



Phenotyping of new hybrid citrus rootstocks under water deficit reveals conserved and novel physiological attributes of drought tolerance

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Abstract

The present study aimed to investigate, under controlled conditions, the physiological attributes of drought tolerance induced by new hybrid citrus rootstocks with demonstrated potential to enhance productive efficiency in sweet orange under water-limiting conditions in the field. Twelve-month-old ‘Valencia’ sweet orange grafted on four new hybrid rootstocks were cultivated in plastic pots filled with 1.5 kg dm⁻³ of soil, under greenhouse conditions, and subjected to different intensities of drought stress defined on the basis of predawn leaf water potential. The results showed that the net rate of photosynthesis, stomatal conductance, transpiration and intercellular carbon concentration did not differentiate between plants maintained under moderate and severe stress conditions, evidencing high water use efficiency. The rootstocks were also able to induce osmotic adjustment, cell wall stiffening, decreased osmotic potential at the turgor loss point (Ψ_{TLP}) and stimulation of guaiacol peroxidase (GPX) activity and gene expression in leaves of the drought-stressed plants, allowing the maintenance of cell turgor, oxidative status, carboxylation efficiency and photosynthesis, irrespective of the intensity of drought stress. The rootstocks also showed a constitutively or drought-induced high density of fine roots, contributing to the soil–water uptake. These results revealed various attributes underlying drought tolerance in citrus and provided a valuable reference for developing drought-tolerant citrus rootstocks.

Keywords Water use efficiency · Osmotic adjustment · Elastic adjustment · Chlorophyll *a* fluorescence · Antioxidant enzymes · Root length density

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Introduction

Drought is a major abiotic stress limiting crop productivity and distribution of plant species across different environments (Kramer and Boyer 1995). The increasing scarcity of water for crop irrigation and the impacts of global climate change on rainfall patterns and temperature extremes make the genetic improvement for drought tolerance an absolute requirement to maintain agricultural productivity despite the predicted increasing occurrences of drought (White et al. 2004). Understanding the responses of plants to low soil water potential provides a valuable framework for assessing genetic resources and identifying corresponding key traits under water-limiting conditions.

The avoidance/tolerance model has been most commonly used, and revisited in several studies since its publication by Levitt (1972), to describe low soil water potential responses at the levels of the whole plant and the cell (Verslues et al.

2006). In stress avoidance, the plant balances water uptake and water loss to avoid an effect of stress on tissue water potential and water content. It is achieved in the short term mainly by stomatal closure and improved water use efficiency (WUE), and in the long term by increased root/shoot ratio and deepening of the root system. If the stress becomes internalized to the plant tissue, then adjustments termed ‘dehydration avoidance’ take place to decrease cell water potential to values lower than those of soil water potential to avoid water loss (Verslues et al. 2006). Its main mechanisms are accumulation of solutes (osmotic adjustment) and cell wall hardening. Finally, adjustments termed ‘dehydration tolerance’ occur when dehydration stress becomes more severe and mechanisms are activated to protect cellular structures from the effects of dehydration, which include protective solutes (e.g., proline) and proteins (e.g., LEA proteins and chaperones), ROS detoxification (e.g., antioxidant enzymes) and metabolic changes (Verslues et al. 2006).

Citrus is a genus that encompasses several commercially important cultivated species, such as *C. sinensis* (sweet oranges), *C. reticulata* (mandarins and tangerines), *C. limon* (lemons/limes), *C. grandis* (pummelos) and *C. paradisi* (grapefruits), which are top ranked among all the fruit crops (FAO 2015). Citrus are mainly cultivated in regions of the world where water supply is restricted and evaporative demand is high, often leading to the appearance of drought stress symptoms that ultimately affect fruit productivity and fruit quality (Carr 2012). Drought tolerance has been achieved in commercial citrus groves by grafting scions of economic interest onto drought-tolerant citrus rootstocks, making it possible to grow citrus plants under water-limited environments. Unfortunately, there are a quite limited number of drought-tolerant rootstock cultivars commercially available for citrus growers around the world, thus limiting genetic variability and making citrus crops vulnerable to the emergence of new pests and diseases. The generation of new citrus rootstock cultivars that combine drought tolerance, among other attributes, to increase the genetic diversification of the orchards is not an easy task, mainly because of the complex reproductive biology within the *Citrus* genus (Khan and Kender 2007) and our poor understanding of the physiological and genetic basis of the rootstock-induced drought tolerance (Gonçalves et al. 2016). So far, the specifically identified rootstock-induced physiological attributes of drought tolerance includes improved plant root system distribution (Castle and Krezdorn 1975), efficient water and nutrient uptake (Castle and Krezdorn 1977; Pérez-Pérez et al. 2008), increased root length and biomass (Magalhães Filho et al. 2008; Pedroso et al. 2014), improved water use efficiency (Pérez-Pérez et al. 2008; García-Tejero et al. 2011), increased root vessel element diameter (Vasconcellos and Castle 1994; Rodríguez-Gamir et al. 2010a), higher root hydraulic conductivity (Syvertsen and Graham 1985;

Medina et al. 1998; Rodríguez-Gamir et al. 2010a), greater osmotic adjustment (Rodríguez-Gamir et al. 2010b; Gonçalves et al. 2016) and changes in cell wall elasticity (Gonçalves et al. 2016).

Novel hybrid rootstocks have been systematically generated by some citrus breeding programs across the world, providing a valuable material for identifying both conserved and novel drought tolerance attributes that could be targeted for the future selection of new drought-tolerant citrus rootstocks. Ramos et al. (2015) have evaluated the performance of new hybrid rootstocks combined with ‘Valencia’ sweet orange cultivated in the field under rainfed conditions, for 3 consecutive years. The authors observed that the hybrids ‘(LCR×TR)–001’, ‘(TSKC×CTSW)–041’, ‘TSKC×(LCR×TR)–059’ and ‘HTR–069’ induced higher production efficiency of fruits with higher or equivalent quality in comparison with fruits on conventional rootstocks such as ‘Rangpur’ lime, a well-known and widely used drought-tolerant rootstock. These hybrids were also highlighted by their elevated potential for inducing high tolerance to drought based on visual assessment (Ramos et al. 2015). In another study aiming to evaluate drought-tolerant rootstocks alternative to ‘Rangpur’ lime for ‘Pera’ sweet orange cultivation under rainfed conditions, de Carvalho et al. (2016) also observed that the hybrid ‘TSKC×(LCR×TR)–059’ was among the rootstocks that induced high drought tolerance and productive efficiency numerically higher than that of ‘Rangpur’ lime. Thus, the objective of the present study was to investigate, in controlled conditions, the physiological attributes of drought tolerance induced by these novel hybrid citrus rootstocks, aiming to further improve the current knowledge potentially applicable to the development of new drought-tolerant citrus rootstocks.

Materials and methods

Plant material and experimental conditions

The experiment was carried out in an anti-aphid greenhouse, in the period of December 2015 to October 2016, at *Embrapa Cassava & Fruits*, located in the municipality of Cruz das Almas, state of Bahia, Brazil (latitude: 12°40′39″S, longitude; 39°06′23″W, Altitude, 225 m). Data on temperature and relative humidity (RH) were obtained from the National Institute for Meteorology (INMET), located at Embrapa Cassava & Fruits, with average temperature of 24.9 °C and average RH of 79.8% during the experimental period. Twelve-month-old ‘Valencia’ sweet orange (*Citrus sinensis* L. Osb.) were grafted onto the hybrid rootstocks ‘(LCR×TR)–001’, ‘(TSKC×CTSW)–041’, ‘TSKC×(LCR×TR)–059’ and ‘HTR–069’ (hereinafter

referred as '001', '041', '059' and '069', respectively), which have been selected by the citrus breeding program at Embrapa Cassava & Fruits based on their potential for tolerance to drought and productive efficiency under rain-fed conditions in the field (Rodrigues et al. 2015; Ramos et al. 2015). The hybrids '001', '041', '059' and '069' were selected from crosses, respectively, between 'Rangpur' lime (*C. limonia* L. Osb.) and 'Trifoliata' orange (*Poncirus trifoliata* L. Raf.), 'Sunki' mandarin (*C. sunki* hort. ex Tanaka) and Swingle citrumelo (*C. paradisi* Macf. × *P. trifoliata* L. Raf.), 'Sunki' mandarin and a hybrid of 'Rangpur' lime × 'Trifoliata' orange, and 'Trifoliata' orange and an unknown *Citrus* species.

All plants were transplanted to 100 L plastic pots, with holes drilled in the bottom and previously divided into four compartments with anti-aphid net and filled with 89.94 dm³ of soil at a density of 1.5 kg dm⁻³. Each compartment represented an experimental plot with 22.49 dm³ of soil, and each pot therefore contained four experimental plots with four citrus plants, one from each hybrid rootstock under evaluation. The pots were covered with PVC and aluminum foil to ensure soil water loss only through transpiration. The soil used in the experiment was collected at a depth of 0–50 cm in an experimental area at Embrapa Cassava & Fruits and classified as Dystrophic Cohesive Yellow Latosol. Based on the chemical and granulometric analysis of the soil [Electronic Supplementary Material (ESM) Table S1], liming was carried out aiming to increase the base saturation to 70% with the use of dolomitic limestone and the application of 100 mg dm⁻³ of P₂O₅ and 50 mg dm⁻³ of K₂O, both in single dose. Nitrogen fertilization was divided in three applications of 50 mg dm⁻³ at 20, 40 and 60 days after transplanting, and castor cake was used as a source of micronutrients. Thirty days after limestone incubation, the citrus plants were transplanted to the plastic pots with the soil. Pruning and defoliation were carried out 15 days after transplanting, standardizing the plants with fifteen centimeters of height from the insertion of the graft.

Treatments consisted of a combination of four rootstocks and four water regimes in a 4 × 4 factorial design. The different rootstocks ('001', '041', '059' and '069') and water regimes (control—CO, moderate drought stress—MO, severe drought stress—SE and rehydrated—RE) were combined in a completely randomized design with 5 replicates, totaling 80 plots that were represented by 20 pots. Each experimental plot was equal to ¼ of a pot. The water regimes were defined based on predawn leaf water potential (Ψ_L). In the CO treatment, the soil moisture was always set near the soil field capacity and Ψ_L was maintained between -0.1 and -0.5 MPa. In the drought treatments, irrigation was withheld and the stress was characterized when leaves reached Ψ_L values between -1.0 and -1.5 MPa for the MO treatment and ≤ -2.5 MPa for SE treatment. In the RE treatment,

the soil moisture was brought back to soil field capacity after the plants reached $\Psi_L < -3.5$ MPa, and the analyses were performed 24 h after rewatering. The leaf area was standardized among the plants in each pot, before suspending irrigation. All the physiological assessments, sampling and harvests of plant material were carried out simultaneously when the four plants of each pot were within the range of Ψ_L that characterized the corresponding treatment. The volume of water used for irrigation was determined by the Van Genuchten equation, according to the soil water retention curve. A matric potential of -10 kPa was taken as corresponding to the field capacity. Soil moisture was determined from the volumetric soil moisture measurements in each experimental plot obtained with the use of TDR (time domain reflectometry), with corrected values based on the calibration curve of the probes in undisturbed soil samples of the experimental units. The average volumetric soil moisture value in the four compartments of each pot was used to calculate the water volume to be applied in irrigation, with readings performed every 2 days to aid irrigation management and characterization of soil drying process.

A second experiment was carried out with the same hybrid rootstocks to specifically assess root growth characteristics under control and drought stress conditions. Initially, seeds of the four hybrid rootstocks were germinated in plastic tubes (290 cm³) containing Plantmax (Eucatex Agro., Brazil) substrate, under greenhouse conditions. Four months after germination, seedlings of nucellar origin were transplanted to plastic pots (7.5 × 30 cm) containing washed sand and transferred to a Fitotron growth chamber. Plants were maintained in a 12 h day length, with a day and night temperature and RH of 26 °C/70% and 21 °C/80%, respectively, during the experimental period. All the pots were maintained at the maximum field capacity of the substrate (0.15 cm³ cm⁻³) during the plant acclimatization period of 20 days and irrigated with nutritive solution (Forth Solúveis Inicial; Forth Aqua Micros and Forth Aqua Calcio, Tiete, Brazil). After that, the plants were subjected to two treatments: (1) control, in which irrigation was applied to substrate field capacity daily and (2) drought, in which irrigation was withheld until the development of leaf rolling symptoms. The experimental design was a completely random design with 4 × 2 factorial arrangement of hybrids and water regime, with at least five replicates per treatment.

Fraction of transpirable soil water (FTSW)

The fraction of transpirable soil water (FTSW) was calculated according to the methodology adopted by Sinclair and Ludlow (1986), according to Eq. (1):

$$\text{FTSW} = \frac{\theta_{\text{day}} - \theta_{\text{Final}}}{\theta_{\text{Initial}} - \theta_{\text{Final}}}, \quad (1)$$

where FTSW is the fraction of transpirable soil water, θ_{Initial} is the volumetric soil moisture at the time when irrigation was withheld, θ_{day} is the volumetric soil moisture on the day in which readings were taken and θ_{Final} is the volumetric soil moisture at end of the period characterizing each treatment.

The normalized transpiration rate (NTR) was calculated for each individual drought-stressed plant by dividing its transpiration rate determined from the differences in daily soil moisture by the average transpiration rate of CO-treated plants for each rootstock. The change of NTR was then plotted against the FTSW using a sigmoidal non-linear model (Muchow and Sinclair 1991): $Y = 1/[1 + a \times \exp(-b \times X)]$, where Y is NTR, X is FTSW and 'a' and 'b' are empirical coefficients estimated by the non-linear model. The FTSW threshold was calculated, using the non-linear regression, as the FTSW value for which the NTR equals to 0.95 (Sadras and Milroy 1996).

Predawn leaf water potential (Ψ_L)

The second or third fully expanded and mature leaf from the apex of the plants was used to determine the predawn leaf water potential (Ψ_L), in the period between 2:00 AM and 4:00 AM, using a Scholander pressure chamber (M670, PMS Instrument Co., Albany, OR, USA), according to the methodology described by Scholander et al. (1965).

Leaf relative water content (RWC)

The leaf relative water content (RWC) was estimated according to Barrs and Weatherley (1962). Ten 2-cm-diameter leaf disks from pooled samples were cut out with a cork borer and immediately weighed to determine their fresh weight (FW). The leaf disks were then immersed in distilled water, stored in a dark environment at 4 °C for 24 h, and weighed after that to determine their turgid weight (TW). Next, the leaf disks were dried in a forced air circulation oven at 70 °C until constant weight and the dry weight (DW) were determined. RWC was calculated according to Eq. (2):

$$\text{RWC (\%)} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100. \quad (2)$$

Leaf turgor potential (Ψ_p)

The leaf turgor potential (Ψ_p) was determined using the Wiltmeter[®], developed by Embrapa Instrumentação, and estimated from Eq. (3), as proposed by Calbo et al. (2010):

$$\Psi_p = P_y - f_w, \quad (3)$$

where Ψ_p is leaf turgor potential (MPa), P_y is pressure recorded at the time of reading and f_w is Wiltmeter factor.

Leaf osmotic potential (Ψ_π)

The leaf osmotic potential (Ψ_π) was measured using a VAPRO 5520 vapor pressure osmometer (Wescor Inc., Logan, UT, USA). The leaves were squeezed and 10 μL of the solution was collected for osmotic potential analysis. The osmotic potential values were obtained from the osmolality (mmol L^{-1}) in the sap of the leaf tissue, using the equation by Van't Hoff (Eq. 4):

$$\Psi_\pi = -R \times T \times C \quad (4)$$

where Ψ_π is osmotic potential of the solution (MPa), R is the constant universal for gases ($0.00831 \text{ kg MPa mol}^{-1} \text{ K}^{-1}$), T is the absolute temperature of the solution (K) and C is the content of solutes in the solution (mmol L^{-1}).

Pressure–volume (PV) curve

The pressure–volume (PV) curve analysis was carried out as previously described (Gonçalves et al. 2016). Three leaves were collected from each plant (technical replicates) from the four experimental plots (biological replicates) for each treatment and used in the analysis. The petiole was cut at approximately 0.5 cm from the abscission zone and immediately immersed in distilled water for a period of 24 h in a dark environment, at 4 °C, for complete rehydration of the leaves. After rehydration, leaves were weighed in a precision balance (0.0001 g) to obtain their turgid weight, and then Ψ_L was determined using a Scholander-type pressure chamber (M670, PMS Instrument Co., Albany, OR, USA) according to the methodology described by Scholander et al. (1965). The fresh leaf mass and the Ψ_L measurements were taken at 1-h intervals, and the procedure was completed when the leaves reached $\Psi_L \leq -3.50 \text{ MPa}$. In the intervals between the weight and Ψ_L measurements, the leaves were left on the bench for dehydration by the process of free transpiration at room temperature. Subsequently, the dry mass was determined after oven drying the leaves in forced air circulation at 75 °C until constant weight. With the obtained data, the pressure–volume relationships were obtained as detailed by DaMatta et al. (2003). The calculations of the parameters for relative water content at the turgor loss point (RWC_{TLP}), osmotic potential at full turgor (Ψ_0), osmotic potential at the turgor loss point (Ψ_{TLP}) and bulk modulus of elasticity (ϵ) were carried out using the analysis sheets as described by Koide et al. (2000) and by Sack et al. (2003). Osmotic adjustment (OA) was calculated as the difference between Ψ_0 of CO and MO plants.

Chlorophyll *a* fluorescence

Measurements of chlorophyll *a* fluorescence emission were performed on mature leaves between 8:00 AM and 10:00 AM using a portable pulse-modulated fluorometer (Opti-Sciences, model OS5p, Hudson, USA). The selected leaves were submitted to a dark adaptation period for 20 min, sufficient for the complete oxidation of the reaction centers. The fluorescence signals were recorded in the apparatus, which automatically calculates the minimum fluorescence (F_0), the maximum fluorescence (F_m), as well as the maximum potential quantum yield of photosystem II (F_v/F_m).

Leaf gas exchange

The rates for net photosynthesis per leaf unit area (A), stomatal conductance to water vapor (g_s), leaf transpiration (E) and intercellular carbon concentration (C_i) were estimated based on the CO_2 and air humidity variation values inside the chamber, determined by the LCpro-SD IRGA portable analyzer (ADC Biotech-Scientific Limited, UK) under saturating light conditions of $1150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and ambient CO_2 concentration. Measurements were performed on fully expanded mature leaf located at the middle third of each plant, between 8:00 AM and 10:00 AM. Intrinsic water use efficiency (A/g_s) was calculated by the ratio between the net photosynthetic rate (A) and stomatal conductance (g_s). Instantaneous water use efficiency (A/E) was calculated by the ratio of the net photosynthetic rate (A) to the transpiration rate (E). The ratio between the intercellular and atmospheric concentrations of CO_2 (C_i/C_a) was also determined.

Antioxidant enzyme activity and gene expression

Enzyme activity and quantitative real-time RT-PCR (qPCR) expression analysis of superoxide dismutase (SOD, EC 1.15.1.1) and guaiacol peroxidase (GPX, EC 1.11.1.7) were performed as previously described (Gonçalves et al. 2016). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of reduction of nitroblue tetrazolium (NBT) measured at 560 nm on a spectrophotometer (SPECTRAMax Paradigm, Molecular Devices, Sunnyvale, USA). One GPX unit was defined as the amount of enzyme that produces $1 \mu\text{mol min}^{-1}$ oxidized guaiacol. All qPCR procedures, including tests, validations and experiments were carried out following the recommendations of Applied Biosystems. Glyceraldehyde-3-phosphate dehydrogenase C2 (GAPC2) was amplified along with the target genes (*SOD* and *GPX*) as an endogenous control to normalize expression among different samples, using the primer sequences as described in Gonçalves et al. (2016). Gene expression was quantified using the comparative methods C_t , $2^{-\Delta C_t}$ and $2^{-\Delta\Delta C_t}$, with data obtained from a pool

of at least three biological replicates that were individually validated.

Analysis of root growth characteristics

Roots were carefully washed and the measurement of total root length, mean diameter and root length density per class of diameter was conducted using the scanning equipment and imaging software WinRizho (WinRizhoTM Pro, Regent Instruments Inc., Quebec City, QC, Canada). Leaves, shoot and roots were also separated and kept in an air circulation stove at 75°C until a constant weight was obtained. These materials were used to determine the leaf, shoot and root dry biomass and their relations.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) and Tukey's test for multiple comparisons at 5% significance level was used. All statistical analyses were performed with the aid of the SISVAR software (Ferreira 2011).

Results

Transpiration responses to progressive soil drying

To investigate whether there are variations in the soil moisture thresholds where transpiration starts to decline among sweet orange plants grafted on the different rootstocks, changes in NTR during the progressive soil drying were expressed as a function of FTSW using a sigmoidal non-linear model that describes the typical relationship between these two variables (Muchow and Sinclair 1991; Sadras and Milroy 1996). The pattern of NTR response to FTSW was observed to differ in sweet orange plants, depending on the rootstock (Fig. 1). The transpiration ratio of plants on '069' and '059' started declining at lower FTSW threshold values (0.63 and 0.81, respectively) than those on '041' and '001' (0.88 and 0.96, respectively), indicating a significant effect of rootstock on the transpiration response to soil drying in sweet orange.

Leaf–water relations

The different water treatments have significantly affected the leaf relative water content (RWC) of plants, with no significant differences observed among rootstocks and their interaction (ESM Table S2). The RWC values decreased significantly according to the drought stress intensity, but they were partially recovered in the RE treatment (Fig. 2). This result indicates that more than 24 h after rehydration was required

Fig. 1 Relationships between normalized transpiration rate (NTR) and fraction of transpirable soil water (FTSW) in ‘Valencia’ sweet orange grafted onto rootstocks ‘001’, ‘041’, ‘059’ and ‘069’ during the soil dry-down period. The lines describe the fitted equation for the pooled data sets using a sigmoidal non-linear model (Muchow and Sinclair 1991)

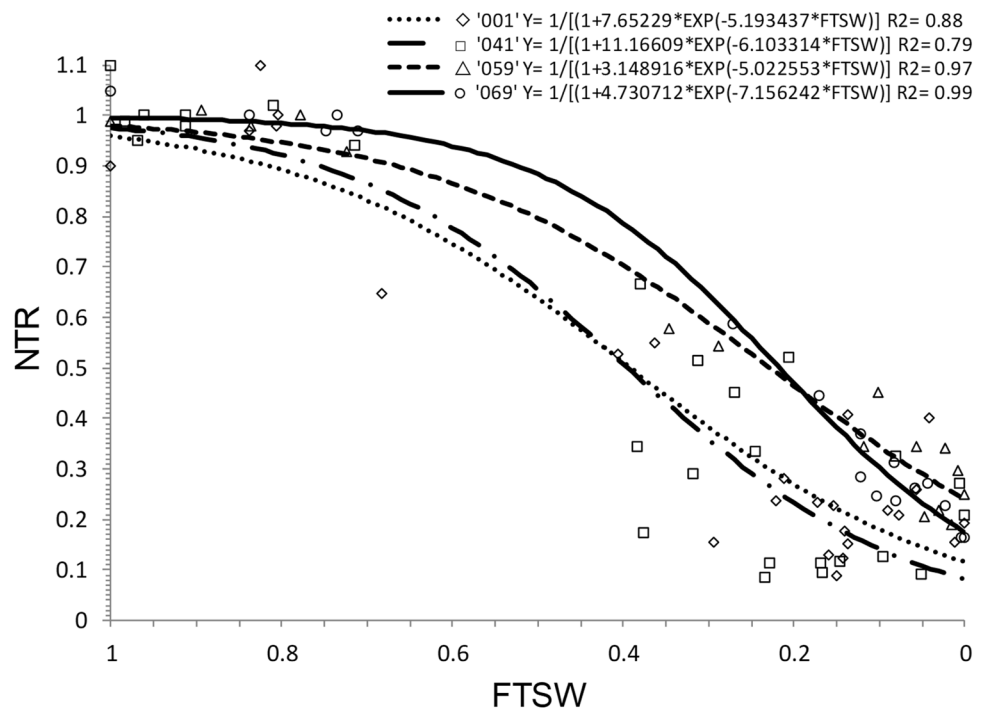
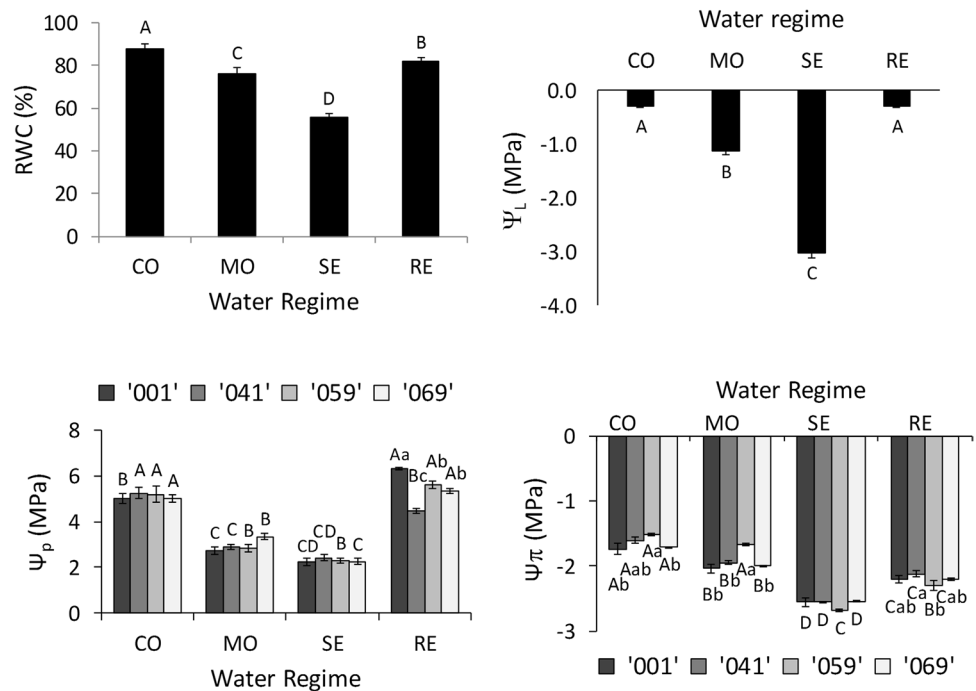


Fig. 2 Relative water content (RWC), water potential (Ψ_L), turgor potential (Ψ_p) and osmotic potential (Ψ_π) in leaves of ‘Valencia’ sweet orange grafted onto rootstocks ‘001’, ‘041’, ‘059’ and ‘069’, subjected to the CO, MO, SE and RE water treatments. Different uppercase letters indicate significant differences among water regimes within each rootstock, and distinct lowercase letters indicate significant differences among rootstocks within each water regime by the Tukey’s test ($P \leq 0.05$). Bars represent standard error



for the complete RWC recovery. Significant differences in Ψ_L were also observed among the water regimes, but not among the rootstocks and their interaction (ESM Table S2). The average Ψ_L value for MO treatment was -1.13 MPa, differing significantly from SE treatment, with an average of -3.01 MPa (Fig. 2). On the other hand, Ψ_L did not differ significantly between CO and RE treatments, which showed

average values between -0.25 and -0.35 MPa, indicating the ability of the different rootstocks to reestablish Ψ_L within 24 h after rehydration.

Analysis of Ψ_p showed significant interaction effects between rootstock and water treatment (ESM Table S2). Ψ_p values significantly decreased with the drought stress intensity, with no significant differences observed among the

rootstocks under CO, MO and SE treatments (Fig. 2). However, plants on '059' and '069' showed the least decrease in Ψ_p values under MO treatment, with the former not showing further significant decrease in its Ψ_p values under SE treatment. All plants were able to recover completely or nearly their Ψ_p values in the RE treatment (Fig. 2). However, Ψ_p values in this treatment differed significantly among the rootstocks, with plants on '001' showing the highest values and those on '041' the lowest.

Significant interaction effects between rootstock and water treatment were also observed for Ψ_π (ESM Table S2). Except for '059' under MO treatment, the Ψ_π values decreased significantly with the increase of drought stress intensity for all rootstocks (Fig. 2). Interestingly, plants on '059' showed the highest Ψ_π values in CO and MO conditions, but the lowest in SE treatment. RE treatment increased Ψ_π again, but to values lower than those of the CO treatment, irrespective of the rootstock (Fig. 2).

PV curve relations

Analysis of the PV curve parameters indicated significant effects of water regime, rootstock or its interaction (ESM Table S2). RWC_{TLP} and ϵ increased significantly in plants subjected to the SE and RE treatments, irrespective of the rootstock (Table 1). Ψ_0 and Ψ_{TLP} values tended to decrease in SE treatment and in the RE treatment returned partially or completely to those values of CO treatment, depending on the rootstock. Plants on '001' and '069' had a significant decrease in the Ψ_0 values under SE treatment, indicating the occurrence of osmotic adjustment (OA), whereas those

on '041' and '059' showed negligible differences in the Ψ_0 values between CO and SE treatments (Table 1).

Chlorophyll *a* fluorescence relations

Analysis of the chlorophyll *a* fluorescence data indicated an isolated effect of water regime on F_0 and F_v/F_m , but significant interaction effects between these two factors on F_m (ESM Table S2). F_0 values decreased significantly only in the MO treatment, irrespective of the rootstock (Table 2). F_m values decreased significantly in the MO treatment only for '059', and for all the rootstocks in the SE treatment (Table 2). F_m remained significantly decreased in the RE treatment. On the other hand, F_v/F_m values were significantly affected only in the highest drought stress intensity (SE treatment), and continued significantly decreased in the RE treatment, irrespective of the rootstock (Table 2).

Leaf gas exchange relations

Analysis of the leaf gas exchange parameters indicated significant effects only of the water regime, but not of the rootstock and its interaction, in the values of A , g_s , E , C_i , A/g_s , A/E and C_i/C_a (ESM Table S2). MO treatment was sufficiently effective to induce a significant reduction in A , g_s and E , but not in C_i , in comparison with the CO treatment (Fig. 3). On the other hand, no significant differences were observed between MO and SE treatments for all the leaf gas exchange variables analyzed.

Differences in the leaf gas exchange parameters among the rootstocks were not detected, probably because there was no significant variation in their average volumetric soil moisture

Table 1 Pressure–volume (PV) curve parameters in 'Valencia' sweet orange grafted onto rootstocks '001', '041', '059' and '069', subjected to the CO, SE and RE water treatments

Water regime	Rootstock	Ψ_0		Ψ_{TLP}		RWC_{TLP}	ϵ		OA	
CO	'001'	-1.77 ± 0.53	AB ^{ns}	-2.67 ± 0.24	A ^{ns}	60.08 ± 3.25	Bb	08.00 ± 0.86	C ^{ns}	0.89
SE		-2.66 ± 0.08	B ^{ns}	-3.02 ± 0.10	B ^{ns}	81.27 ± 1.20	A ^{ns}	15.06 ± 0.70	B ^{ns}	
RE		-2.27 ± 0.14	A ^{ns}	-2.80 ± 0.07	ABb	84.99 ± 1.37	A ^{ns}	16.82 ± 0.99	A ^{ns}	
CO	'041'	-2.51 ± 0.09	AB ^{ns}	-2.96 ± 0.02	B ^{ns}	75.95 ± 1.91	Ca	10.80 ± 0.80	C ^{ns}	0.01
SE		-2.52 ± 0.14	B ^{ns}	-3.01 ± 0.05	B ^{ns}	82.62 ± 1.05	B ^{ns}	14.79 ± 0.34	B ^{ns}	
RE		-2.00 ± 0.13	A ^{ns}	-2.57 ± 0.05	Aab	88.11 ± 1.07	A ^{ns}	16.61 ± 0.85	A ^{ns}	
CO	'059'	-2.51 ± 0.08	AB ^{ns}	-2.78 ± 0.06	AB ^{ns}	74.58 ± 1.78	Ba	10.74 ± 0.73	C ^{ns}	0.03
SE		-2.54 ± 0.16	B ^{ns}	-3.05 ± 0.07	B ^{ns}	82.38 ± 1.11	A ^{ns}	14.88 ± 1.59	B ^{ns}	
RE		-2.24 ± 0.11	A ^{ns}	-2.72 ± 0.10	Aab	86.49 ± 0.89	A ^{ns}	16.72 ± 0.62	A ^{ns}	
CO	'069'	-2.39 ± 0.04	AB ^{ns}	-2.88 ± 0.05	B ^{ns}	71.49 ± 0.78	Ca	09.33 ± 0.16	C ^{ns}	0.25
SE		-2.65 ± 0.11	B ^{ns}	-2.94 ± 0.07	B ^{ns}	82.31 ± 1.40	B ^{ns}	16.09 ± 0.19	B ^{ns}	
RE		-2.01 ± 0.06	A ^{ns}	-2.41 ± 0.05	Aa	88.95 ± 0.44	A ^{ns}	18.22 ± 0.48	A ^{ns}	

The data are average \pm SE of the osmotic potential at full turgor (Ψ_0), osmotic potential at the turgor loss point (Ψ_{TLP}), relative water content at the turgor loss point (RWC_{TLP}), bulk modulus of elasticity (ϵ) and osmotic adjustment (OA). Different uppercase letters indicate significant differences among water regimes within each rootstock, and distinct lowercase letters indicate significant differences among rootstocks within each water regime by the Tukey's test ($P \leq 0.05$). ^{ns}Not significant by the *F* test

Table 2 Chlorophyll *a* fluorescence parameters in ‘Valencia’ sweet orange grafted onto rootstocks ‘001’, ‘041’, ‘059’ and ‘069’, subjected to the CO, MO, SE and RE water treatments

Water regime	Rootstock	F_0		F_m		F_v/F_m	
CO	‘001’	144.00 ± 6.37	AB ^{ns}	752.40 ± 26.53	A ^{ns}	0.808 ± 0.00	A ^{ns}
MO		134.00 ± 2.05	B ^{ns}	723.00 ± 06.72	ABab	0.814 ± 0.00	A ^{ns}
SE		150.00 ± 3.29	A ^{ns}	664.20 ± 11.28	B ^{ns}	0.774 ± 0.00	B ^{ns}
RE		138.60 ± 3.98	AB ^{ns}	569.20 ± 08.63	Cbc	0.758 ± 0.01	B ^{ns}
CO	‘041’	142.40 ± 5.64	AB ^{ns}	719.80 ± 19.55	A ^{ns}	0.802 ± 0.00	A ^{ns}
MO		141.00 ± 4.67	B ^{ns}	748.20 ± 14.38	Aa	0.810 ± 0.00	A ^{ns}
SE		146.20 ± 4.83	A ^{ns}	631.00 ± 14.55	B ^{ns}	0.776 ± 0.00	B ^{ns}
RE		143.60 ± 2.62	AB ^{ns}	560.00 ± 11.80	Cc	0.759 ± 0.01	B ^{ns}
CO	‘059’	143.80 ± 4.82	AB ^{ns}	775.00 ± 16.59	A ^{ns}	0.814 ± 0.00	A ^{ns}
MO		124.75 ± 2.35	B ^{ns}	675.75 ± 04.53	Bb	0.815 ± 0.00	A ^{ns}
SE		145.60 ± 4.96	A ^{ns}	622.40 ± 27.67	B ^{ns}	0.764 ± 0.01	B ^{ns}
RE		141.80 ± 1.59	AB ^{ns}	644.40 ± 14.60	Ba	0.780 ± 0.00	B ^{ns}
CO	‘069’	136.40 ± 5.11	AB ^{ns}	712.60 ± 24.96	A ^{ns}	0.808 ± 0.01	A ^{ns}
MO		137.20 ± 3.09	B ^{ns}	727.60 ± 13.21	Aab	0.811 ± 0.00	A ^{ns}
SE		138.20 ± 3.61	A ^{ns}	626.80 ± 18.55	B ^{ns}	0.779 ± 0.00	B ^{ns}
RE		146.20 ± 1.46	AB ^{ns}	627.40 ± 09.05	Bab	0.768 ± 0.00	B ^{ns}

The data are average ± SE of the minimum (F_0 , non-dimensional) and maximum (F_m , non-dimensional) fluorescences of dark-acclimated leaves and on photosystem II (PSII) maximum fluorescence efficiency (F_v/F_m , non-dimensional). Different uppercase letters indicate significant differences among water regimes within each rootstock, and distinct lowercase letters indicate significant differences among rootstocks within each water regime by the Tukey’s test ($P \leq 0.05$). ^{ns}Not significant by the *F* test

(0.138 cm³ cm⁻³) since the first level of drought stress intensity (MO treatment) assessed. To test this hypothesis, we evaluated the leaf gas exchange patterns in all plants throughout the progressive soil dry-down period (Fig. 4). Significant differences in *A*, *g_s* and *E* values were observed among the rootstocks between the 5th and 14th day after water withholding, when the average volumetric soil moisture was in the range between 0.204 and 0.156 cm³ cm⁻³. Upon these conditions, plants on ‘001’ showed the lowest *A*, *g_s* and *E* values, and those on ‘069’ or ‘059’ the highest (Fig. 4).

Water use and carboxylation efficiencies

Significant effects of water regime, but not of rootstock, were observed for the water use and carboxylation efficiencies (ESM Table S2). The intrinsic (*A/g_s*) water use efficiency significantly increased in the MO, SE and RE treatments in comparison with the CO treatment (Fig. 3). The instantaneous (*A/E*) water use efficiency was significantly reduced in the MO treatment, but increased again in the SE and RE treatments to values similar to that of the CO treatment (Fig. 3). The *C_i/C_a* ratio remained relatively constant across the different water regimes (Fig. 3).

Enzyme activity and gene expression of antioxidant enzymes

Sweet orange plants grafted on ‘001’ and ‘059’ were further selected for analysis of SOD and GPX enzyme activity and

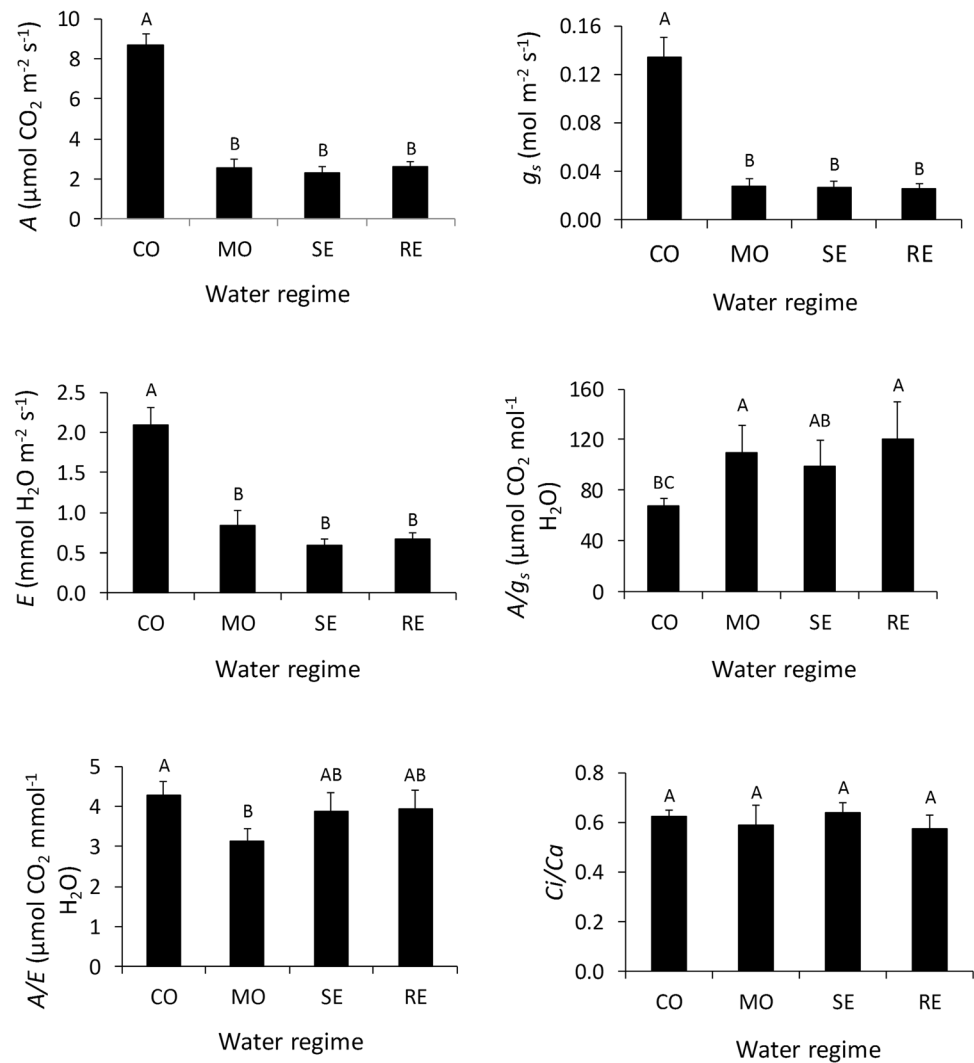
gene expression based on their more differentiated responses to drought stress among the rootstocks evaluated. No significant effects of rootstock and water regime were observed for SOD activity, whereas an isolated effect of both factors was observed for GPX activity (ESM Table S2). In comparison with the CO treatment, GPX activity was significantly increased in plants under MO treatment, irrespective of the rootstock (Fig. 5a). When comparing both rootstocks, plants on ‘001’ exhibited a significantly higher GPX activity than those on ‘059’, regardless of the water treatment.

Significant interaction effects of rootstock and water regime were observed for SOD and GPX mRNA expression (ESM Table S2). SOD expression was significantly upregulated by MO treatment in plants on ‘001’, but remained essentially unaltered in plants on ‘059’ (Fig. 5b). On the other hand, GPX expression was significantly upregulated by MO treatment in plants on both rootstocks.

Root growth characteristics

Root growth characteristics of the hybrid rootstocks under control and drought stress conditions were specifically addressed in a second experiment. We observed significant interaction effects between rootstock and water regime for root dry biomass, mean root diameter and root length density in the 0- to 0.5-mm diameter class, isolated effects of both factors for aerial shoot to root dry biomass and an isolated effect of only rootstock for total root length and root length density in the diameter classes of 0.5–1.0 mm, 1.0–1.5 mm

Fig. 3 Leaf gas exchange parameters in ‘Valencia’ sweet orange grafted onto rootstocks ‘001’, ‘041’, ‘059’ and ‘069’, subjected to the CO, MO, SE and RE water treatments. The data are average \pm SE for the net photosynthesis per leaf unit area (A), stomatal conductance to water vapor (g_s), leaf transpiration (E), intrinsic (A/g_s) and instantaneous (A/E) water use efficiencies and ratio of internal and atmospheric concentrations of CO_2 (C_i/C_a). Different letters indicate significant differences among water treatments by the Tukey’s test ($P \leq 0.05$)



and > 1.5 mm (ESM Table S3). An increase in the root dry biomass under drought condition was observed for ‘041’, whereas no significant changes between control and drought conditions were observed for the other rootstocks (Fig. 6). A significant decrease in the relation shoot to root dry biomass was observed for all the rootstocks under drought treatment (Fig. 6). Drought treatment did not significantly affect total root length of the rootstocks, with ‘041’ and ‘059’ showing a significantly higher root length than ‘001’ and ‘069’, irrespective of the water regime (Fig. 6). However, drought treatment changed the mean root diameter in the rootstocks of higher root length, which was decreased in ‘041’ but increased in ‘059’. An increase of the root length density in the diameter class of 0–0.5 mm was observed for ‘001’ and ‘041’ under drought treatment, whereas it decreased for ‘059’ or remained constant for ‘069’ (Fig. 6). However, ‘059’ showed the highest root length density in the diameter class of 0–0.5 mm among the rootstocks evaluated, irrespective of the water treatment. No significant changes in the

root length density of other diameter classes were observed between water treatments (Fig. 6).

Discussion

The distinct patterns of transpiration response (Fig. 1) and leaf gas exchange rates (Fig. 4) to the fraction of transpirable soil water (FTSW) among the rootstocks illustrate their significant effects on the adaptive strategy to the soil drying-down conditions in sweet orange. Plants on ‘001’ and ‘041’ showed FTSW threshold values higher than those on ‘059’ and ‘069’, which in turn were higher than those reported for ‘Sunki Maravilha’ mandarin (~ 0.60) and ‘Rangpur’ lime (~ 0.40) (Neves et al. 2013). Genotypes with higher FTSW threshold values for the onset of NTR decline show a ‘conservative’ strategy, which leads to water conservation at the expense of photosynthesis, in contrast with the ‘productive’ strategy, which allows to maintain prolonged photosynthetic

Fig. 4 Variations in the leaf gas exchange parameters among rootstocks during the soil dry-down period. The data are average for the net photosynthesis per leaf unit area (A), stomatal conductance to water vapor (g_s), leaf transpiration (E) and volumetric soil moisture (θ) over 26 days after water withholding

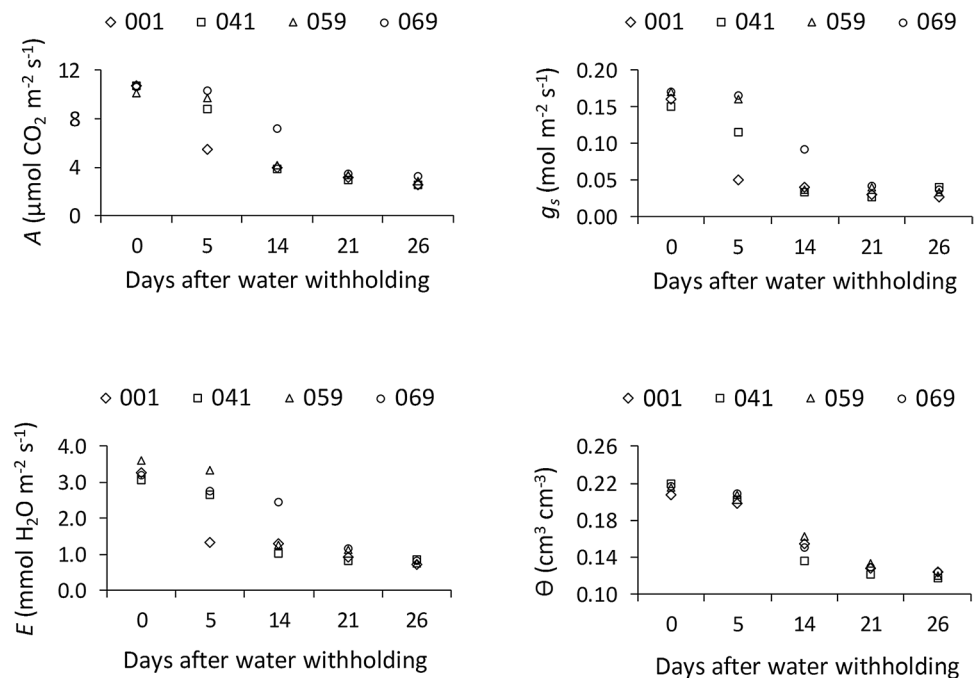
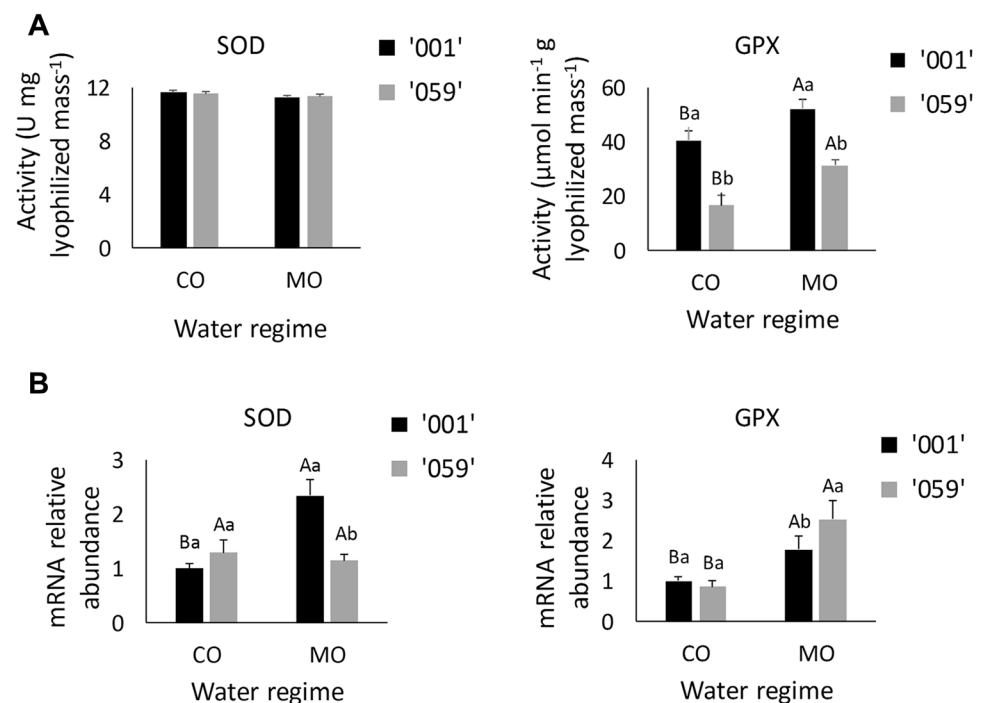


Fig. 5 Enzyme activity (a) and gene expression (b) of the antioxidant enzymes superoxide dismutase (SOD) and guaiacol peroxidase (GPX) in leaves of 'Valencia' sweet orange grafted onto rootstocks '001' and '059', subjected to the CO and MO water treatments. Different uppercase letters indicate significant differences among water regimes within each rootstock, and distinct lowercase letters indicate significant differences among rootstocks within each water regime by the Tukey's test ($P \leq 0.05$). The data are mean \pm SE of three biological replicates ($n = 3$)

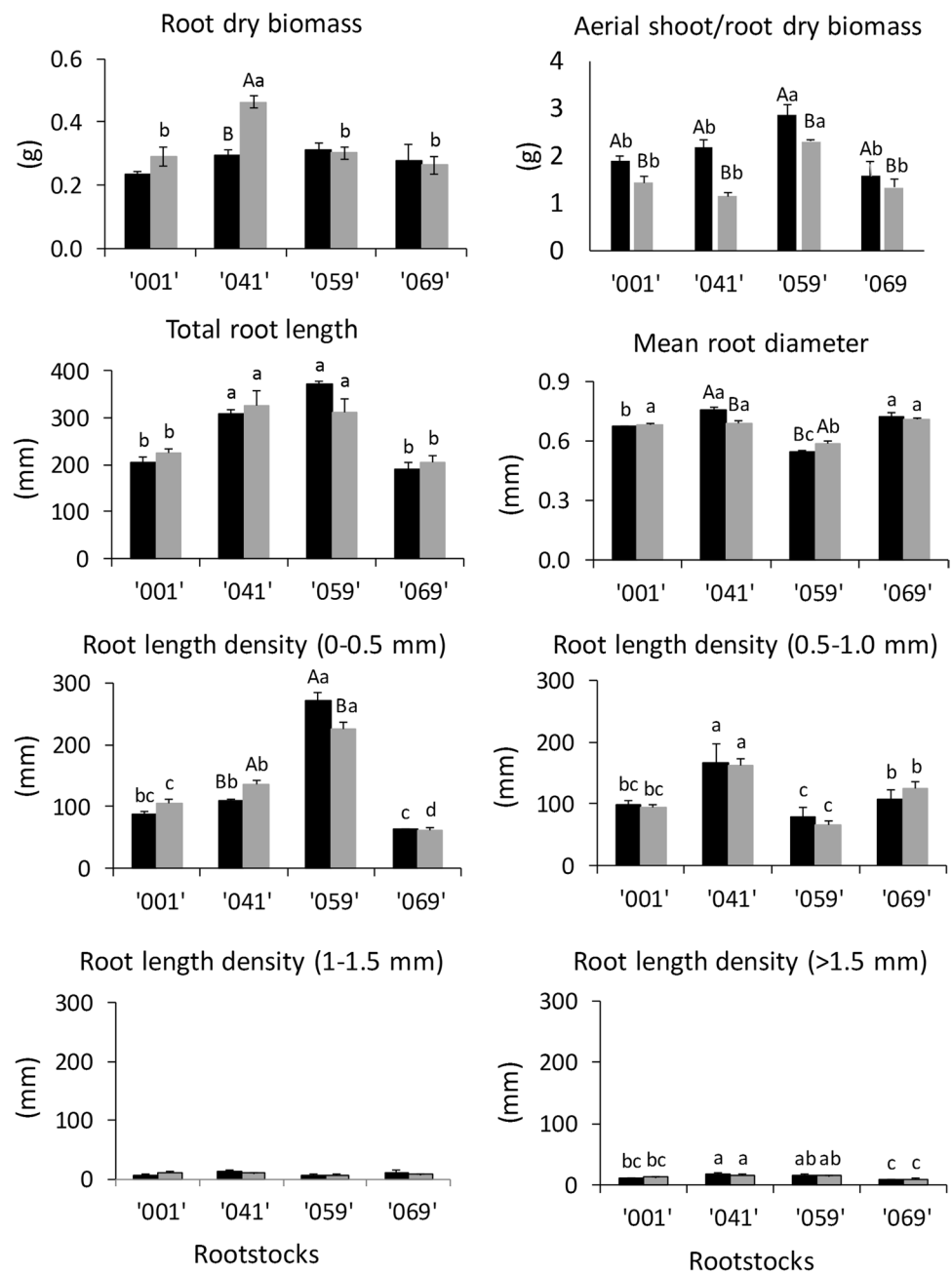


activity until the soil becomes very dry, in anticipation that there will be rain or irrigation before severe water deficits develop (Sinclair and Muchow 2001). Therefore, genotypes with a 'conservative' strategy are potentially suited to environments with severe water deficits, but not for environments with short, frequent and moderate soil water deficit periods alternating with wet periods, where the 'productive' strategy could result in better agronomic performance (Casadebaig

et al. 2008). Taken together, our results suggest that significant genotypic differences in the FTSW threshold values exist among citrus rootstocks, which may allow citrus cultivation across a broad range of environments with different rainfall patterns.

Leaf RWC, Ψ_L , Ψ_p and Ψ_π values decreased significantly as the drought stress intensity increased, but they recovered completely or nearly after rehydration, irrespective of

Fig. 6 Root growth characteristics of the hybrid rootstocks '001', '041', '059' and '069', under control (black filled square) and drought stress (gray filled square) conditions. Root dry biomass, aerial shoot to root dry biomass, total root length, mean root diameter and root length density per diameter class (0–0.5 mm, 0.5–1.0 mm, 1–1.5 mm and > 1.5 mm) were measured in individual plants 48 days after the treatments. Different uppercase letters indicate significant differences among water regimes within each rootstock, and distinct lowercase letters indicate significant differences among rootstocks within each water regime by the Tukey's test ($P \leq 0.05$). The data are mean \pm SE of five biological replicates ($n = 5$)



the rootstock (Fig. 2). Similar observations have been also previously reported for 'Valencia' sweet orange grafted on 'Rangpur' lime, which affected some of these leaf–water relations (Medina and Machado 1998; Machado et al. 1999; Santana-Vieira et al. 2016). The leaf RWC and Ψ_L values decreased more quickly and deeply in plants on 'Rangpur' lime than those on 'Trifoliata' orange under drought stress, and they were fully recovered within 24 h after rehydration for both rootstocks (Medina and Machado 1998; Machado et al. 1999). Plants on 'Rangpur' lime also showed a significant decrease in the Ψ_π values under drought treatment, in contrast with those on 'Sunki Maravilha', which showed

constant Ψ_π values between the different water treatments (Santana-Vieira et al. 2016).

The RWC_{TLP} and ϵ values increased significantly in plants exposed to drought stress, whereas the opposite was observed for Ψ_0 and Ψ_{TLP} , regardless of the rootstock (Table 1). These PV curve parameters have been correlated with various aspects of drought tolerance (Lenz et al. 2006) and shown to be significantly affected by the citrus rootstock (Gonçalves et al. 2016). An increase in ϵ indicated that the cell walls became more rigid by drought stress treatment, a strategy that leads to large reduction in Ψ_L for only small decline in RWC, thus increasing the

soil–plant gradient of water potential and hence the water uptake (Lenz et al. 2006). The decreased Ψ_{TLP} observed in drought treatment for all the rootstocks is indicative of greater drought stress tolerance, since the tissues can support the adverse conditions longer before the cells reach plasmolysis (Lenz et al. 2006). Significant decrease in Ψ_0 , as observed in plants on ‘001’ and ‘069’, is driven by net accumulation of solutes in the symplast, or osmotic adjustment. Thus, cell wall stiffening, decreased Ψ_{TLP} and osmotic adjustment contributed to the maintenance of Ψ_p (Fig. 2) and, hence, to the turgor-dependent processes like A , g_s and E , regardless of the intensity of drought stress (Fig. 3).

Significant effects of water treatment in F_0 values were observed only in the MO treatment, whereas F_m and F_v/F_m values were significantly decreased in the SE and RE treatments in all the rootstocks (Table 2). These results may indicate the occurrence of an increased nonphotochemical quenching and photoinhibitory damage in the photosystem II (PSII) reaction centers (Baker and Rosenqvist 2004) caused by drought stress, which was not recovered within 24 h after rehydration. Despite these significant variations, the F_v/F_m values in SE and RE treatments were within the indicative range of intact photosynthetic apparatus (Maxwell and Johnson 2000).

A , g_s and E rates were significantly decreased in MO treatment, but maintained stable at further drought stress intensity (SE treatment), whereas A/g_s increased and A/E and C_i/C_a did not change as the drought stress intensity increased, irrespective of the rootstock (Fig. 3). These results indicate the activation of stress avoidance mechanisms (Verslues et al. 2006) at the first level of drought stress, which together with dehydration avoidance mechanisms activated at increased drought stress intensity (Table 1), contributed to the maintenance of CO_2 assimilation and carboxylation efficiencies and increased intrinsic water use efficiency (Fig. 3). Lower A/g_s and A/E values than those found in the present study were reported for ‘Valencia’ grafted on ‘Rangpur’ lime, ‘Trifoliolate’ orange, ‘Cleopatra’ mandarin and ‘Sunki Maravilha’ mandarin under control (irrigated) condition, which were further decreased by drought stress treatment regardless of the rootstock, except for ‘Sunki Maravilha’ (Machado et al. 1999; Rodríguez-Gamir et al. 2010b; Santana-Vieira et al. 2016). On the other hand, plants of ‘Valencia’ grafted on the hybrid rootstock ‘FA-5’, which was shown to be more drought tolerant than its parents, ‘Cleopatra’ and ‘Trifoliolate’, exhibited A/E values as higher as those found in the present study, in both control and drought conditions (Rodríguez-Gamir et al. 2010b). These results suggest that water use efficiency is an important component of drought tolerance in citrus. After a 24 h rewatering period, leaf gas exchange rates had been only partially recovered (Fig. 3), as also evidenced in ‘Valencia’ grafted on ‘Rangpur’ and

‘Trifoliolate’, which fully recovered A and g_s values only after a 3-day rewatering period (Machado et al. 1999).

SOD and GPX enzyme activity and gene expression were differentially regulated by the rootstock and water treatment (Fig. 5). As expected, a proportional correlation between gene expression and the corresponding total enzyme activity was not so evident due to the presumed additional levels of post-transcriptional regulation (Gonçalves et al. 2016). In contrast with SOD, both GPX activity and gene expression were significantly increased in MO treatment, irrespective of the rootstock (Fig. 5). The importance of GPX in the rootstock-induced drought stress tolerance was previously demonstrated in citrus (Gonçalves et al. 2016), in which it was activated as a mechanism of ‘dehydration tolerance’ to alleviate the extended oxidative process triggered by drought stress in sweet orange plants grafted on ‘Flying Dragon’ trifoliolate orange.

Several root system traits are considered to be important in maintaining plant productivity under drought stress, including increased root:shoot ratio, small fine root diameters, long specific root length and considerable root length density (Comas et al. 2013). All the rootstocks exhibited an increased root:shoot ratio under drought stress, in which one of them (‘041’) was caused by a significant increment in the root dry biomass (Fig. 6). This is a key trait of interest since it can compensate for water shortage by improving the acquisition of water and nutrients while minimizing transpiration loss (Diaz-Espejo et al. 2012). Except for ‘069’, the rootstocks also exhibited a high root length density in the diameter class of 0–0.5 mm, which was produced constitutively (‘059’) or induced by drought stress (‘001’ and ‘041’) (Fig. 6). Fine roots are the most active portion of the root system in water uptake, comprising the majority of the length and surface area of the root systems in citrus and other woody and herbaceous plants (Rewald et al. 2011; Comas et al. 2013).

Taken together, our results indicate that the four new hybrid rootstocks evaluated induce various physiological mechanisms of drought tolerance that are conserved in other citrus rootstock varieties, such as osmotic adjustment (Rodríguez-Gamir et al. 2010a; Gonçalves et al. 2016), cell wall stiffening (Gonçalves et al. 2016), increased root biomass (Pedroso et al. 2014), improved water use efficiency (Pérez-Pérez et al. 2008; García-Tejero et al. 2011) and activation of GPX (Gonçalves et al. 2016), as well as novel drought tolerance mechanisms, such as decreased Ψ_{TLP} and high density of fine roots. These mechanisms ensured the maintenance of soil water uptake, cell turgor, oxidative status, carboxylation efficiency and photosynthesis under progressive soil–water deficit. These results also pinpoint the importance of the combination of diverse physiological attributes in the rootstocks to effectively enhance drought stress tolerance in citrus. Finally, these new hybrids constitute alternative

drought-tolerant citrus rootstocks either for immediate use as improved rootstock varieties or as parental material for conventional cross-breeding aiming the diversification of orchards.

Author contribution statement MCS, WSSF, ASG, MACF and MGCC conceived and designed the experiments. MCS, AROS and ESC conducted the experiments. MCS, AROS, ESC and AFS analyzed the data and drafted the manuscript. WSSF, ASG, MACF and MGCC supported the project. MGCC and MACF polished the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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