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Impact of tetraploidization on morphophysiological leaf traits in the drought tolerant 'de Ramellet' tomato landrace

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Abstract

Tetraploidization was induced in the drought-tolerant tomato landrace 'de Ramellet' to evaluate its physiological and anatomical response under well-watered (WW) and water-deficient (WD) conditions. Under WW, tetraploid plants exhibited approximately 40% lower stomatal density and approximately 80% larger stomata than diploids. Net photosynthetic rate (P_N), intrinsic water-use efficiency, and intercellular CO_2 concentration remained unchanged between diploids and tetraploids. Under WD, both genotypes reduced P_N and stomatal conductance by similar proportions; however, only diploids decreased leaf area and adjusted stomatal density and size, whereas the tetraploid maintained stomatal traits similarly to those in WW conditions. However, both genotypes maintained similar photosynthetic capacity under WD despite different stomatal display and total pore area, which suggests the involvement of morphophysiological mechanisms beyond stomatal traits, such as root traits and hydraulic regulation.

Keywords: drought stress; gas exchange traits; *Solanum lycopersicum*; stomatal traits; tetraploidization.

Introduction

The process of polyploidization involves the multiplication of an organism's chromosome set, frequently leading to the emergence of new species with distinct traits compared to their ancestors (Zhang *et al.* 2019). It can be the result of hybridization (*i.e.*, allopolyploidization, resulting from merging structurally different chromosome sets,

Highlights

- Changes in stomatal size and density did not affect photosynthetic performance
- The capacity to modify leaf size and stomatal traits under drought stress was limited
- Drought adaptation in 'de Ramellet' is driven by factors other than total pore area

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Abbreviations: a_{max} – maximum stomatal pore area; C_i – intercellular CO_2 concentration; D – stomatal density; ET_C – crop evapotranspiration; g_s – stomatal conductance; g_{smax} – theoretical maximum stomatal conductance; L – stomatal length (considering guard cell length); LA – leaf area; LMA – leaf mass per area; PAI – pore area index; P_N – net photosynthetic rate; P_{Nmax} – maximum net photosynthetic rate; RH – relative humidity; S – stomatal size; SAI – stomatal area index; WD – water deficit; WUE_i – intrinsic water-use efficiency; WW – well-watered.

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commonly from different species), and from cell anomalies at the individual plant level (*i.e.*, autopolyploidy, resulting from multiplying a particular chromosome set) (Tate *et al.* 2004). It is considered a major evolutionary force, as all flowering plants have at least one, if not numerous, ancient polyploidy events in their evolutionary history (OTPTI 2019). Natural polyploidization is also associated with plant domestication in relevant crops and is a relatively common strategy in artificial crop improvement, leading to novel crop varieties with higher vigour and resistance to both biotic and abiotic stressors (Sattler *et al.* 2016, Trojak-Goluch *et al.* 2021). It is the case of crops such as durum and bread wheat (tetraploid -4x- and hexaploid -6x-, respectively; Feldman *et al.* 1995, Levy and Feldman 2022), strawberry (octoploid -8x-; Edger *et al.* 2019), oats (6x; Tomaszewska *et al.* 2022), sweet potato (4x, 6x; Zhang *et al.* 2025), alfalfa (4x; Capomaccio *et al.* 2010), cotton (4x; Chen *et al.* 2020), and coffee (4x; Scalabrin *et al.* 2020) among others.

The enlargement of the cell nucleus as a direct consequence of polyploidization results in increased cell size and larger stomata (Aryavand *et al.* 2003, Beaulieu *et al.* 2008, Wilson *et al.* 2021). Guard cell size in fossil plants has been used as evidence for the origin of many angiosperms through polyploidization events (Masterson 1994). The role of polyploidy in enhancing plant evolution under stressful conditions has been previously reported (Van de Peer *et al.* 2021). In this regard, one of the factors associated with the widespread radiation of angiosperms across most land environments and stressful conditions is the variation of several orders of magnitude in the ratio between stomatal size and density (Franks and Beerling 2009).

Maximum stomatal pore area (*i.e.*, fully open stomata) drives leaf gas-exchange capacity (Franks and Beerling 2009). Across most plant lineages, it has been described a negative correlation between stomatal size and density, and a theoretical maximum limit for stomatal size for a given density (Franks and Beerling 2009). Furthermore, despite a given maximum stomatal pore area can be achieved either with a low number of large stomata, or a large number of small stomata, the latter is more frequently observed in drought-adapted plants (Chaves *et al.* 2016, Hetherington and Woodward 2003, Peppe *et al.* 2011). The reasons behind this are basically two. First, smaller stomata allow higher responsiveness and control capacity, given that large stomata react later and take longer time to open and close than small ones, which has a direct impact in water-use efficiency (Franks and Farquhar 2007, Drake *et al.* 2013, Lawson and Blatt 2014, McAusland *et al.* 2016, Lawson and Vialet-Chabrand 2019) and energetic cost in stomatal movements is lower in small stomata (Assmann and Zeiger 1987, Srivastava *et al.* 1995). Second, smaller stomata have shorter stomatal pore depth, which results in shorter diffusion path length for gases and thus, higher stomatal conductance for the same maximum pore area (Franks and Beerling 2009). However, there are physical constraints to maximum stomatal number, since the largest number of stomata that can be packed into a given leaf area depends on its size and on the ratio of stomata to epidermal cells; yet stomatal size and density are the only epidermal characteristics determining maximum gas-exchange capacity (Franks and Beerling 2009, Haworth *et al.* 2023).

Plant adaptive strategies to drought (and to other abiotic stressors) involve morphophysiological adaptations that can lead to suboptimal performance under non-stressing conditions. This includes the reduction of the theoretical maximum stomatal density, for example, by becoming hypostomatous (*e.g.*, Muir 2015), and at the extreme, losing leaf capacity for gas exchange, which is performed exclusively by modified stems (*e.g.*, some Cactaceae and Euphorbiaceae; Lüttge 2010). Polyploidy can contribute to these adaptations, providing additional mechanisms to cope with water limitation. Hence, there is evidence of plant lineages with variable ploidy levels in which ploidy increases from equator to poles (Rice *et al.* 2019), and with a higher frequency of polyploids in extremely dry environments. In such lineages, and also in domesticated plant species, the impact of ploidy in enhancing abiotic stress tolerance goes beyond direct stomatal traits, affecting also hormone regulation and gene expression patterns (reviewed in Van de Peer *et al.* 2021).

Consequently, increasing stomatal size through polyploidization in a drought-adapted plant may lead to two alternative scenarios when this plant is cultivated under optimal conditions. On the one hand, if the diploid plant commonly operates under suboptimal conditions due to a stomatal size and density devoted to tolerate drought, not to maximise growth, *e.g.*, small stomata in low density, polyploidization might enhance its growth capacity under optimal conditions, since increased stomatal size may not reduce density, but may increase gas-exchange capacity. On the other hand, if the diploid has stomatal size and density allowing maximized growth capacity in suboptimal conditions, *e.g.*, small stomata in the highest density, the increased stomatal size in the polyploid might be detrimental when grown under optimal cultivation conditions, since it may not increase gas-exchange capacity, but may reduce density to fit larger stomata. Nonetheless, it has also been described that polyploid plants with lower stomatal density than their diploid counterparts can tolerate severe drought better (Van Laere *et al.* 2011). This suggests that crop improvement with greater drought tolerance might, to some extent, be directed towards breeding with lower ploidy level ancestors (Haworth *et al.* 2023).

To test the impact of tetraploidization in a drought-tolerant crop, we induced (auto)tetraploidy in the Mediterranean tomato (*S. lycopersicum* L.) landrace 'de Ramellet', which has demonstrated high capacity to tolerate drought when cultivated outdoors under Mediterranean summer conditions (*e.g.*, Fullana-Pericàs *et al.* 2017, 2019; Galmés *et al.* 2011, 2013). As compared to commercial and non-drought-adapted tomato varieties, under optimal growth conditions, 'de Ramellet' has higher WUE_i and a proportionally lower yield reduction (Fullana-Pericàs *et al.* 2019). Given that autotetraploid and diploid individuals have the same genetic background,

leaf gas-exchange comparisons between both genotypes must be primarily explained by differences observed at the morphological and stomatal level.

Materials and methods

Plant material and tetraploid induction: The 'de Ramellet' Balearic tomato landrace accession UIB1-28 was selected to perform this experiment due to its exceptional performance under water stress conditions. Tetraploidization was induced *via* colchicine treatment by adapting Praça *et al.* (2009) protocol. Briefly, fifty seeds were surface-sterilized in 70% ethanol for 3 min, followed by a treatment with 50% sodium hypochlorite solution and one drop of *Tween 20* (*PanReac AppliChem*, Barcelona, Spain) under continuous shaking for 20 min. Seeds were rinsed with sterile distilled water. Sterilized seeds were placed on germination medium (GM) containing Murashige & Skoog (MS), including MES buffer and vitamins (*Duchefa Biochemie*, Haarlem, The Netherlands) (2.5 g L^{-1}), sucrose (*Duchefa Biochemie*, Haarlem, The Netherlands) (15 g L^{-1}), and plant agar (*Duchefa Biochemie*, Haarlem, The Netherlands) (10 g L^{-1}) in a final pH of 5.8. Seeds were kept in dark conditions at 25°C for 3 d and subsequently exposed to a 16:8-h photoperiod.

Shoot tips were excised from one-week-old seedlings as follows. Under the laminar flow hood and using a *MOTIC SFC-11C-N2GG* binocular (40× magnification), the apical leaf primordium (2 mm) was isolated using sterilized tweezers and scalpels. Shoot tips were gently shaken for 96 h at room temperature and dark conditions in polyploidization medium whose composition was the same as GM medium except for plant agar, and supplemented with 5 mM colchicine (*Sigma-Aldrich*, St. Louis, MO, USA). Then, they were rinsed with sterile distilled water and placed into elongation medium containing MS including vitamins and MES buffer (4.9 g L^{-1}), glucose (*Labkem*, Barcelona, Spain) (20 g L^{-1}), plant agar (10 g L^{-1}), and trans-zeatin (*Gold Biotechnology*, St. Louis, MO, USA) (0.1 mg L^{-1}) in a final pH 5.8, for 15 d at 25°C and 16:8-h photoperiod. Elongation medium (EM) was refreshed every two weeks. Shoot tips with clear evidences of development (1 to 3 cm growth) were placed into rooting medium containing MS including vitamins and MES buffer (4.9 g L^{-1}), glucose (20 g L^{-1}), plant agar (10 g L^{-1}), and IBA (*Duchefa Biochemie*, Haarlem, The Netherlands) (0.2 mg L^{-1}) in a final pH 5.8, and were kept at the same growth conditions as in EM. When shoots developed a robust radicular system, they were placed into an alveolar tray with 4:1 peat:perlite, watered with 25% Hogland solution, and kept covered with a plastic box for one week to acclimate to soil conditions and maintain high air humidity. After this period, plants had developed 4 to 5 leaves, and tetraploidization was confirmed by flow cytometry analysis from leaf tissue.

In vitro-originated polyploid plants confirmed by flow cytometry (G0) were planted into 20-L pots and grown until tomato production, to obtain the tetraploid plants resulting from seeds (G1). To ensure self-fertilization, plants were grown in a phytotron, ensuring no pollinator/pest presence, and flowers were manually vibrated. Seeds from G0 plants were cleaned from fruits using a 1.5 N HCl solution for 20 min, rinsed with tap water and planted in seed trays with horticultural peat soil. First-generation seed plants (G1) were grown in a greenhouse, and tetraploidization was confirmed by flow cytometry from leaf tissue. The G1 plants with confirmed tetraploidy were transplanted to the field and used in the experiments (*see* below).

Ploidy screening by flow cytometry: Samples were processed by flow cytometry using propidium iodide (PI)-stained nuclei to unravel DNA ploidy levels (Suda *et al.* 2006). Leaf tissues from each specimen, either G0 or G1, were preserved in ziplock plastic bags at 4°C before analysis. About 1 cm^2 of freshly collected leaf material of the study species and the calibration standard species (*Petunia hybrida* Vilm. 'PxPc6', 2C = 2.85 pg) were chopped together in 2 mL of 'general purpose isolation buffer' (GPB; Loureiro *et al.* 2007) supplemented with 3% PVP-40 following the one-step procedure described in Pellicer *et al.* (2021) in a small Petri dish, then filtered through 30-µm nylon mesh *Celltric* filter (*Sysmex*, Barcelona, Spain), and stained by adding in 50 µL of 1 mg mL^{-1} PI solution. Nuclei suspensions were then incubated for about 10 min on ice. The genome sizes were estimated using a *CyflowSL Partec* flow cytometer (*Sysmex Partec GmbH*, Görlitz, Germany) fitted with a 100-mW green (532 nm) solid-state *Cobolt Samba* laser (*Cobolt AB*, Solna, Sweden), measuring at least 1,000 particles. The resulting flow histograms were analyzed using *Partec* software (*FloMax v. 2.7*). Ploidy levels were allocated based on the sample/standard fluorescence index (*i.e.,* ratio), taking into account the FCM profile of a preliminary test in which a confirmed diploid individual of *S. lycopersicum* was analyzed (Fig. 1S, *supplement*).

Experimental design and field conditions: The experiment was performed outdoors at the experimental field of the University of the Balearic Islands (Mallorca, Balearic Islands, 39°38'N, 2°38'E, altitude 87 m a.s.l) during summer 2024. Field soil was enriched with 230 kg ha^{-1} of granulated fertilizer (20% total N, 10% total P_2O_5, 10% total K_2O) before transplantation. Two different genotypes were used: the diploid (2x) genotype, corresponding to the original UIB1-28 'de Ramellet' accession, and the tetraploid (4x) genotype, corresponding to G1 plants obtained by tetraploidization of the UIB1-28 accession. Five plants per genotype were distributed in two blocks separated by 5 m, one block for the well-watered treatment (WW) and another for the water-deficient treatment (WD). The WW block was irrigated, covering the daily ET_C, calculated as in Fullana-Pericàs *et al.* (2019), whereas the WD treatment was irrigated with only 35% of the WW water volume, considering previous results in Fullana-Pericàs *et al.* (2019) for 'de Ramellet' under water stress in an open field. Plants were irrigated with a plastic-covered dripping system (*AzudPro*, 0.33 m emitter spacing, 1 mm thickness, 2.0 L h^{-1} at 100 kPa).

Field transplantation was performed in mid-April, and both treatments' blocks were irrigated as described for WW during the first month, to ensure proper field establishment of plantlets. The WD treatment was established in mid-May, maintaining the WW treatment as described previously during the whole experiment. Gas-exchange and stomatal anatomy measurements were performed in the last week of July.

Monthly average, maximum and minimum temperatures (obtained from averaging daily average and absolute values for maximum and minimum temperatures, respectively), and monthly relative air humidity were, respectively: 18.8°C, max. 28.9°C, min. 8.1°C and 68.2% in May, 22.9°C, max. 34.0°C, min. 12.6°C and 65.8% in June, and 26.3°C, max. 39.1°C, min. 15.9°C and 62.7% in July. Rainfall during the experiment was 10.2 mm, 19.6 mm, and 2.6 mm in May, June, and July, respectively, and such volumes were subtracted from the irrigation dose in both treatments at the first irrigation event after the rain.

Gas-exchange measurements: Leaf gas exchange and chlorophyll fluorescence measurements were performed simultaneously with an open infrared gas analyzer equipped with a leaf chamber fluorometer (*Li-6800*, *Li-Cor Inc.*, Nebraska, USA). All measurements were performed from 9:30 to 12:00 h in the terminal leaflet of young fully expanded leaves.

Environmental conditions in the leaf chamber consisted of an air flow of 500 mmol(air) min^{-1} for WW and 300 mmol(air) min^{-1} for WD. A photosynthetic photon flux density (PPFD) of 1,500 µmol m^{-2} s^{-1} (with 10% blue light) and, given the temperatures at the measuring period, maintaining leaf temperature at 30 ± 1°C. Relative humidity in the leaf chamber was set to 50%, and vapour pressure deficit (VPD) ranged between 2.0 and 3.0 kPa in all measurements.

Measurements consisted of curves of photosynthesis response to varying substomatal CO_2 concentration (P_N/C_i). Measurements were performed after inducing steady-state photosynthesis for at least 5 min for punctual, and 30 min for P_N/C_i curves, at an ambient CO_2 concentration (C_a) of 400 µmol(CO_2) mol^{-1}(air). P_N/C_i curves consisted of 13 measurements per curve with C_a set at: 400, 200, 100, 50, 0, 400, 600, 800; 1,000; 1,200; 1,500; 1,800; and 2,000 µmol(CO_2) mol^{-1}(air). Corrections for CO_2 leakage of the leaf chamber of the *Li-6800* were applied to all gas-exchange data, as described by Flexas *et al.* (2007).

Maximum photosynthesis (P_{Nmax}) was determined from the saturating portion of the P_N/C_i curves at high CO_2 C_a concentration.

LA, LMA, and biomass measurements: Leaf mass per area (*LMA*) and leaf area per leaf (LA) were measured in a young fully expanded leaf per plant, excluding the leaf rachis. Selected leaves were scanned using a *LiDE220* (*Canon Inc.*, Tokyo, Japan) and images analyzed to obtain LA using *ImageJ* (*ver. 1.54f*, https://imagej.net/ij/). Leaf dry mass was determined by oven-drying the leaflets until a constant mass (70°C, 72 h). For each leaf, LMA was calculated as the ratio of leaf dry mass to the corresponding LA.

Stomata anatomical measurements, SAI, PAI, and theoretical maximum stomatal conductance (g_{smax}): The stomatal density (D) and guard cell length (L) were measured in five plants per genotype and treatment, at both adaxial and abaxial sides of the leaves used for gas exchange. Measurements were performed on the terminal and the two adjacent leaflets in each plant, using scaled images taken with an *Olympus BX60* optical microscope with an integrated digital camera (*Olympus Corporation*, Tokyo, Japan). The leaflet epidermis was flayed from the central-right part of the leaf blade, avoiding the main veins. The sample was mounted on a microscope slide and kept hydrated. Images were taken at 200× magnification. Three D measurements were taken per plant and leaf side at different leaflets, and four random L measurements were taken per plant and leaf side at the terminal leaflet. Stomatal size (S) was calculated for each stoma by multiplying L × L/2 (Franks and Beerling 2009). Stomatal area index (SAI) was obtained as the product of D and S, expressed in mm^2 stomata per mm^2 leaf.

The theoretical maximum stomatal conductance to water vapour (g_{smax}) was calculated according to Franks and Beerling (2009) as:

$$g_{smax} = \frac{D \times d_w \times a_{max}}{v\left(l + \frac{\pi}{2}\sqrt{\frac{a_{max}}{\pi}}\right)}$$

where d_W is the diffusivity of water vapour in air (2.6301×10^{-5} m^2 s^{-1} at 30°C according to Massman (1998), v is the molar volume of air (0.02486 L mol^{-1} at 30°C and 1 atm), a_{max} is the maximum area of the open stomatal pore, calculated as π $(p/2)^2$ according to Franks and Beerling (2009), where the stomatal pore length (p) was approximated as L/2 according to Franks and Farquhar (2007), and the stomatal pore depth for fully open stomata (l) was assumed to be L/4, considering that guard cells inflate to a circular cross-section (Franks and Beerling 2009). Values for d_w and v were considered at 30°C to coincide with the gas-exchange measurement conditions at leaf level, allowing proper comparison to measured g_s and g_{smax} values. The g_{smax} was obtained independently for each leaf surface, and both summed to obtain a global g_{smax} per unit leaf area.

The pore area index (PAI) was calculated as the product of D and a_{max}.

Statistical analysis: Statistical analyses were performed using the *Student*'s *t*-test. Comparisons were made between genotypes within each treatment and between treatments within each genotype. The level of significance was set at $p<0.05$. All analyses and graphical representations were conducted in *R version 3.2.2* (*R Core Team* 2024).

Results

Leaf gas exchange under well-watered conditions in diploid and tetraploid plants: Under common agricultural conditions such as the one performed for

'de Ramellet' tomato in commercial production practices with irrigation, diploid and tetraploid plants exhibited similar behaviour in photosynthetic parameters such as net CO_2 assimilation rate (P_N), stomatal conductance to water (g_s), substomatal CO_2 concentration (C_i), and intrinsic water-use efficiency (WUE_i), with non-significant differences between diploid and tetraploid plants in any of the aforementioned parameters (Table 1).

P_N/C_i curves performed to determine the maximum carbon assimilation rate (P_{Nmax}) showed that P_N at increased CO_2 doubled the values measured at ambient CO_2 concentration (*i.e.*, 400 ppm), with P_{Nmax} reaching 41.0 and 44.5 µmol(CO_2) m^{-2} s^{-1} in diploid and tetraploid plants, respectively, with non-significant differences between genotypes (Table 1). In both cases, P_{Nmax} was achieved at approximately 1,000 ppm of CO_2, and again, no significant differences between diploid and tetraploid plants were observed at any of the CO_2 concentrations measured (Fig. 1).

Differences in leaf morphology and stomata between diploid and tetraploid plants at WW conditions: Under WW conditions, non-significant differences were found between genotypes in leaf dry mass, leaf area per leaf (LA), and leaf mass per area (LMA) (Table 2). On the contrary, total stomatal density considering both leaf sides (D) was higher (p<0.01) in the 2x (191.8 stomata mm^{-2}) than the 4x (123.8 stomata mm^{-2}), due to a larger density in both the adaxial (D_{ad}) and the abaxial (D_{ab}) leaf sides (Table 2). For both genotypes, the density of stomata in the abaxial side was larger than in the adaxial side (Table 2), with the abaxial side having a percent of the total stomatal density of 73% in the diploid and 75% in the tetraploid.

Regarding stomatal length (L) and area (S), both traits were higher (p<0.01) in the 4x than in the 2x (Table 2). In the adaxial and abaxial leaf sides, L was 30–40% larger in the 4x as compared to the 2x. In turn, on the adaxial and abaxial leaf sides, respectively, S was 69% and 95% larger in the 4x. Consequently, when considering maximum stomatal aperture, the stomatal pore area (a_{max}) was also significantly higher in the 4x as compared to the 2x (Table 2). On the other hand, when comparing L, S, and a_{max} between both leaf sides within each genotype, there were non-significant differences in the 2x in any of the traits, but the 4x had significantly larger stomata (L, S, and a_{max}) in the abaxial as compared to the adaxial leaf sides. The stomatal area index (SAI) and stomatal pore area index (PAI) resulted in non-significant differences between 2x and 4x, neither considering each leaf side independently, nor averaging both leaf sides (Table 2).

Table 1. Net assimilation rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), intrinsic water-use efficiency (WUE_i as P_N/g_s), and maximum net assimilation rate (P_{Nmax}) as obtained from the P_N/C_i curves for the diploid (2x) and the tetraploid (4x) genotypes under well-watered (WW) and water-deficit (WD) conditions. Values are means ± standard error of five replicates per accession and treatment. *Asterisks* in the WW columns denote significant differences between treatments for each genotype, as determined by a *t*-test at p<0.05 (one asterisk) or at p<0.01 (two asterisks). There are no significant differences between genotypes within each treatment.

	WW		WD	
	2x	4x	2x	4x
P_N [µmol(CO_2) m^{-2} s^{-1}]	21.59 ± 3.33**	21.43 ± 1.40**	7.29 ± 1.28	10.72 ± 1.24
g_s [mol(H_2O) m^{-2} s^{-1}]	0.409 ± 0.103**	0.340 ± 0.028**	0.085 ± 0.010	0.132 ± 0.021
C_i [µmol(CO_2) mol^{-1}(air)]	276.33 ± 13.35	268.99 ± 6.04*	242.40 ± 12.39	241.72 ± 5.63
WUE_i [µmol(CO_2) mol^{-1}(H_2O)]	59.10 ± 9.32	63.87 ± 3.84	85.17 ± 7.70	82.92 ± 4.20
P_{Nmax} [µmol(CO_2) m^{-2} s^{-1}]	40.95 ± 2.74	44.46 ± 1.14	-	-

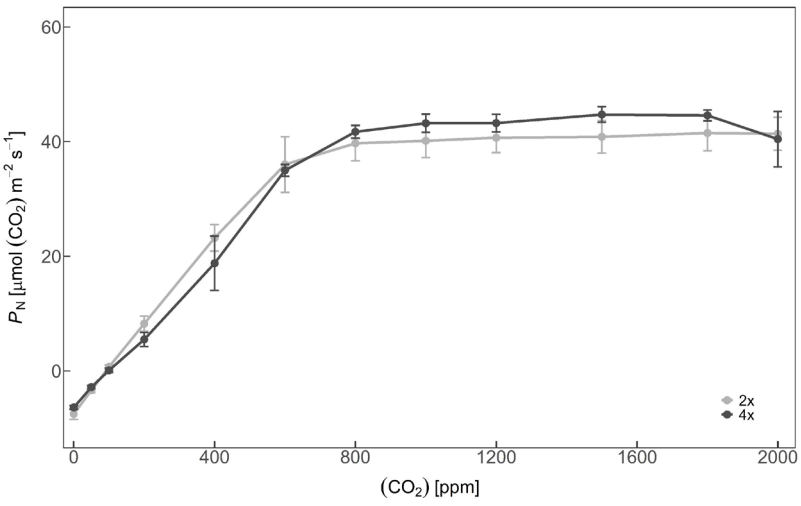

Fig. 1. Performance of the net assimilation rate (P_N) at varying CO_2 concentrations between 0 and 2,000 ppm for the diploid (2x) and the tetraploid (4x) plants. Points are mean values ± standard error for each of the CO_2 concentrations. The two-tailed *Student*'s *t*-test (p<0.05) denoted the lack of significant differences between 2x and 4x at any of the measured CO_2 concentrations.

Table 2. Leaf and stomatal traits in the diploid (2x) and tetraploid (4x) genotypes grown under well-watered (WW) and water-deficient (WD) conditions. Leaf traits include leaf dry mass, leaf area (LA), and leaf mass per area (LMA). Stomatal traits include stomatal density (D), stomatal length (L; considering guard cell length), stomatal size (S), stomatal area index (SAI), maximum stomatal pore area (a_{max}), pore area index (PAI), and theoretical maximum stomatal conductance calculated from stomatal traits (g_{smax}). Stomatal traits are shown globally, considering both leaf sides, and also independently for each leaf side. In the latter case, parameters detailed above include a subindex "ad" for adaxial and "ab" for abaxial leaf sides. Values are means ± standard error (n = 5 for leaf morphology and gas-exchange measurements, n = 15 for density-related traits, and n = 20 for stomatal size traits). *Asterisks* in 2x denote statistically significant differences between genotypes under the same treatment (* for p<0.05 and ** for p<0.01), *empty circles* in WW denote significant differences between treatments for the same genotype (° for p<0.05 and °° for p<0.01), and *dark boxes* in adaxial traits denote differences between adaxial and abaxial within the same genotype and treatment (▪ for p<0.05 and ▪▪ for p<0.01), as denoted by t-test.

	WW		WD	
	2x	4x	2x	4x
Leaf dry mass [g]	1.36 ± 0.21°	1.32 ± 0.15	0.70 ± 0.06**	1.24 ± 0.12
LA [cm^2]	154.86 ± 24.67°	154.43 ± 18.34	85.19 ± 5.31**	148.86 ± 15.88
LMA [g m^{-2}]	67.19 ± 5.82	69.40 ± 3.48	60.13 ± 3.08	66.76 ± 2.49
D [stomata mm^{-2}]	191.80 ± 16.12**°°	123.80 ± 17.97	271.39 ± 26.80**	129.40 ± 15.29
L [µm]	34.55 ± 1.01**°°	46.63 ± 1.15	30.90 ± 1.69**	48.63 ± 2.65
S [µm^2]	603.28 ± 35.21**°°	1,102.84 ± 53.80	487.20 ± 53.27**	1,216.11 ± 141.20
SAI [mm mm^{-2}]	0.116 ± 0.014	0.134 ± 0.015	0.130 ± 0.015	0.149 ± 0.009
a_{max} [µm^2]	236.91 ± 13.83**°°	433.09 ± 21.13	191.32 ± 20.92**	477.57 ± 55.45
PAI [mm mm^{-2}]	0.046 ± 0.006	0.053 ± 0.006	0.051 ± 0.006	0.059 ± 0.004
g_{smax} [mol(H_2O) m^{-2}s^{-1}]	2.16 ± 0.21	1.87 ± 0.24	2.71 ± 0.24*	2.01 ± 0.18
Adaxial				
D_{ad} [stomata mm^{-2}]	52.09 ± 7.02**°°▪▪	30.92 ± 7.88▪▪	86.67 ± 14.82**▪▪	36.66 ± 6.08▪▪
L_{ad} [µm]	33.60 ± 0.93**°°	43.56 ± 2.35▪▪	30.40 ± 1.60**	47.35 ± 4.75
S_{ad} [µm^2]	568.20 ± 32.27**°°	961.53 ± 104.96▪▪	470.20 ± 48.97**	1,173.58 ± 254.04
SAI_{ad} [mm mm^{-2}]	0.030 ± 0.005▪▪	0.029 ± 0.007▪▪	0.041 ± 0.007▪▪	0.038 ± 0.005▪▪
a_{max_ad} [µm^2]	223.13 ± 12.67**°°	377.59 ± 41.22▪▪	184.65 ± 19.23**	460.86 ± 99.76
PAI_{ad} [mm mm^{-2}]	0.012 ± 0.002▪▪	0.011 ± 0.003▪▪	0.016 ± 0.003▪▪	0.015 ± 0.002▪▪
g_{smax_ad} [mol(H_2O) m^{-2}s^{-1}]	0.57 ± 0.09▪▪	0.43 ± 0.11▪▪	0.86 ± 0.14▪▪	0.53 ± 0.08▪▪
Abaxial				
D_{ab} [stomata mm^{-2}]	139.71 ± 10.81**°°	92.88 ± 10.64	184.72 ± 13.21**	92.75 ± 9.35
L_{ab} [µm]	35.50 ± 1.57**°°	49.69 ± 1.41	31.40 ± 1.93**	49.90 ± 1.79
S_{ab} [µm^2]	638.35 ± 54.47**°°	1,244.16 ± 70.02	504.20 ± 61.58**	1,258.65 ± 88.31
SAI_{ab} [mm mm^{-2}]	0.090 ± 0.011	0.115 ± 0.013	0.091 ± 0.009	0.116 ± 0.014
a_{max_ab} [µm^2]	250.68 ± 21.39**°°	488.58 ± 27.50	198.00 ± 24.18**	494.27 ± 34.68
PAI_{ab} [mm mm^{-2}]	0.035 ± 0.004	0.045 ± 0.005	0.036 ± 0.003	0.045 ± 0.005
g_{smax_ab} [mol(H_2O) m^{-2}s^{-1}]	1.61 ± 0.16	1.49 ± 0.16	1.86 ± 0.09	1.50 ± 0.15

Despite differences in stomatal size and density, the theoretical maximum stomatal conductance calculated from stomatal traits (g_{smax}) showed non-significant differences between 2x and 4x plants under well-watered conditions, reaching theoretical average values of 2.16 and 1.87 mol(H_2O) m^{-2} s^{-1}, respectively (Table 2).

Impact of water deficit on photosynthesis, leaf morphology and stomata in diploid and tetraploid plants: Permanent reduction of irrigation in the WD treatment resulted in significant differences between treatments in both the 2x and the 4x genotypes for P_N and g_s, but not WUE_i (as P_N/g_s). Differences in C_i between treatments were significant only in the 4x (Table 1).

Different from WW conditions, under WD conditions, 2x plants showed *ca*. 50% smaller leaves and lower dry biomass per leaf as compared to the 4x (Table 2). Indeed, there were significant differences between treatments in leaf dry mass and LA in the 2x, but not in the 4x, denoting that the treatment had an impact on these traits only in the 2x. Despite this, there were no differences in LMA between genotypes in WD and thus, this parameter remained very similar across genotypes and treatments (Table 2).

Coinciding with WW, under WD, the D (considering both leaf sides) in the 2x was higher than in the 4x, with an even larger difference in this treatment (*i.e.*, 36% under WW and 52% in WD). This was the result of a significant increase in stomatal density in the 2x due to WD (42% larger), since the differences between treatments in the 4x were non-significant (Table 2). Results were coincident when considering D independently for each

leaf side and, in this case, D increased in the 2x under WD by 66% in the adaxial and a 32% in the abaxial leaf sides, also without significant differences in D in the 4x due to WD (Table 2).

Similar results were obtained for stomatal L, S, and a_{max}. For the three traits, considering both leaf sides together and also independently, differences between the 2x and the 4x were significant under both treatments. Under WD, L was a 57% larger in the 4x as compared to the 2x considering both leaf sides (56% larger in the adaxial, and 59% larger in the abaxial); whereas S, and consequently a_{max} (which is proportional to S) were a 150% larger in the 4x considering both leaf sides (being also *ca.* 150% larger in the adaxial and in the abaxial leaf sides) (Table 2). However, the differences between treatments were significant only in the 2x, denoting that WD did not affect stomatal traits in the 4x (Table 2). As compared to WW, under WD the 2x had stomata with 10.6% smaller L and 19% smaller S when considering both leaf sides, which resulted from L a 10% smaller in the adaxial and a 12% smaller in the abaxial leaf sides, and from S a 17% smaller in the adaxial and a 21% smaller in the abaxial leaf sides (Table 2). Therefore, in the 2x, the WD had a proportionally larger impact on D than on L and S.

Coinciding with WW, under WD, there were no differences between 2x and 4x in the SAI and in the PAI. Moreover, differences between treatments were not significant in any of the genotypes (Table 2). However, contrary to WW, under WD there were differences ($p<0.05$) in the g_{smax} (considering both leaf sides) between genotypes, being a 26% lower in the 4x as compared to the 2x. Indeed, despite the lack of significant differences between treatments in the 2x and in the 4x, the highest average value corresponded to the 2x under WD, with up to 2.73 mol(H_2O) m^{-2} s^{-1} (Table 2).

Finally, the comparison between adaxial and abaxial leaf sides, the differences between genotypes described under WW also occurred under WD, except L, S, and a_{max} in the 4x. Thus, these traits were smaller in the adaxial side as compared to the abaxial side under WW but were very similar in both leaf sides under WD (Table 2).

Discussion

Tetraploidization led to larger stomata and lower density, albeit with no impact on photosynthesis: Under WW conditions, tetraploidization caused significant differences in stomatal density (D) and size (S), but not in the stomatal area and pore area indexes (SAI, PAI) (Table 2). Reduced D in the 4x was compensated by increased S, maintaining similar stomatal area and theoretical maximum conductance (g_{smax}; calculated from stomatal traits) as that in the 2x (Table 2). Moreover, the differences in stomatal traits between genotypes were independent of leaf size (LA), dry mass per leaf and leaf mass per area (LMA) (Table 1). Increased P_N in angiosperms has been correlated to increased g_{smax}, which is achieved with high density of small stomata (Sack and Buckley 2016, Xiong and Flexas 2020). However, our results in tetraploidized tomato show that a significant difference in D and S can result in very similar g_{smax}, the same measured P_{Nmax} (Fig.1), and similar P_N and g_s under field conditions (Table 1). It is worth noting that widespread correlations across plant phylogeny relating leaf anatomy with gas-exchange traits may not occur within small phylogenetic groups, as previously demonstrated in wild tomatoes and relatives (Muir *et al.* 2017).

When considering leaf sides independently, D decreased in 4x more in the adaxial (*ca*. 41%) than the abaxial (*ca*. 34%) side, whereas S increased by less in the adaxial (*ca*. 69%) than the abaxial (95%) side. Yet, SAI and g_{smax} per leaf surface were similar between genotypes (Table 2), which denotes that the compensation between D and S also occurs independently on each leaf side. In angiosperms, shifting from hypostomy to amphistomy allowed maximizing P_N, resulting from a double transpiring surface and enhanced mesophyll conductance due to shorter CO_2 pathways (Haworth *et al.* 2018). Indeed, variation in the distribution of stomata in both leaf sides may explain large variations in photosynthetic capacity across species (Muir 2015, 2018; Drake *et al.* 2019, Xiong and Flexas 2020). Therefore, variable changes in D and S in the adaxial and abaxial sides might have an additional effect explaining the lack of photosynthetic differences between 2x and 4x. Furthermore, adaxial and abaxial stomata have different sensitivity and regulation; the adaxial stomata have higher sensitivity to drought stress (Mott 2007, Wang *et al.* 2011, Richardson *et al.* 2017, Driesen *et al.* 2023), which might be an additional factor in the observed behaviour.

Nevertheless, larger stomatal size in the 4x may result in operational differences related to stomatal responsiveness and to gas exchange diffusion. In terms of responsivity, larger stomata require a longer time to close, with higher energy cost (Srivastava *et al.* 1995), whereas a smaller number of large stomata does not allow a smooth control of g_s (Lawson and Blatt 2014). Regarding diffusion, for the same pore area, smaller stomata achieve higher g_s since the stomatal pathway is shorter (Franks and Beerling 2009). Therefore, the lack of differences in g_{smax} responds to proportional differences in total pore area and to differences in pore depth.

Theoretical g_{smax} values above 2 mol(H_2O) m^{-2} s^{-1} in the 2x (Table 2) are similar to those reported in the tomato crop for g_{smax} (*e.g.*, Song *et al*. 2023). Common operational g_s in open-field conditions for 'de Ramellet' ranges between 0.4 and 0.6 mol(H_2O) m^{-2} s^{-1} (Fullana-Pericàs *et al.* 2017, 2022), close to the observed in the 2x this study (Table 1). Consequently, g_s/g_{smax} ratios (0.188 in the 2x, and 0.180 in the 4x) are between those reported for herbaceous angiosperms (0.12; Xiong and Flexas 2020), and for chamber-grown *Arabidopsis* (0.31; Dow *et al.* 2014). Nevertheless, studies comparing theoretical g_{smax} and operational g_s have noted a non-significant relationship across angiosperms, suggesting that g_s regulation is in a large proportion determined by further unknown traits (*e.g.*, Russo *et al.* 2010, McElwain *et al.* 2016, Xiong and Flexas 2020). Under WW, 2x and 4x showed also no differences in P_N, WUE_i, and C_i (Table 1), indicating that tetraploidization neither improved nor

impaired physiological performance. In this regard, when considering tetraploidization as an improving strategy, it may be noted that examples in tomato polyploids show low fertility at early generations (Lindstrom and Humphrey 1933, Nilsson 1950). Observations here (not shown) indicate that this was also the case in some of the G1 plants of the 4x 'de Ramellet'. It remains elusive if this impairment is due to low fertility, increased sensitivity to high temperatures in the 4x (*i.e.*, tomato sensitivity to high temperatures; Sato *et al.* 2002, Firon *et al.* 2006), or to further factors, and if this can be overcome after several self-fertilization generations.

Tetraploid stomatal size limits increasing stomatal density under water-stress conditions: To assess whether larger stomata due to tetraploidization constrain drought adaptation, both the 2x and the 4x were grown outdoors under permanent water shortage for three months (WD treatment). Both genotypes reduced P_N and g_s, and, coinciding with the previously described for WW, there were no differences between the 2x and the 4x in P_N, g_s, WUE_i, and C_i (Table 1).

Under WD, the 2x reduced leaf size (LA) and dry mass, increased D (41.5%), and reduced S (19.2%) (Table 2), as previously reported in 'de Ramellet' (Galmés *et al.* 2011, 2013; Fullana-Pericàs *et al.* 2017, 2019, 2022). Conversely, the 4x showed no treatment differences in leaf morphology (LA, leaf dry mass, and LMA) and stomatal traits (D and S) (Table 2), suggesting limited capacity of the 4x to deploy adaptations observed in the 2x under WD. Given the similarity in the physiological response (Table 1, Fig. 1), alternative adaptive traits may be involved, which remain elusive in this study.

Moreover, there was a proportionality between D and S, ensuring a similar SAI and PAI irrespective of the treatment and not affected by tetraploidization, also occurring when considering each leaf side independently (Table 2). Studies on rice mutants with different D and S under drought showed that stomata display largely impacted g_s but not growth capacity and yield, so that mutants with lower D reduced water use, due to higher capacity to maintain water content in leaves (Caine *et al.* 2019, Pitaloka *et al.* 2022). On the contrary, lack of differences in WUE_i, g_s, and P_N between 2x and 4x under WD, despite having large stomatal differences, suggests that it may be crop-dependent. In this regard, rice has very small stomata and is very water-demanding (Bertolino *et al.* 2019), contrasting with a landrace selected for centuries under harsh Mediterranean summer conditions (Conesa *et al.* 2020). Therefore, root traits, cell biochemistry, and management of hydraulic failure risk may play relevant roles in defining stomatal display under drought (Lu *et al.* 2020). This is reinforced by the lack of treatment differences in g_{smax} in the 2x and in the 4x, since either a large impact of WD on D and S in the 2x, and a low impact in the 4x, results in similar g_{smax} (Table 2).

Reduction of LA is a common strategy to reduce transpiring surface, yet stomatal adaptations shown tend to the opposite (*i.e.*, increase maximum transpiration capacity per unit leaf area; Franks and Beerling 2009). Consequently, as compared to the 2x, the lack of LA reduction under WD might pose a water-saving constraint in the 4x. However, the total stomatal number per leaf (considering both leaf sides), based on D and LA, renders in the 2x, 2.96×10^6 and 2.31×10^6 stomata/leaf in WW and WD, respectively, and in the 4x 1.87×10^6 stomata/leaf in both treatments. Therefore, the differences in D and LA across genotypes and treatments (Table 2) result in a similar number of stomata per leaf in both treatments ($p<0.05$; not shown), suggesting that the leaf size reduction could be an additional factor promoting D increase, as observed in basil (Driesen *et al.* 2023).

Conclusion: Overall, tetraploidization of the Mediterranean drought-adapted 'de Ramellet' tomato limited its adaptive capacity to modify leaf size and stomatal traits under WD. However, the proportion of stomata per area remained stable across water treatments and genotypes, suggesting that common adaptation to drought in this landrace is not governed by total pore area, but rather related to stomatal functioning (sensitivity, closure kinetics), and to determining factors with higher impact on drought adaptation than stomata. Nevertheless, the ultimate factors determining stomatal functioning and contribution to drought adaptation remain to be elucidated, and this aspect deserves further investigation.

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