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Chlorophyll Fluorescence, Photosynthesis and Growth of Tomato Plants as Affected by Long-Term Oxygen Root Zone Deprivation and Grafting

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Abstract: A greenhouse experiment was conducted to study the effects of the O_2 root zone level and grafting on chlorophyll fluorescence, photosynthesis and growth of cherry tomato grown in a hydroponic system. Two O_2 concentrations in the root zone, namely Ox (saturation level) and Ox- (2–3 mg L^{-1}), were applied for 30 days on self-grafted cherry tomato *Dreamer* or grafted onto the hybrids *Arnold*, *Beaufort*, *Maxifort* and *Top Pittam*. Root hypoxia increased minimum fluorescence (by 10%) while it decreased variable fluorescence and the maximum quantum yield of PSII (up to 16 and 8%, respectively). Moreover, it reduced leaf photosynthesis, transpiration and stomatal conductance (by 12, 17 and 13%, respectively), whereas it increased leaf electrolyte leakage (by 2.1%). The graft combinations showed a different ability in buffering the effects of root hypoxia on plant growth and related components, and these differences were related to their root biomass. The minimum fluorescence was negatively correlated to plant growth, so it may be a useful indicator to select tolerant rootstocks to root hypoxia. Our results suggest the occurrence of both diffusive and metabolic constraints to tomato photosynthesis under root hypoxia, a condition that can be mitigated by selecting rootstocks with a more developed root system.

Keywords: tomato; rootstock; oxygen starvation; chlorophyll fluorescence; photosynthesis; stomatal conductance

1. Introduction

Higher plants are obligate aerobic organisms needing molecular oxygen (O_2) to accomplish the oxidation reactions supporting their life. Nevertheless, they can experience excess water in the growth substrate in either natural or agricultural ecosystems, because of erratic rainfall and/or incorrect irrigation scheduling associated to climate change. This can flow in flooding, which is a major abiotic stress threatening growth and yield of many economically important crops [1]. Flooding conditions cause O_2 starvation in roots, which arises from the slow diffusion of gases in water and from O_2 consumption by microorganisms and plant roots [2]. Oxygen deprivation results in an arrest of aerobic respiration, leading to an energy deficit in plants, having, in turn, severe impacts on root activity and on photosynthetic metabolism [3]. Greenhouse crops are generally selected for their high

yield in optimum growth conditions, so tolerance to many abiotic stressors can be partially or totally neglected. Indeed, adaptation to abiotic stressors often require extra energy consumption at the expense of yield potential. This also concerns sensitivity to hypoxia in the root environment [4].

Tomato (*Solanum lycopersicum* L.) is a widely consumed vegetable crop throughout the World, with an estimated production of about 160 Mt from more than 4.8 Mha cropland [5]. In the coastal regions of the Mediterranean Basin, it is one of most important field and greenhouse vegetable crops [6]. Over recent years, the greenhouse tomato has experienced a progressive transition to soilless culture, in order to meet the growing demands to produce vegetables with improved quality, health properties and ecological profile [7]. Tomato is considered susceptible to excessive substrate moisture [8], a condition to which plants are often exposed during growth stages following transplants. In soilless tomato cultivation of the Mediterranean Basin, this arises from the combination of the fast and scarcely predictable changes in water vapor pressure deficit inside the greenhouse, with the need to oversize the irrigation volumes to avoid salts accumulation in the rhizosphere [9]. As a result, young tomato plants often display suboptimal growth, with subsequent impairment of their biological performances.

An effective available means of adapting plants to environmental stressors is grafting commercial cultivars onto selected rootstocks [2]. Grafting is nowadays regarded as a rapid alternative tool to the relatively slow breeding methodology aimed at increasing the environmental stress tolerance of many crops, including root O_2 deprivation [10,11]. According to the literature, there is a close correlation among the root size and the biological performances of many crops subjected to abiotic stressors [8,12,13], but this evidence is still lacking when tomato plantlets subjected to long-term O_2 root deprivation are concerned. For this reason, more experimental evidence is needed before proclaiming root vigor as one of the most desirable traits in tomato-compatible rootstocks showing tolerance to O_2 root deprivation. This condition could occur in Mediterranean greenhouses' soilless cultivation, based on organic substrate (e.g. coconut coir) that is already used for other cultivation cycles and thus, with a lower air capacity [14]. Moreover, a better understanding of the physiological modifications subtended by the increased grafted tomato tolerance to root hypoxia may represent a useful framework in directing the selection of the best-adapted rootstock genotypes.

Over the last decades, development in instrumentations and methodologies have improved the precision estimates of chlorophyll a (Chl) fluorescence and leaf gas exchanges. As a result, significant advances have been made in improving selection criteria in a multitude of crops. Chl fluorescence is a quantitative and qualitative indicator of light-dependent photosynthetic processes, which has been suggested as a screening method for heat tolerance in chickpeas [15] and durum wheat [16], for drought tolerance in barley [17] or for flooding tolerance in some leguminous and cereals crops [18]. The combined measurements of Chl fluorescence and leaf gas exchange have been exploited to describe the adaptability and plant-ageing pattern in subterranean clover genotypes exposed to shading strain [19].

The aim of the current research was to investigate the response of different tomato grafting combinations to root O_2 suboptimal condition, by measuring at an early stage their Chl fluorescence, photosynthetic rate, leaf electrolyte leakage and plant growth variables. We also set out to explore the possibility of identifying physiological parameters, which could be employed as predictive tools in breeding programs for flooding-tolerant tomato rootstocks.

2. Materials and Methods

2.1. Experimental Site, Plant Material and Growing Conditions

A greenhouse experiment was conducted in 2018, at the experimental farm of the University of Catania (37°24′26″ N, 15°03′37″ E, 6 m a.s.l.), in an area characterized by a semi-arid/Mediterranean climate. An 810 m^2, east–west oriented, multi-aisle greenhouse was used, having a steel tubular structure with adjustable windows on the roof and along the sides, and covered with polycarbonate slabs. Self-grafted cherry tomato plants, cultivar *Dreamer* (Nunhems BV, Haelen, The Netherlands) (control) or grafted onto the hybrid rootstocks *Arnold* (Syngenta, Basel, Switzerland), *Beaufort*,

Maxifort (both from De Ruiter Seeds, Bergschenhoek, The Netherlands) and *Top Pittam* (TSI Italia s.r.l., Foggia, Italy) were used in the experiment. A detailed description of the rootstocks is reported in Table 1. The rootstocks genotypes were chosen on the basis of our previous observation (unpublished data), revealing a higher root size in *Beaufort* and *Maxifort*, whereas a lower one in *Arnold* and *Top Pittam*. Plants were obtained from a specialized nursery, were the splice-grating technique was applied, followed by the application of plastic clips to secure the graft union. Before the start of the experiment, plants were selected for homogeneous size. On 9 April 2018, plantlets at the stage of two true leaves, were placed in a rectangular format (0.25 × 0.35 m) in a floating system constituted by six separate metallic tanks (2.20 × 2.20 × 0.20 m), which constituted the main plots of the split-plot design (three tanks per main plot), in which optimal and reduced O_2 concentrations were applied. For each tank, one replicate of 6 plants (net sub-plot) per grafting combination was included. The hypoxia treatment started 15 days after planting (DAP), to allow plants to adapt to the hydroponic conditions. In the control tank, the O_2 level (hereafter referred Ox) was kept at saturation level by continuous forced aeration through a serious of Jeneca AP-9800 air pumps. In the low-O_2 treatment (Ox-), aeration was started only when root respiration reduced the O_2 content of the nutrient solution to 2 mg L^{-1}, and stopped again when a concentration of 3 mg L^{-1} was reached. In both cases, constant movement of the nutrient solution was maintained by submerged small pumps to avoid the rapid O_2 decrease in the liquid layers close to the roots. Dissolved O_2 concentration was monitored in the center and along the diagonals of each tank, through CS511-L sensors (Campbell Scientific, Inc., Logan, UT, USA), made up of a self-polarizing galvanic cell that generated a millivolt signal proportional to the amount of O_2 present in the nutrient solution. Mean air temperature, relative humidity (RH), global radiation and vapor pressure deficit (VPD) inside the greenhouse were recorded on an hourly basis, by means of two sets of sensors in the center of each main plot. All sensors were connected to a CR-510 data logger (Campbell Scientific, Inc., Logan, UT, USA) that also controlled the aeration pumps. In order to minimize the influence of the external conditions, the experimental plots were placed in the center of the greenhouse. The nutrient solution (150 L m^{-2}) had the following composition (in mmol L^{-1}): 14.2 NO_3^-, 1.9 $H_2PO_4^-$, 6.0 K^+, 4.25 Ca^{2+}, 1.75 Mg^{2+}, 1.0 NH_4^+, 0.75 SO_4^{2-} and microelements. The EC of the nutrient solution was 2.8 dS m^{-1} and the pH was maintained between 5.5 and 6.5 by adding H_2SO_4 (95% concentration, 1.83 kg L^{-1} at standard temperature and pressure). Fresh nutrient solution was added once a week, to restore plant uptake. The gross experimental area inside the greenhouse was 44.46 m^2 (8.6 × 5.4 m), including 336 plants (180, excluding border plants), divided into 30 net subplots (2 O_2 levels × 5 grafting combinations × 3 replicates), each containing 6 plants.

Table 1. Main characteristics of the rootstock genotypes used in the experiment.

Rootstock	Genetic Background	Vigor	Resistance [1]
Arnold F_1	*S. lycopersicum* × *S. habrochaites*	Low	*Fulvia fulva*; *Fusarium oxysporum* f. sp. *lycopersici*; *F. oxysporum* f. sp. *radicis-lycopersici*; *Verticillium albo-atrum*; *V. dahliae*; Tomato Mosaic Virus.
Beaufort F_1	*S. lycopersicum* × *S. habrochaites*	Medium	*F. oxysporum* f. sp. *lycopersici*; *F. oxysporum* f. sp. *radicis-lycopersici*; *Pyrenochaeta lycopersici*; *V. albo-atrum*; *V. dahliae*; Tomato Mosaic Virus.
Maxifort F_1	*S. lycopersicum* × *S. habrochaites*	High	*F. oxysporum* f. sp. *lycopersici*; *F. oxysporum* f. sp. *radicis-lycopersici*; *Pyrenochaeta lycopersici*; *V. albo-atrum*; *V. dahliae*; Tomato Mosaic Virus.
Top Pittam F_1	*S. lycopersicum* × *S. peruvianum*	Medium to high	*F. oxysporum* f. sp. *lycopersici*; *F. oxysporum* f. sp. *radicis-lycopersici*; *Pyrenochaeta lycopersici*; *V. albo-atrum*; *V. dahliae*; Tomato Mosaic Virus.

[1] declared by Seed Company.

2.2. Chl Fluorescence and Gas Exchange Measurements

Photosystem II (PSII) efficiency was measured 30 days after the beginning of the hypoxia treatment through Chl fluorescence analysis using an OS1-FL fluorometer (Opti-Sciences

Corporation, Tyngsboro, MA). Chlorophyll fluorescence excitation was performed by a 660 nm solid-state light source coupled with filters able to block λ above 690 nm; the modulated light intensity was adjusted from 0 to 1 μE. Fluorescence detection was performed between 700 and 750 nm using a PIN silicon photodiode coupled with appropriate filtering to remove extraneous light. Saturation of PSII was provided by a filtered 35 W halogen lamp (350–690 nm), which performed an 800 milliseconds light pulse. F_0 (minimum fluorescence), F_V (variable fluorescence), F_m (maximum fluorescence) and the ratio F_V/F_M were measured. All the measurements were performed after a 30 minutes leaf dark-adaptation through OS cuvettes (Liu et al., 2005). Gas exchange measurements were performed on the same day on the uppermost fully expanded leaves through an LCi Portable Photosynthesis System (ADC BioScientific Ltd.). Instantaneous net photosynthesis (A_N, μmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), stomatal conductance (gs, mmol m^{-2} s^{-1}) and substomatal CO_2 concentration (Ci, μmol CO_2 mol^{-1}) were measured around midday (local solar time), by recording duplicate measurements from the same leaf of four plants per sub-plot. Measurements were taken on a clear sunny day, with a PAR in the range 1700–1900 μmol photons m^{-2} s^{-1}, an average air CO_2 concentration inside the greenhouse of 345 ppm, and an average temperature and relative humidity inside the cuvette equal to 28.6 °C and 42.8%, respectively.

2.3. Electrolyte Leakage

Electrolyte leakage of leaves was measured after 30 days of the onset of the hypoxia treatment, before removing plants for destructive analysis, according to the procedure described by Bajji et al. [20], with modifications. For each measurement, 20 leaf discs (1 cm^2 each, taken from the sixth fully expanded leaf from the bottom) were collected and placed in a 50 mL tube containing 20 mL of millipore water. The tubes were placed in a shaker at 100 r.p.m. for 24 h at 25 °C. A first EC reading (EC_1) was performed at the end of the 24 h shaking, then the test tubes were placed in an autoclave at 120 °C for 20 min and left to cool again at 25 °C for the second EC reading (EC_2). Electrolyte leakage (EL) was calculated as the ratio between EC_1 and EC_2, and expressed on a percentage basis.

2.4. Biometric Measurements

Plant height, number of leaves (LN) and leaf area (LA) were measured on day 30 after the onset of the stress treatment, by harvesting seven plants per replicate. Dry weight (DW) of roots, stem and leaves was determined by using a thermo-ventilated oven at 70 °C until constant weight was reached. Specific leaf area (SLA) was calculated as the ratio of LA to leaves' DW, whereas the leaf area ratio (LAR) was calculated as the ratio of LA to plant DW.

2.5. Statistical Analysis

All data were subjected to Shapiro–Wilk and Levene's test, in order to check for normal distribution and homoscedasticity, respectively, then to a factorial 'O_2 level x rootstock' analysis of variance (ANOVA), according to the split-plot experimental layout adopted in the experiment. Percentage data were Bliss transformed before the ANOVA (untransformed data are reported and discussed), whereas multiple mean comparisons were performed through Fisher's protected least significant difference (LSD) test ($p = 0.05$). Pearson's correlation analysis was also performed, in order to define possible relationships among variables. All calculations were performed using Excel version 2016 (Microsoft Corporation, Redmond, WA) and Minitab version 16.1.1 (Minitab Inc., State College, PA, USA).

2.6. Greenhouse Microclimate and Root Zone O_2 Concentration during the Trial

During the experiment, the average mean temperature was 21.2 °C, progressively increasing from 18.0 to 24.0 °C (on 3 and 43 DAP, respectively), whereas the average relative humidity varied between 67.6% and 39.8% (on 13 and 27 DAP, respectively) (Figure 1A). The average global solar radiation over the experimental period was 12.4 MJ m^{-2}, and oscillated between 3.1 and 18.0 MJ m^{-2} (on 13 and 38 DAP, respectively), whereas the vapor pressure deficit oscillated between 0.7 (at 13

DAP) and 2.0 kPa (at 27 DAP) (Figure 1B). When the hypoxia treatment started (at 15 DAP), in the Ox- treatment the average root zone, O_2 sharply dropped from 8.0 to 2.2 mg L^{-1}, then slightly oscillated around 2.4 mg L^{-1} up to the end of the experiment, whereas in the Ox treatment, it never dropped below 7.1 mg L^{-1} (Figure 1C). As regards the spatial variability among main plots, the differences in terms of mean temperature, relative humidity, solar radiation and O_2 concentration in the root zone never exceeded 0.2 °C, 1.9%, 0.3 MJ m^{-2} and 0.3 mg L^{-1}, respectively.

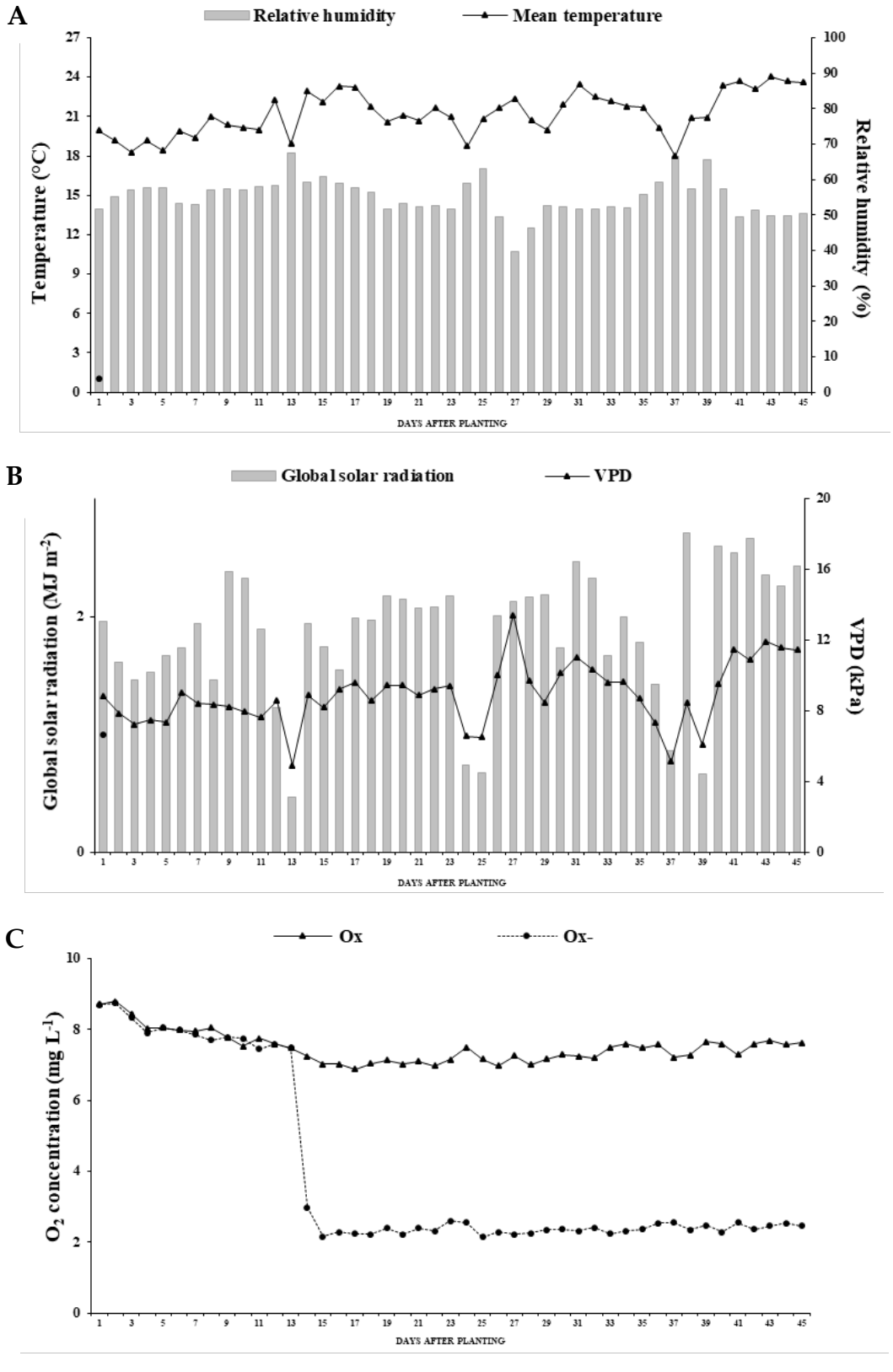

Figure 1. Microclimate conditions inside the greenhouse (**A**, **B**) and O_2 concentration in the nutrient solution (**C**) during the experimental period. VPD: vapor pressure deficit.

3. Results

3.1. Chlorophyll Fluorescence

The O_2 availability at root level had significant effects on the chlorophyll fluorescence variables in a way that, in most cases, was rootstock-dependent (Table 2). Indeed, passing from optimal- to low-O_2 level in roots, *Dreamer* showed a significant F_0 increase when was self-grafted (from 171 to 197) or grafted onto *Arnold* (from 164 to 191), while it showed no significant variation in the other grafting combinations (Table 3). The F_V values were higher under Ox (776, on average) than under limited Ox- conditions (652), while regarding the rootstock genotype, this variable proved to be higher when *Dreamer* was grafted onto *Maxifort* (743), intermediate in *Arnold* (717) and lower in the remaining graft combinations (704, on average) (Table 3). Similarly, F_M was significantly reduced, passing from Ox to Ox- treatment (from 938 to 832), whereas it reached the highest level in the grafting combination *Dreamer*/*Maxifort* (904) and the lowest one in *Dreamer*/*Top Pittam* (864) (Table 3). As regards the ratio F_V/F_M, passing from Ox- to Ox treatment, a significant decrease was recorded in all the grafting combinations, with the sharpest drops observed when *Dreamer* self-grafted (from 0.824 to 0.754) or grafted onto *Arnold* and *Top Pittam* (from 0.826 to 0.777, on average) (Table 3).

3.2. Leaf Gas Exchanges and Electrolyte Leakage

Significant interactions between O_2 level in the root zone and rootstock were observed for all the gas exchange variables (Table 2). Under low-O_2 conditions, A_N significantly decreased when *Dreamer* was self-grafted (−17%) and grafted onto *Arnold* and *Top Pittam* (−15%, on average), whereas it proved to be constant when grafted onto *Beaufort* and *Maxifort* (Table 4). The O_2-limiting conditions proved to decrease, in a rootstock-dependent way, as well as the E values of the scion (Table 2). Indeed, passing from Ox to Ox- treatment, a sharper E decrease was recorded when *Dreamer* was grafted onto *Top Pittam* (−29%), *Arnold* (−22%) and self-grafted (−23%), whereas no significant E variation was recorded on *Maxifort* (Table 4). The gs response to root O_2 starvation proved to be rootstock-dependent too. Indeed, passing from Ox to Ox- treatment, this variable showed the strongest decrease in self-grafted *Dreamer*, or grafted onto *Arnold* and *Top Pittam* (−18%, −16% and −15%, respectively), whereas on *Beaufort* and *Maxifort*, it underwent the least reduction (−9%, on average) (Table 4). Ci showed a general decrease, passing from optimal- to low-O_2 root availability, with the highest drop recorded in *Dreamer* grafted on *Beaufort* (−18%) and *Maxifort* (−17%) (Table 4). All the main factors under study, namely O_2 level and rootstock combination, significantly affected the electrolyte leakage, without interactive effect (Table 2). Indeed, passing from Ox- to Ox treatment, electrolyte leakage increased from 24.2% to 26.3%, while comparing the rootstock genotypes, this variable showed the highest values in *Dreamer* self-grafted or grafted onto *Arnold* and *Top Pittam* (26.2%, on average), and the lowest value in that grafted onto *Maxifort* (23.4%) (Table 4).

Table 2. *F*-values related to the main factors and their interaction on the observed and calculated variables, with the significance resulting from the ANOVA. NS: not significant; *, **, *** significant at $p \leq 0.05$, 0.01 and 0.001, respectively. F_0: minimum fluorescence, F_V: variable fluorescence, F_M: maximum fluorescence, A_N: net photosyntesis, E: leaf transpiration rate, gs: stomatal conductance, C_i: substomatal CO_2 concentration.

Variable	Oxygen Level (O)	Rootstock (R)	O × R
F_0	22.1 ***	3.9 *	5.3 **
F_V	12.0 **	4.1 *	3.1 *
F_M	4.3 NS	5.2 **	1.6 NS
F_V/F_M	13.3 **	0.6 NS	3.0 *
A_N	117.3 ***	7.7 ***	3.3 *
E	56.6 ***	11.0 ***	3.5 *

gs	225.2 ***	9.4 ***	3.7 *
Ci	32.2 ***	8.1 ***	3.1 *
Electrolyte leakage	16.1 ***	6.7 ***	0.7 NS
Plant dry biomass	49.5 ***	31.7 ***	3.3 *
Shoot dry biomass	30.4 ***	25.6 ***	3.8 *
Root dry biomass	69.6 ***	13.1 ***	4.2 *
Root : shoot ratio	4.8 *	1.6 NS	7.7 ***
Leaves number	7.6 *	6.0 **	0.7 NS
Leaf area	84.1 ***	72.0 ***	19.2 ***
Leaf area ratio	6.4 *	6.8 **	6.7 **
Specific leaf area	66.9 ***	3.8 *	5.0 **

Table 3. Chlorophyll fluorescence variables of tomato plants as affected by oxygen level and rootstock (mean ± standard error). Different letters within main factors indicate significantly different means according to Fisher's protected LSD test (p = 0.05).

Variable	Oxygen Level	Rootstock						
		Dreamer F1 (control)	*Arnold* F1	*Beaufort* F1	*Maxifort* F1	*Top Pittam* F1	Mean	LSD Interaction (p = 0.05)
F_0	Ox	171 ± 8	164 ± 8	159 ± 7	158 ± 7	160 ± 7	162 b	23
	Ox-	197 ± 9	191 ± 9	170 ± 8	164 ± 8	178 ± 8	180 a	
	Mean	184 a	178 ab	165 bc	161 c	169 bc		
F_V	Ox	800 ± 17	774 ± 7	772 ± 13	768 ± 14	766 ± 12	776 a	NS
	Ox-	605 ± 9	659 ± 8	655 ± 13	718 ±10	623 ± 17	652 b	
	Mean	703 b	717 ab	714 b	743 a	695 b		
F_M	Ox	971 ± 35	938 ± 34	931 ± 33	926 ± 33	926 ± 38	938 a	NS
	Ox-	802 ± 27	850 ± 29	825 ± 28	882 ± 31	801 ± 27	832 b	
	Mean	887 b	894 ab	878 bc	904 a	864 c		
F_V/F_M	Ox	0.824 ± 0.003	0.825 ± 0.009	0.829 ± 0.004	0.829 ± 0.004	0.827 ± 0.003	0.827 a	0.031
	Ox-	0.754 ± 0.001	0.775 ± 0.001	0.794 ± 0.002	0.814 ± 0.003	0.778 ± 0.001	0.783 b	
	Mean	0.789 c	0.800 bc	0.812 ab	0.822 a	0.803 bc		

Table 4. Leaf gas exchange variables and electrolyte leakage of tomato plants as affected by oxygen level and rootstock (mean ± standard error). Different letters within main factors indicate significantly different means according to Fisher's protected LSD test (p = 0.05).

Variable	Oxygen Level	Rootstock						
		Dreamer F1 (control)	*Arnold* F1	*Beaufort* F1	*Maxifort* F1	*Top Pittam* F1	Mean	LSD Interaction (p = 0.05)
A_N (μmol CO_2 m^{-2} s^{-1})	Ox	21.9 ± 0.4	22.0 ± 0.6	22.3 ± 0.8	22.6 ± 0.6	22.8 ± 0.7	22.3 a	1.2
	Ox-	18.1 ± 0.7	18.7 ± 0.7	20.4 ± 0.6	21.2 ± 0.7	19.6 ± 0.6	19.6 b	
	Mean	20.0 c	20.3 bc	21.4 a	21.9 a	21.2 ab		
E (mmol H_2O m^{-2} s^{-1})	Ox	8.0 ± 0.5	8.8 ± 0.2	7.5 ± 0.4	8.6 ± 0.4	7.8 ± 0.3	8.1 a	0.9
	Ox-	6.2 ± 0.2	6.9 ± 0.2	6.4 ± 0.3	8.4 ± 0.5	5.5 ± 0.2	6.7 b	
	Mean	7.1 b	7.8 a	6.9 b	8.5 a	6.7 b		
gs (mmol m^{-2} s^{-1})	Ox	528 ± 26	531 ± 19	536 ± 25	545 ± 21	531 ± 19	534 a	23
	Ox-	433 ± 18	448 ± 26	488 ± 36	493 ± 28	451 ± 30	462 b	
	Mean	480 b	490 b	512 a	519 a	491 b		
Ci (μmol CO_2 mol^{-1})	Ox	298 ± 5	293 ± 8	289 ± 4	279 ± 6	290 ± 6	290 a	24
	Ox-	290 ± 7	278 ± 4	238 ± 9	232 ± 8	268 ± 9	261 b	
	Mean	294 a	286 a	263 bc	255 c	279 ab		
Electrolyte leakage (%)	Ox	25.8 ± 0.5	23.8 ± 0.6	23.3 ± 0.7	23.0 ± 0.5	24.9 ± 0.7	24.2 b	NS
	Ox-	28.4 ± 0.8	27.0 ± 0.7	24.8 ± 0.7	23.8 ± 0.6	27.5 ± 0.8	26.3 a	
	Mean	27.1 a	25.4 ab	24.0 bc	23.4 c	26.2 a		

3.3. Plant Growth Variables

Plant, shoot and root dry biomass, as well as the ratio between root and shoot dry biomass, were all significantly affected by the root O_2 availability, in a rootstock-dependent way (Table 2). Indeed, passing from Ox to Ox- treatment, plant biomass showed the highest drop in self-grafted *Dreamer* (from 56.2 to 37.9 g plant^{-1}, −33%), followed by *Dreamer* grafted onto *Top Pittam* (from 63.9 to 52.7 g plant^{-1}, −18%) and *Arnold* (from 60.5 to 52.4 g plant^{-1}, −13%) (Table 5). Differently, no significant variation in whole plant biomass was recorded on *Beaufort* and *Maxifort* rootstocks (Table 5). A similar response was recorded in shoot biomass, for which a significant reduction was recorded under O_2 starvation mainly in self-grafted *Dreamer* (from 46.7 to 30.6 g plant^{-1}, −34%), then, in *Dreamer* grafted onto *Top Pittam* (from 52.4 to 44.5 g plant^{-1}, −15%) and onto *Arnold* (from 50.8 to 43.1 g plant^{-1}, −15%) (Table 5). Differently, with the only exception of *Arnold*, all the rootstocks under study showed a significant reduction of root dry biomass, with variations oscillating from −5% to −30%, in *Arnold* and *Top Pittam*, respectively (Table 5). Overall, root hypoxia acted to reduce the root:shoot ratio, with the highest drop recorded in the grafting combinations *Dreamer*/*Maxifort* (−30%) and *Dreamer*/*Top Pittam* (−18%), whereas a significant increase was recorded in self-grafted *Dreamer* (+20%) (Table 5).

3.4. Leaf Growth Variables

The root O_2 availability significantly influenced LN (Table 2), which, on the average of grafting combinations, was lowered by 4.5% passing from Ox to Ox- treatment (Table 6). *Beaufort*, *Maxifort* and *Top Pittam* were the rootstocks that maximized the scion LN (20.3, on average), followed by *Arnold* (19.2) then by self-grafted *Dreamer* (18.3) (Table 6). LA showed a rootstock-dependent response to the lowered O_2 root availability, with a significant reduction recorded in self-grafted *Dreamer* (from 5607 to 2628 cm^2 plant^{-1}, −53%), followed by the grafting combinations *Dreamer*/*Top Pittam* (from 5770 to 4465 cm^2 plant^{-1}, −23%) and *Dreamer*/*Arnold* (from 5140 to 4578 cm^2 plant^{-1}, −11%). On the contrary, no significant LA variation was recorded when *Dreamer* was grafted onto *Beaufort* and *Maxifort* (Table 6). Differently, LAR proved to be responsive to root O_2 concentration only in self-grafted *Dreamer*, in which it decreased by 31% (from 100.3 to 69.2 cm^2 g^{-1} DW plant^{-1}) passing from Ox to Ox- treatment (Table 6). SLA was significantly reduced in response to O_2 starvation, with the highest drops recorded when *Dreamer* was grafted onto *Top Pittam* (from 229.7 to 191.5 cm^2 g^{-1} DW leaf^{-1}, −17%) and self-grafted (from 227.5 to 190.2 cm^2 g^{-1} DW leaf^{-1}, −16%) (Table 6).

3.5. Correlation among Variables

The results of the correlation analysis are reported in Table 7. Overall 136 correlations were analyzed, of which 78 (57% of total) showed significance, highlighting 57 positive and 21 negative relationships. In the case of chlorophyll fluorescence variables, 27 out of 64 correlations (42% of total) were significant, while there were 34 out 80 (43%) for gas exchange and electrolyte leakage and 17 out of 112 (15%) for plant and leaf growth variables (Table 7). Among the negative correlations, the highest significance was found among F_0 and F_V/F_M (−0.783***), electrolyte leakage and plant dry biomass (−0.742***), shoot dry biomass (−0.734***) and gs (−0.708***), and among electrolyte leakage and LA (−0.665***) (Table 7). The strongest relationships in the dataframe of positive correlations were found among F_V and F_M (0.951***), plant and shoot dry biomass (0.933***), A_N and gs (0.909***), plant dry biomass and LA (0.887***) and among LA and LAR (0.846***) (Table 7).

Table 5. Plant growth variables of tomato plants as affected by oxygen level and rootstock (mean ± standard error). Different letters within main factors indicate significantly different means according to Fisher's protected LSD test ($p = 0.05$). DW: dry weight.

Variable	Oxygen Level	Rootstock						
		Dreamer F1 (control)	*Arnold* F1	*Beaufort* F1	*Maxifort* F1	*Top Pittam* F1	Mean	LSD Interaction ($p = 0.05$)
Plant biomass	Ox	56.2 ± 6.0	60.5 ± 5.9	64.4 ± 3.9	72.9 ± 5.9	63.9 ± 4.4	63.6 a	6.4
(g DW plant^{-1})	Ox-	37.9 ± 5.0	52.4 ± 7.0	59.1 ± 4.6	68.2 ± 4.7	52.7 ± 5.1	54.0 b	
	Mean	47.0 d	56.5 c	61.8 b	70.6 a	58.3 bc		
Shoot biomass	Ox	46.7 ± 5.8	50.8 ± 5.8	53.4 ± 3.4	59.6 ± 4.7	52.4 ± 3.9	52.6 a	6.2
(g DW plant^{-1})	Ox-	30.6 ± 5.0	43.1 ± 5.3	50.0 ± 3.6	58.5 ± 5.2	44.5 ± 4.2	45.4 b	
	Mean	38.7 d	47.0 c	51.7 b	59.1 a	48.5 bc		
Root biomass	Ox	9.5 ± 0.2	9.7 ± 0.7	11.0 ± 0.5	13.3 ± 1.1	11.5 ± 0.9	11.0 a	1.3
(g DW plant^{-1})	Ox-	7.2 ± 0.2	9.2 ± 0.4	9.2 ± 0.4	9.7 ± 0.5	8.1 ± 0.1	8.7 b	
	Mean	8.4 c	9.5 b	10.1 b	11.5 a	9.8 b		
Root:shoot	Ox	0.20 ± 0.02	0.19 ± 0.02	0.21 ± 0.02	0.23 ± 0.02	0.22 ± 0.01	0.21 a	0.04
(adimensional)	Ox-	0.24 ± 0.03	0.21 ± 0.01	0.18 ± 0.01	0.16 ± 0.01	0.18 ± 0.02	0.19 b	
	Mean	0.22 a	0.20 a	0.20 a	0.20 a	0.20 a		

Table 6. Leaf growth variables of tomato plants as affected by oxygen level and rootstock (mean ± standard error). Different letters within main factors indicate significantly different means according to Fisher's protected LSD test ($p = 0.05$). LN: number of leaves; LA: leaf area; LAR: leaf area ratio; SLA: specific leaf area.

Variable	Oxygen Level	Rootstock						
		Dreamer F1 (control)	*Arnold* F1	*Beaufort* F1	*Maxifort* F1	*Top Pittam* F1	Mean	LSD Interaction ($p = 0.05$)
LN	Ox	19.2 ± 1.2	19.5 ± 1.3	20.5 ± 0.9	20.8 ± 0.7	20.5 ± 1.0	20.1 a	NS
(n. plant^{-1})	Ox-	17.3 ± 1.3	18.9 ± 0.7	20.3 ± 0.6	19.7 ± 0.4	19.8 ± 0.7	19.2 b	
	Mean	18.3 c	19.2 bc	20.4 a	20.3 ab	20.2 ab		
LA	Ox	5607 ± 439	5140 ± 383	5209 ± 396	7361 ± 497	5770 ± 405	5818 a	552
(cm^2 plant^{-1})	Ox-	2628 ± 203	4578 ± 318	5134 ± 391	6892 ± 432	4465 ± 397	4739 b	
	Mean	4118 c	4859 b	5171 b	7127 a	5118 b		
LAR	Ox	100.3 ± 5.2	85.0 ± 5.1	81.8 ± 9.0	102.7 ± 10.9	90.2 ± 8.1	92.0 a	11.7
(cm^2 g^{-1} DW plant^{-1})	Ox-	69.2 ± 3.2	87.0 ± 7.8	87.0 ± 7.1	100.6 ± 8.5	84.7 ± 9.9	85.7 b	
	Mean	84.8 b	86.0 b	84.4 b	101.6 a	87.4 b		
SLA	Ox	227.5 ± 9.2	224.3 ± 7.4	231.8 ± 13.3	231.7 ± 16.3	229.7 ± 14.2	229.0 a	21.0
(cm^2 g^{-1} DW leaf^{-1})	Ox-	190.2 ± 13.5	196.7 ± 10.8	199.7 ± 12.7	203.6 ± 13.2	191.5 ± 13.6	196.3 b	
	Mean	208.9 b	210.5 ab	215.8 ab	217.7 a	210.6 ab		

Table 7. Pearson's product-moment correlation coefficients (r) among variables. *, ** and *** indicate significance at $p \leq 0.05$, 0.01 and 0.001, respectively. NS: not significant.

	F_0	F_V	F_M	F_V/F_M	A_N	E	gs	Ci	Electrolyte Leakage	Plant Biomass	Root Biomass	Shoot Biomass	Root: Shoot	LN	LA	LAR
F_V	NS	-														
F_M	NS	0.951 ***	-													
F_V/F_M	−0.783 ***	0.668 ***	0.432 *	-												
A_N	−0.598 ***	NS	NS	0.511 **	-											
E	NS	0.454 *	0.363 *	0.422 *	0.682 ***	-										
gs	−0.550 **	0.534 **	0.375 *	0.568 **	0.909 ***	0.746 ***	-									
Ci	NS	NS	NS	NS	−0.572 **	NS	NS	-								
Electrolyte leakage	0.459 *	−0.564 **	−0.439 *	−0.587 ***	−0.632 ***	−0.550 **	−0.708 ***	NS	-							
Plant biomass	−0.445 *	NS	NS	0.399 *	0.751 ***	0.623 ***	0.726 ***	NS	−0.742 ***	-						
Root biomass	−0.411 *	NS	NS	0.403 *	0.687 ***	0.619 ***	0.733 ***	NS	−0.607 ***	0.797 ***	-					
Shoot biomass	−0.453 *	NS	NS	NS	0.717 ***	0.566 **	0.701 ***	NS	−0.734 ***	0.933 ***	0.654 ***	-				
Root: shoot	NS	NS	NS	NS	NS	NS	NS	0.538 **	NS	NS	NS	NS	-			
LN	−0.533 **	NS	NS	0.370 *	0.492 **	NS	0.488 **	NS	−0.571 ***	0.707 ***	0.604 ***	0.642 ***	NS	-		
LA	−0.505 **	NS	NS	0.402 *	0.667 ***	0.648 ***	0.678 ***	NS	−0.665 ***	0.887 ***	0.753 ***	0.845 ***	NS	0.653 ***	-	
LAR	−0.382 *	NS	NS	NS	0.431 *	0.538 **	0.508 **	NS	−0.457 *	0.577 ***	0.527 **	0.533 **	NS	0.417 *	0.846 ***	-
SLA	NS	NS	NS	NS	0.442 *	0.436 *	0.515 **	0.692 ***	NS	NS	0.383 *	NS	0.480 **	NS	NS	NS

4. Discussion

One of the earliest responses of tomato plants exposed to root hypoxia is the reduced ability of roots to take up water and nutrients from the growth substrate. This is followed by a variety of physiological dysfunctions concerning plant growth, photosynthesis, hormonal balances, distribution of carbohydrates, nutrient uptake, early senescence or injury in organs, which sometimes precede plant death [21]. To cope with this stress typology, tomato plants display an array of metabolic modifications leading to morphological, anatomical and biochemical changes [22].

In the present experiment, the low root O_2 availability affected all the recorded physiological and developmental characteristics of tomato plants, showing systemic effects involving both rootstock and scion. As regards the chlorophyll fluorescence variables of the scion, the main effects of root hypoxia were recorded on minimum fluorescence (F_0), variable fluorescence (F_V) and the ratio F_V/F_M. F_0 represents the basal emission of Chl fluorescence when the redox components of photosystem II (PSII) are fully oxidized, while F_M reflects the reduction at a given time of the primary electron acceptor, which, in the oxidized state, quenches fluorescence [23]. F_V/F_M is a useful ratio which has been shown to be proportional to the quantum yield of PSII photochemistry and exhibits a high degree of correlation with the quantum yield of net photosynthesis [24]. Beyond the differences among rootstock genotypes, root hypoxia acted to promote F_0, a condition that, in turn, lowered F_V and the F_V/F_M ratio. The increase in F_0 and the reduction of F_V/F_M are both associated to possible damage of PSII [8,21]; therefore, our results suggest that under root hypoxia, there was a progressive impairment of photosynthetic machinery, acting to reduce the efficiency of the light-harvesting complexes. The significant correlations among A_N, F_0 and F_V/F_M appeared to corroborate such a hypothesis, while the significant correlations among F_0 and plant, shoot and root dry biomass suggest that this fluorescence variable can represent a simple and non-destructive means to rapidly detect the best growth response of grafted tomato plants to root O_2 starvation in breeding programs. On the other hand, in the present experiment, there was a general reduction of A_N, E, gs and Ci in response to root hypoxia, together with strong correlations among A_N, E and gs, suggesting the occurrence of stomatal limitations to photosynthetic processes. Hence, according to Yan et al. [25] and Yordanova and Popova [26], the working hypothesis is that under CO_2-limited mesophyll availability, surplus reducing power was diverted to the generation of damaging reactive oxygen species, with subsequent alteration of the operational status of light-harvesting complexes [27]. Accordingly, under root hypoxia, we found a significant increase in electrolyte leakage, a condition which is strongly associated to a loss of the integrity of the cell membranes in tomato plants [28]. This hypothesis is consistent with the observed promoting effect of lipid peroxidation in leaf cells under root hypoxia [29]. The present correlation analyses bear this out, as the electrolyte leakage was positively associated to F_0, and negatively to F_V/F_M, A_N, E and gs. Taken together, all these modifications suggest that the primary effect of root hypoxia on tomato photosynthesis lies in the disruption of the fine-tuning among light-dependent processes and stomatal behavior. Indeed, under hypoxic conditions, roots experience a reduction of their hydraulic properties, with subsequent reduction of the stomatal conductance to prevent plant water loss and cavitation vulnerability of the xylem. However, such modifications also induce a decrease in CO_2 availability for the leaves [22]. Interestingly, self-grafted tomato plants, as well as those grafted onto *Arnold* and *Top Pittam*, i.e., those showing the highest F_V/F_M reduction under Ox- conditions, also displayed the highest reduction in gs, E and A_N, but the least reduction in terms of Ci, despite their more pronounced stomatal closure. This suggests the involvement of non-stomatal factors too in determining tomato response to the condition of root O_2 deprivation. This seems to be confirmed by the negative correlation between A_N and Ci we found, indicating a reduced carboxylation potential of tomato leaves undergoing root hypoxia. However, this remains a point that is difficult to interpret in our experiment, since a reduced carboxylation ability can result from either diffusive or metabolic constraints at leaf cellular level [27]. Indeed, there is evidence about the importance of mesophyll conductance in determining the CO_2 transfer from the intercellular leaf spaces to the vicinity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO), a feature that has been demonstrated to be negatively influenced by an insufficient leaf water supply [30,31]. This hypothesis is consistent with the decreased specific leaf area we found in

response to root hypoxia, since this hormonal-mediated anatomical change is associated to a reduction of the mesophyll water (and CO_2) conductance, while also having negative side-effects on leaf photosynthesis rate under O_2-deficient root conditions [32,33]. On the other hand, both leaf photosynthetic rate and substomatal CO_2 concentration are strongly associated to the RuBPCO concentration and activity in leaf cells, which, in turn, are both closely correlated to the leaf N status [19,31]. To this end, it has been demonstrated the depressive effects of waterlogging on tomato N uptake and upward translocation from the roots [33]. Therefore, an alternative (or additional) interpretation about the negative correlation we found among A_N and Ci relies on an insufficient N supply to the actively growing leaf tissues, deriving from the drop of the root hydraulic conductance. To this end, it must also be taken into account that the substrate O_2-deficient conditions promote the conversion of $N–NO_3^-$ into $N–NH_4^+$, so increasing the loss of gaseous N, while lowering the plants' N absorption [34]. In any case, the correlation coefficients we found suggest the prevalence of stomatal limitations in determining tomato A_N response under root O_2 starvation.

When compared to self-grafted test, grafting *Dreamer* onto the interspecific hybrids proved to enhance, particularly under O_2 starvation, the photosynthetic rate and stomatal conductance of the scion, as well as to reduce the substomatal CO_2 concentration. These overall better photosynthetic performances were mirrored in better growth performances of these grafting combinations in terms of plant, shoot and root dry biomass. In particular, the analysis of correlation revealed that root biomass had a pivotal role in promoting the whole plant growth under Ox- treatment, as well as in buffering the scion proneness to the electrolyte leakage. Indeed, the grafting combination *Dreamer*/*Maxifort* (i.e., that showing the highest root biomass) was characterized by the highest whole plant growth, as well as by the lowest leaf electrolyte leakage, especially under conditions of root O_2 starvation; therefore, highlighting *Maxifort* as the most suitable rootstock to limit the detrimental effects of root hypoxia, followed by *Beaufort*. Therefore, the outcome of this experiment strongly suggests a close relationship existing among root biomass and functionality under hypoxic stress, likely as a consequence of a better exploitation of the O_2 available in the substrate and its subsequent storage in more developed aerenchyma [2].

Under conditions of root O_2 stress, it was recorded an average 15%, 14% and 21% decrease of plant, shoot and root biomass, respectively, together with a modification of the root:shoot ratio, overall indicating a decrease of the synthesis of carbohydrate and an alteration of the photosynthates allocation into the plant, respectively. In particular, this last feature was associated to a dramatic loss of the scion's photosynthetic potential, as can be inferred from the significant reduction in leaves number and overall leaf area per plant. Self-grafted *Dreamer* showed the highest reduction of both leaf area and leaf area ratio; therefore, indicating the highest vulnerability of its photosynthetic apparatus to the root hypoxia. Contrastingly, all the interspecific rootstocks were able to buffer such detrimental effects of low-O_2 availability, particularly *Maxifort*, a response that finds its potential explanation once more in the significant correlation between these scion developmental variables and root biomass. Interestingly, the root:shoot biomass ratio appeared to be a discriminant variable among grafting combinations in response to the stress condition, as the self-grafted test was the only in which this ratio significantly increased under root hypoxia. It has been demonstrated that under long-term waterlogging, tomato roots tend to adapt by producing new roots with increased aerenchyma formation [33,35]. Indeed, the primary effect of soil flooding is to slow down O_2 transfer to the roots, which results in a degradation process and in the death of at least a part of root tissues. This leads to limited aerobic respiration and dramatically alters the root cells' turnover, a condition which is sustained by diverting more photosynthates toward roots [36]. Hence, the response of self-grafted *Dreamer* is likely attributable to its higher need for energy and carbohydrate to sustain root cell turnover and regeneration, leading roots to act as priority organs competing with the shoot for carbohydrate allocation, a condition that was buffered in the interspecific rootstocks.

5. Conclusions

The outcome of this experiment shows that grafting onto compatible interspecific hybrids is an effective technique for improving tomato tolerance to root-zone hypoxia. Grafted *Dreamer* plants onto

S. lycopersicum × *S. habrochaites* and *S. lycopersicum* × *S. peruvianum* rootstocks exhibited superior photosynthetic and growth performances under conditions of O_2 stress, because of the reduced impairment of both light-dependent and dark reactions of photosynthesis, the latter likely deriving from a better diffusive and metabolic response of the leaf mesophyll. F_0 proved to be highly correlated to the growth and photosynthetic response of grafting combinations to O_2 starvation, so it could represent a rapid and non-destructive means to select the grafting combinations most suitable to thrive in root O_2-limited conditions. In our experiment, *Maxifort* and *Beaufort* were the most suitable rootstocks in buffering the negative effects of root hypoxia in terms of plant growth and photosynthetic potential. These superior performances were correlated to a higher root dry biomass under O_2 deprivation, which, in turn, proved to have a direct link with a better root functionality. This implies that the overall root biomass is a discriminant trait in defining the rootstock tolerance to the O_2 starvation, likely as it influences the ability to absorb larger O_2 volumes from the substrate. In this view, further investigations on tomato rootstocks are needed, in order to highlight possible relationships between the root dimension and the development of aerenchyma and lenticels system under flooding conditions.

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