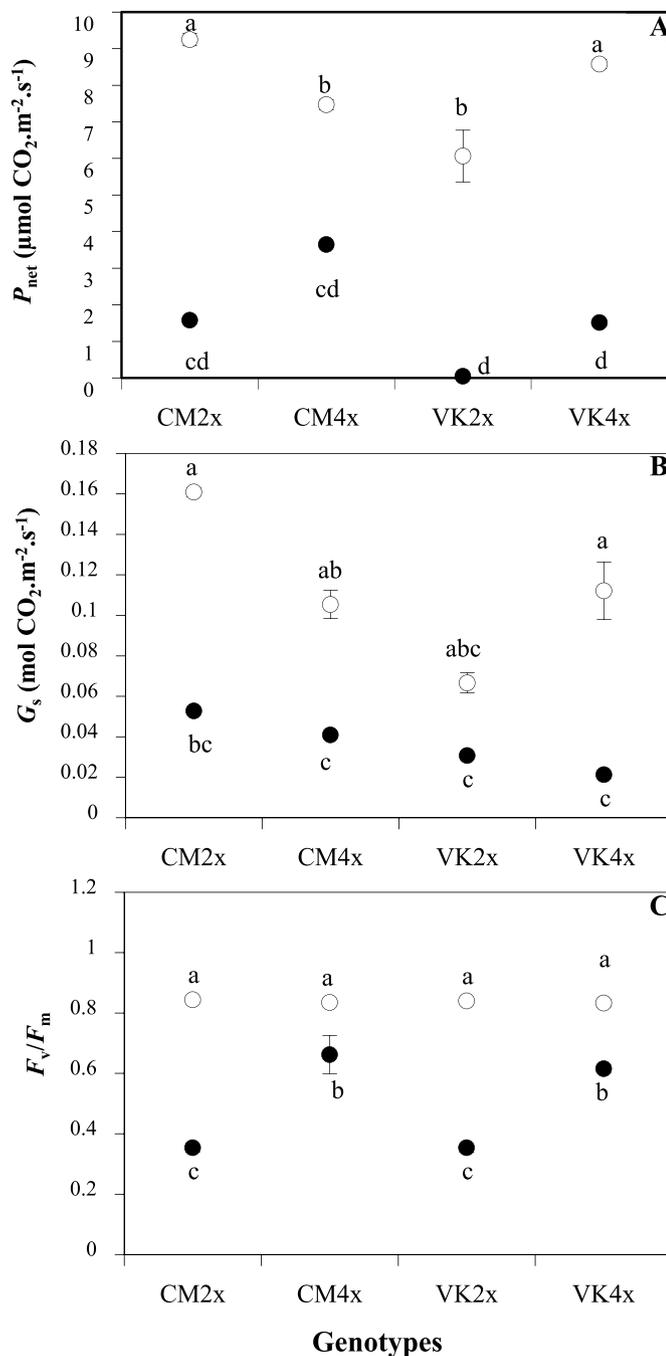
**Table 3**

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

Values are mean ( ± 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).



**Fig. 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.

independent measurements on 5 different leaves of each variety and each genotype were analyzed using Adobe Photoshop software in order to estimate the stomata density. The number of stomata was counted on photographs and taken into account the stomata cut only on two sides of the photograph.

## 2.6. Transmission electron microscopy (TEM)

Leaf pieces (typically  $1\text{ mm}^2$ ) were cut with a razor blade from mid-laminar areas of leaves and fixed in cold ( $4\text{ }^\circ\text{C}$ ) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2, rinsed in a 0.1 M cacodylate buffer at pH 7.2, post-fixed in cold ( $4\text{ }^\circ\text{C}$ ) 1% osmium tetroxide in the same buffer for 1 h, dehydrated through a graded ethanol series (70% and 100%) and propylene oxide, embedded in Spurr, and polymerized at  $60\text{ }^\circ\text{C}$  for 24 h. Ultra-thin sections (60–90 nm) were cut using an Power tome PC ultramicrotome (RMC Boeckeler, Tuscon, U.S.A.). The sections were placed on 200- and 300-mesh copper grids and stained with UranylLess (Delta Microscopies, France) and lead citrate. Sections were examined on a Hitachi H-7650 (Hitachi High-Technologies Corporation, Tokyo, Japan) at an accelerating voltage of 80 kV at the University of Corsica (Corte, France). TEM measurements were realized using the software of the microscope. 10 cells of each variety, each genotype and each mesophyll type were analyzed in order to estimate length, width and thickness of cells and length, width and number of chloroplasts, starches, plastoglobuli and mitochondria. 30 independent measurements of each variety, each genotype and each mesophyll type were analyzed in order to estimate length and width of chloroplasts, starches, plastoglobuli, mitochondria, grana, number of grana per cell section, and number of thylakoids per granum.

## 2.7. Assay of oxidation markers

Malondialdehyde (MDA) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) contents and antioxidant enzyme activities (superoxide dismutase, catalase and ascorbate peroxidase) were determined using the method described by Santini et al. (2013). A V-630 spectrophotometer (Jasco Inc., Tokyo, Japan) was used to measure the absorption spectra of the samples.

## 2.8. Statistical analyses

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

## 3. Results and discussion

### 3.1. Anatomical properties of doubled-diploid versus diploid genotypes under control conditions

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

Doubled-diploid genotypes had more chloroplasts but their size remained broadly similar to their 2x counterparts (Figs. 2 and 3; Tables 2 and 3; Sup. Tables 2 and 3), in line with previous reports (Jellings and Leech, 1984; Mathura et al., 2006). Conversely, ploidy level did not influence number and size of starches and plastoglobuli or number of grana per chloroplasts, which were approximately identical between

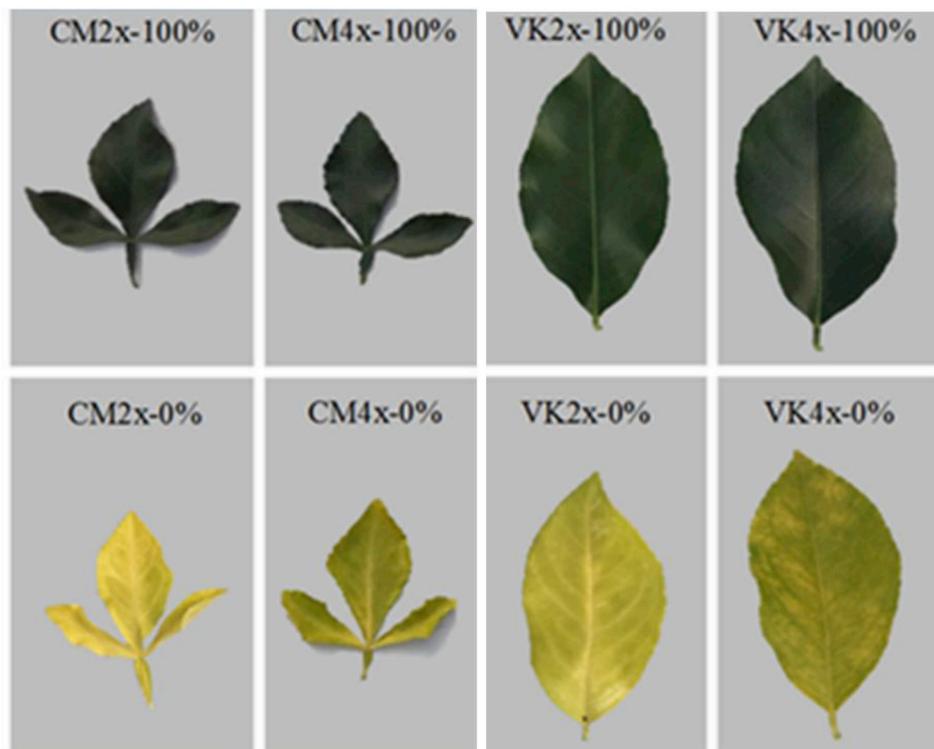


Fig. 5. Leaf damages after 210 days of total nutrient deficiency.

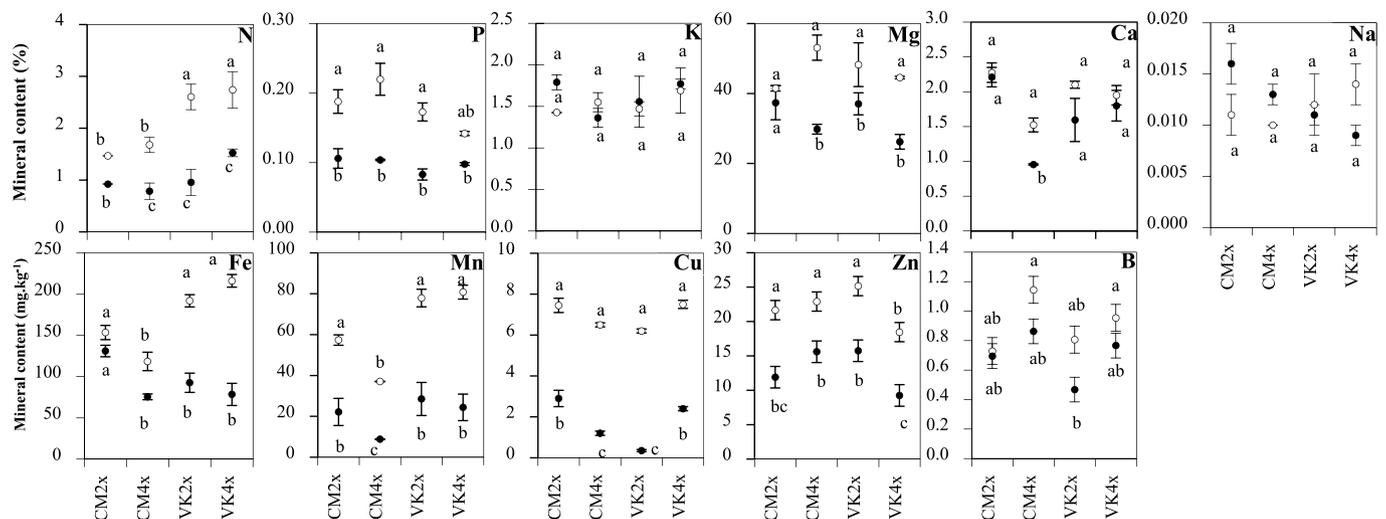


Fig. 6. Total content of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), sodium (Na), iron (Fe), manganese (Mn), copper, zinc (Zn) and boron (B) in leaves of diploid (CM2x) and doubled-diploid (CM4x) Citrumelo 4475 and diploid (VK2x) and doubled-diploid (VK4x) Volkamer lemon 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 analyzed using ANOVA and Fisher LSD tests ( $P < 0.05$ ). Different letters indicate significant differences between genotypes and treatments.

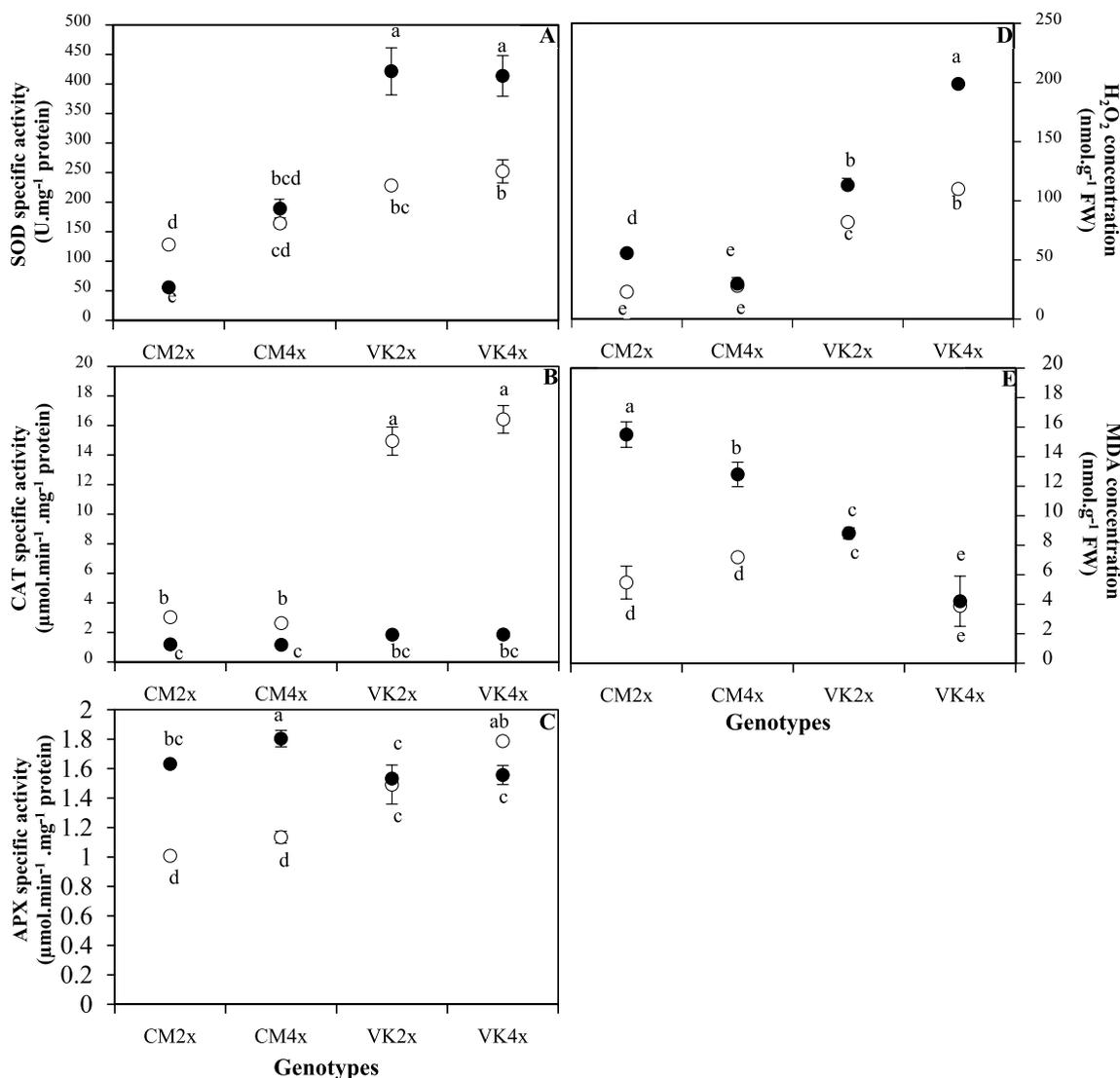
the different ploidy levels and whatever the parenchyma (Figs. 2 and 3; Tables 2 and 3; Sup. Tables 2 and 3). This result was also observed in 4x black locust (*Robinia pseudoacacia* L.) (Meng et al., 2014).

### 3.2. Impact of tetraploidization on photosynthetic properties under nutrient deficiency

Deficiency of one or more essential minerals is known to affect the photosynthetic process (Lima et al., 1999; Huang et al., 2004; Boussadia et al., 2010). The decrease in  $P_{net}$  was associated with a decrease in  $G_s$

in all genotypes (Fig. 4A and B; Sup. Table 4), suggesting a stomatal limitation (Zhao et al., 2005) that would lead to down-regulation of the  $CO_2$  accumulation (Flexas et al., 2013).

$P_{net}$  was higher in 4x honeysuckle (*Lonicera japonica* Thunb.) (Li et al., 2009), spathe flower (*Spathiphyllum wallisii*) (Van Laere et al., 2011) and in Carrizo citrange citrus seedlings (*Citrus sinensis* (L.) Osbeck  $\times$  *Poncirus trifoliata* (L.) Raf) (Oliveira et al., 2017) than their 2x counterparts under drought stress, due to higher maintained degree of stomatal opening. However, nutrient deficiency caused less pronounced inhibition of photosynthesis in 4x seedlings than in related 2x (Fig. 4C;



**Fig. 7.** Determination of specific activities of (A) superoxide dismutase (SOD), (B) catalase (CAT) and (C) ascorbate peroxidase (APX) and (D) hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) 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 (± 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.

Sup. Table 4) possibly due to an upregulation of non-stomatal factors (biochemical) (Klamkowski and Treder, 2008; Shahid et al., 2011) or a decrease in protective non-radiative energy dissipation, photodamage of the PSII centers, or both (Osmond, 1994). Photoinhibitory impacts on PSII occurred proportionally more in 2x honeysuckle (*Lonicera japonica* Thunb.) under salt stress (Yan et al., 2015) and heat stress (Li et al., 2011) than in 4x counterparts. The lower decrease of photosynthetic rate in 4x genotypes under nutrient deficiency could be due to ultrastructural changes in leaves (Parisod et al., 2010; Xiong et al., 2006).

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

In each genotype, nutrient deficiency decreased the total content of all minerals except for K (Fig. 6) and was associated with leaf alteration (Fig. 5). Despite a similar decrease in minerals content (Fig. 6), leaves in 2x genotypes had a greater proportion of chlorotic leaves than in their related 4x (Fig. 5), suggesting a substantial loss of chlorophyll pigments (Zhang et al., 2016). However, taking in account the long-term nutrient

deficiency, there was no large change in stomata size and density in either 2x or 4x genotypes (Fig. 1; Table 1; Sup. Table 1), suggesting that stomata do not play a major role in response to nutritional stress.

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

The lower deterioration of thylakoids in 4x genotypes could explain their better F<sub>v</sub>/F<sub>m</sub> ratio and, in turn, the higher photosynthetic activity maintained in 4x genotypes than their respective 2x (Fig. 4C; Sup.

Table 4). Interestingly, lower chloroplast damage associated with higher photosynthetic activity was also observed in 4x black locust (*Robinia pseudoacacia* L.) under salt stress (Wang et al., 2013) than in their related 2x.

Plastoglobuli containing products of membrane degradation showed an abnormal increase in size (spongy and palisadic mesophyll cells) in all genotypes except VK4x (Figs. 2 and 3; Tables 2 and 3; Sup. Tables 2 and 3), which may indicate a link between chloroplast disorganization and plastoglobuli (Dou et al., 1999). In the chloroplast stroma, nutrient deficiency led to a decrease in the number as well as the length and width of starch granules in both spongy and palisadic mesophyll cells in all genotypes except VK4x (Figs. 2 and 3; Tables 2 and 3; Sup. Tables 2 and 3). VK4x showed an increase in starch length and width (Figs. 2 and 3; Tables 2 and 3; Sup. Tables 2 and 3), as reported in other studies under nutrient deficiency (Bondada and Syvertsen, 2003; Zhao et al., 2015). The swelling of starch granules could be induced by an up-regulation of transcripts encoding key enzymes involved in starch biosynthesis and a down-regulation of transcripts encoding enzymes involved in starch consumption (Tao et al., 2013). In CM4x, VK2x and CM2x, the decline in length and width of transitory starch (Figs. 2 and 3; Tables 2 and 3; Sup. Tables 2 and 3) would suggest a high mobilization and translocation of starch leading, in turn, to a more advanced senescence process than in VK4x (Lindroth et al., 2002). In leaves, stimulation of the degradation of starch granules would supply the carbon and energy needed for the stress response, especially when photosynthesis becomes too weak, and protect cellular structures by increasing soluble sugars as osmoprotectants (Kaplan and Guy, 2004; Rizhsky et al., 2004; Naeem et al., 2012).

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

Mesenchymal cells, especially chloroplasts, endure ultrastructure damage from abiotic stresses such as salinity (Hernandez et al., 1995; Fidalgo et al., 2004), drought and heat (Grigороva et al., 2011), chilling stress (Kratsch and Wise, 2000), nutrient excess (Papadakis et al., 2004) and nutrient deficiency (Bondada and Syvertsen, 2003; Papadakis et al., 2007). Reduction of chloroplast numbers leads to a decrease in photosynthetic activity under nutrient deficiency. This decrease is probably due to oxidative stress, i.e. an imbalance between ROS accumulation and antioxidant system capacity (Djanaguiraman and Prasad, 2010). Previous studies have reported that nutrient deficiency enhances oxidative metabolism (Cakmak, 1994; Tewari et al., 2006). Here, SOD and APX activities differed between genotypes, but CAT activity decreased with nutrient deficiency in all genotypes (Fig. 7A, B and C; Sup. Table 4). CAT activity decreases in several species under numerous environmental stresses such as salt stress (Tanaka et al., 1999), shading stress (Causin et al., 2015) and nutrient deficiency (Huang et al., 2004; Tewari et al., 2004). The increase in APX only seemed to compensate for the lower CAT activity in CM4x, which limited accumulation of H<sub>2</sub>O<sub>2</sub> (Fig. 7D; Sup. Table 4). The increase in MDA content in CM2x and CM4x (Fig. 7E; Sup. Table 4) could result directly from the transformation of the H<sub>2</sub>O<sub>2</sub> in OH<sup>•</sup> via Fenton or Haber-Weiss reactions, but also from a direct conversion of O<sub>2</sub> to OH<sup>•</sup> from an addition of three electrons. The lower MDA and H<sub>2</sub>O<sub>2</sub> accumulation in CM4x was associated with greater SOD and APX activities. This result could explain that

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2018.12.020>.

CM4x had less ultrastructural damage and decreased less in photosynthetic activity than CM2x. Higher antioxidant capacity was associated to better ultrastructural cell integrity for better protection under drought stress in a tolerant apple rootstock compared to a sensitive one (Wang et al., 2012). In agreement with our results, MDA and H<sub>2</sub>O<sub>2</sub> were also less accumulated in 4x Ignam (*Dioscorea*) under heat stress (Zhang et al., 2010) and in black locust (*Robinia pseudoacacia* L.) under salt stress (Meng et al., 2012) than in their related 2x.

In VK2x and VK4x, the increase in SOD activity and the lower or similar APX and CAT activities could explain their higher accumulation of H<sub>2</sub>O<sub>2</sub>. These results imply that factors other than redox status could explain the stronger ultrastructural degradations in VK2x than VK4x, i.e. transcriptional regulations, phytohormones and nutrient signalling (Woo et al., 2013; Liu et al., 2016).

## 4. Conclusion

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

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

## Disclosures

The authors have no conflicts of interest to declare.

## CRediT authorship contribution statement

**Julie Oustric:** Data curation, Investigation, Formal analysis, Writing - original draft. **Yann Quilichini:** Formal analysis. **Raphaël Morillon:** Supervision, Validation, Writing - original draft. **Stéphane Herbette:** Supervision, Validation, Writing - original draft. **François Luro:** Supervision, Validation. **Jean Giannettini:** Supervision, Validation. **Liliane Berti:** Supervision, Validation. **Jérémie Santini:** Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

## Acknowledgements

Julie Oustric received grant support from the “Collectivité Territoriale de Corse” (CTC). This work was funded by the “Collectivité Territoriale de Corse” as part of the research project “Sélection de porte-greffes pour le développement d'une agrumiculture durable en Corse”. We thank Jean-Claude Ribaut for hosting this study at the AREFLEC experimental station, and Gilles Paolacci and Paul Martin for installing and monitoring the ferti-irrigation system in the greenhouse.

**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 <sup>-15</sup>	0.063	0.015	10 <sup>-14</sup>	10 <sup>-5</sup>
B (ploidy)	10 <sup>-15</sup>	10 <sup>-15</sup>	10 <sup>-15</sup>	10 <sup>-15</sup>	10 <sup>-15</sup>
C (treatment)	0.620	0.708	0.860	10 <sup>-5</sup>	0.225
D (A x B x C)	10 <sup>-15</sup>	10 <sup>-15</sup>	10 <sup>-15</sup>	10 <sup>-15</sup>	10 <sup>-15</sup>

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.

**Table 2**

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

	Cells			Chloroplasts			Starches			Plastoglobuli
	Length (µm)	Width (µm)	Thickness (µm)	Number	Length (µm)	Width (µm)	Number	Length (µm)	Width (µm)	Number
A (genotype)	0.367	0.013	10 <sup>-4</sup>	0.149	10 <sup>-12</sup>	10 <sup>-15</sup>	10 <sup>-3</sup>	0.145	10 <sup>-10</sup>	10 <sup>-4</sup>
B (ploidy)	10 <sup>-8</sup>	10 <sup>-3</sup>	10 <sup>-5</sup>	0.002	0.005	0.505	0.048	0.242	10 <sup>-6</sup>	0.91
C (treatment)	0.093	0.008	0.048	10 <sup>-8</sup>	0.962	0.878	10 <sup>-8</sup>	0.529	0.013	0.714
D (A x B x C)	10 <sup>-8</sup>	10 <sup>-14</sup>	10 <sup>-15</sup>	10 <sup>-10</sup>	10 <sup>-15</sup>	10 <sup>-15</sup>	10 <sup>-15</sup>	0.333	10 <sup>-15</sup>	10 <sup>-5</sup>

	Plastoglobuli		Mitochondria		Grana				
	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)	0.141	0.573	10 <sup>-3</sup>	0.032	0.800	10 <sup>-4</sup>	0.054	10 <sup>-6</sup>	0.010
B (ploidy)	0.876	0.849	0.573	0.535	0.330	10 <sup>-3</sup>	0.389	0.810	0.642
C (treatment)	10 <sup>-4</sup>	10 <sup>-8</sup>	10 <sup>-4</sup>	0.088	0.211	NA	NA	NA	NA
D (A x B x C)	10 <sup>-15</sup>	10 <sup>-15</sup>	10 <sup>-8</sup>	10 <sup>-2</sup>	0.509	10 <sup>-4</sup>	0.159	10 <sup>-6</sup>	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.

**Table 3**

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

	Cells			Chloroplasts			Starches			
	Length (µm)	Width (µm)	Thickness (µm)	Number	Length (µm)	Width (µm)	Number	Length (µm)	Width (µm)	Number
A (genotype)	0.727	0.113	0.455	0.149	10 <sup>-12</sup>	10 <sup>-15</sup>	10 <sup>-3</sup>	0.145	10 <sup>-10</sup>	10 <sup>-10</sup>
B (ploidy)	10 <sup>-4</sup>	10 <sup>-7</sup>	10 <sup>-7</sup>	0.002	0.005	0.505	0.048	0.242	10 <sup>-6</sup>	10 <sup>-6</sup>
C (treatment)	0.446	0.743	0.743	10 <sup>-8</sup>	0.962	0.878	10 <sup>-8</sup>	0.529	0.013	0.013
D (A x B x C)	10 <sup>-4</sup>	10 <sup>-11</sup>	10 <sup>-11</sup>	10 <sup>-10</sup>	10 <sup>-15</sup>	10 <sup>-15</sup>	10 <sup>-15</sup>	0.333	10 <sup>-15</sup>	10 <sup>-15</sup>

	Plastoglobuli		Mitochondria		Grana					
	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)	10 <sup>-4</sup>	0.141	0.573	10 <sup>-3</sup>	0.032	0.8	10 <sup>-4</sup>	0.054	10 <sup>-6</sup>	0.01
B (ploidy)	0.91	0.876	0.849	0.573	0.535	0.33	10 <sup>-3</sup>	0.389	0.81	0.642
C (treatment)	0.714	10 <sup>-4</sup>	10 <sup>-8</sup>	10 <sup>-4</sup>	0.088	0.211	NA	NA	NA	NA
D (A x B x C)	10 <sup>-5</sup>	10 <sup>-15</sup>	10 <sup>-15</sup>	10 <sup>-8</sup>	10 <sup>-3</sup>	0.509	10 <sup>-4</sup>	0.159	10 <sup>-6</sup>	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.

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 <sub>2</sub> O <sub>2</sub>	MDA
A (genotype)	0.654	0.243	0.531	10 <sup>-4</sup>	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 <sup>-7</sup>	10 <sup>-4</sup>	10 <sup>-4</sup>	0.082	0.002	0.04	0.078	0.002
D (A x B x C)	10 <sup>-6</sup>	0.008	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	0.000	10 <sup>-5</sup>

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.

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 <sup>-1</sup>	Mn mg.kg <sup>-1</sup>	Cu mg.kg <sup>-1</sup>	Zn mg.kg <sup>-1</sup>	B mg.kg <sup>-1</sup>
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 <sup>-7</sup>	0.309	0.540
D (A x B x C)	10 <sup>-4</sup>	10 <sup>-4</sup>	0.386	0.028	10 <sup>-4</sup>	0.251	0.089	0.318	10 <sup>-4</sup>	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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