

## Deciphering the drought tolerance mechanisms in citrus rootstocks

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### Abstract

An increasing amount of land is becoming unsuitable for citrus cultivation, mainly due to water scarcity. This study evaluated the physiological and biochemical performance of trifoliolate orange (*Poncirus trifoliata*) and rough lemon (*Citrus jambhiri*) seedlings subjected to water deficit for 12 days. Under these conditions, trifoliolate orange was more sensitive than rough lemon and exhibited significant changes in LRWC (64.8% vs 36.7%), gas exchange parameters (71.7% vs 54.3% on average), leaf surface area (18.2% vs 4.9%), chlorophyll fluorescence in dark- and light-adapted leaves (44.5% vs 33.3% on average) and non-photochemical quenching (94.3% vs 28.2%). Moreover, oxidative stress indicators, such as malondialdehyde or hydrogen peroxide, indicated significantly higher values in trifoliolate orange than in rough lemon seedlings. They also showed lower antioxidant defense activation. Other biochemical parameters, such as proline, glycine betaine, antioxidant capacity, phenolic content, and total soluble proteins, showed higher levels in rough lemon than in trifoliolate orange seedlings. Overall, the better performance of rough lemon in arid conditions could be attributed to its improved ability to prevent water loss and maintain tissue water content. In addition, rough lemon has a more robust antioxidant defense to keep production of reactive oxygen species at low levels.

**Keywords:** antioxidant defense; citrus rootstocks; osmotic adjustment; transpiration; water deficits

### Introduction

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Citrus is major fruit crop worldwide, with annual yields exceeding 158,4 million ton (FAOSTAT, 2020), thus being vital to the economies of many developed or developing countries (Alipour *et al.*, 2013). Abiotic factors influence citrus production worldwide, including drought, which severely affects yield and quality. In this warmer climate, short-term water deficiency helps citrus trees to flower, but when these conditions persist, it negatively affects plant growth or productivity (Chaves *et al.*, 2009; Sulistiawati *et al.*, 2014; El-Beltagi *et al.*, 2022a). Drought affects expected growth of plants through disruption of water relations and reduced water utilize efficiency. This leads to a reduction in different metabolic processes and overall development. This has been associated with decreased overall productivity and fruit quality in citrus (Syvertsen and Garcia-Sanchez, 2014; Hussain *et al.*, 2018; Khalid *et al.*, 2021; El-Beltagi *et al.*, 2022b). However, the phenomenon is more complex since many physiological or biochemical responses occur at cellular and whole-organism levels.

The primary response of plants subjected to water shortage is to reduce stress pressure. This prevents the accumulation of detrimental ions and metabolites in sensitive parts of plants. These mechanisms are known as avoidance mechanisms (Verslues *et al.*, 2006), which constitute one of most effective ways to sustain performance of plants under short-duration water deficit stress. Through the induction of mechanisms to evade dehydration, stressed plants maintain their tissue water potential and water content close to controlled unstressed levels. The tissue water potential is primarily maintained by limiting water loss and increasing water uptake by modulation of aquaporin gene expression. This regulates root conductivity to water (Miranda *et al.*, 2022). Other adaptations are related to flexibility in leaf rolling, a higher root-to-shoot ratio caused by dense and deeper root system, drought-triggered leaf biomass reduction, and increased tissue cuticular resistance, all critical factors for crop plants to avoid drought stress (Gowda *et al.*, 2011; Hu and Xiong, 2014; Ramadan *et al.*, 2023). Other avoidance mechanisms include hardening plant cell walls and osmotic adjustment by accumulating compatible osmolytes such as proline and other betaines. These mechanisms depend on plant genotypes, stress type, duration, and intensity (Pérez-Pérez *et al.*, 2009; Colmenero-Flores *et al.*, 2020). These mechanisms decrease cell water potential and maintain water flow into cells to keep cell turgor pressure (Ruiz-Sánchez *et al.*, 1997). Physiological responses affecting respiration rate, stomatal conductivity, and net anabolic rate under water stress are relatively fast processes occurring within hours after stress imposition (Tudela and Primo-Millo, 1992). In addition, abiotic stress affects the redox balance in plant cells; therefore, tolerance mechanisms are also linked to the activation of the antioxidant machinery, including antioxidant metabolites (glutathione and ascorbate) and reactive oxygen species-scavenging enzymes as catalase, superoxide dismutase, peroxidase (Balal *et al.*, 2012).

In modern citriculture, grafting varieties on other citrus species acting as rootstocks is common to overcome productivity issues and confer abiotic and biotic stress tolerance traits (Seday *et al.*, 2014; Rao *et al.*, 2021). Several studies have shown that grafting citrus varieties on different rootstocks impact the morphological, biochemical, and physiological levels, affecting fruit productivity and quality under adverse conditions (Shafqat *et al.*, 2019; Shalaby *et al.*, 2022). For these reasons, there is always a need for new and improved rootstocks with enhanced tolerance to various abiotic stresses to maintain profitability of citriculture. The relative sensitivity or tolerance of the different rootstock genotypes used in citriculture to abiotic stresses has been addressed (Hussain *et al.*, 2022). In this respect, the usage of citrus rootstocks varies according to the pedoclimatic conditions in the different citrus-producing areas. For instance, rough lemon (*Citrus jambhiri*) has been used as a citrus rootstock in many citrus-producing countries owing to its outstanding drought-tolerant traits associated with an extensive root system that colonizes a vast soil extension, enabling the efficient absorption of water, and nutrients and its moderate tolerance to salinity. Trifoliolate orange (*Poncirus trifoliata*) is another valuable species for the citrus industry primarily because it confers cold tolerance to grafted scion (Khalid *et al.*, 2022). Species within the genus *Poncirus* display shallow phenotypic diversity but can generally be divided into two significant groups differing in their ability to withstand stressful conditions. They can be easily distinguished based on the size of aerial organs: Group 1 accessions have large flowers and leaves and are

moderately drought-tolerant, whereas Group 2, with smaller aerial organs, seems more drought-tolerant than Group 1. This enhanced ability to withstand drought appears to rely on their ability to maintain lower tissue water potential under stressful conditions (Jacquemond and Blondel *et al.*, 1986; Yahmed *et al.*, 2015).

The present study explored physiological and biochemical mechanisms adopted by rough lemon (*Citrus jambhiri*) or trifoliolate orange (*Poncirus trifoliata*) citrus rootstocks against water stress. We hypothesized that rough lemon and trifoliolate orange might adopt different mechanisms to cope with water deficit conditions due to their diverse genetic makeup and leaf morphology.

## Materials and Methods

### *Plant material and growth conditions*

Citrus Research Institute in Sargodha provided six-month-old seedlings of rough lemon (*Citrus jambhiri*) or trifoliolate orange (*Poncirus trifoliata*) (100 plants each). Trifoliolate orange seedlings belonged to Group 1 and had larger blooms and leaves (Yahmed *et al.*, 2015). Pots of the same size and weight were chosen and filled with the same amount of dry soil (4 kg of soil in each pot). Seedlings were transplanted into these pots and kept in greenhouse for six months for proper acclimation. From each group, 30 healthy and visually homogeneous seedlings were chosen for the experiment. They were divided into two groups based on treatments: 15 seedlings for control and 15 seedlings for drought stress.

Drought was imposed by ceasing watering, whereas controls were kept at field capacity through irrigation with tap water supplemented with Hoagland nutrient solution. This was done to fulfill the nutritional requirements of plants. Plants were distributed into three experimental units, each containing five seedlings, following a completely randomized design to avoid bias in sampling and physiological parameter analysis. The experiments lasted 12 days until the drought-stressed seedlings displayed apparent wilting symptoms. During the experiments, the average greenhouse day and night temperatures were 28 and 14 °C, respectively, with relative humidity between 50% and 80%. Physiological data were collected every three days beginning on day zero (0, 3, 6, 9, and 12). Two fully expanded leaves from each seedling were chosen to measure gas exchange and chlorophyll fluorescence parameters. The gas exchange was estimated using infrared gas analyzer (LCi-SD, BioScientific Ltd UK) between 09:00 and 11:00, and chlorophyll fluorescence was determined using fluorometer (FluorPen FP-100, Photon Systems Instruments, Czech Republic). At same time, leaf surface area (LSA) was determined by laser leaf area meter (CI-202, CID, USA). When, due to stress, chosen leaves dropped, adjacent intact leaves were used to conduct physiological measurements. At the beginning of the experiment, one seedling from each experimental unit was tagged. This was done to measure pot weight variation and physical and physiological parameters on selected dates. Three plants were randomly chosen, harvested, and pooled on each sampling date. Leaves and roots sampled were immediately frozen in liquid nitrogen and crushed to fine powder with pre-chilled mortar and pestle. Until analyses, samples were stored at -80 °C to halt enzyme activity and prevent degradation. Leaves were weighed fresh, dry, and turgid to measure relative water content (RWC) by formula:

$$(\text{Fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight}) \times 100$$

### *Antioxidant enzymatic activities and total soluble protein analyses*

To extract proteins for enzyme activity, 0.3 g of powdered leaf and root samples from each experimental unit were weighed and extracted in 50 mM sodium phosphate buffer (SPB) (7.8 pH) utilizing pre-chilled mortar and pestle. Resulting protein suspension was used to determine catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR) enzyme activities.

Determination of SOD activity was achieved by following spectrophotometrically the  $O_2^{\bullet}$  dependent reduction of nitroblue tetrazolium (NBT), as described by Giannopolitis and Ries (1977). A reaction solution containing 75 mM ethylenediaminetetraacetic acid (EDTA), 50  $\mu$ M NBT, 1.3  $\mu$ M riboflavin, and 13 mM methionine in 50 mM SPB (pH 7.8) was used. Photochemical  $O_2^{\bullet}$  production was triggered by exposing reaction mixtures to fluorescent light after adding 50  $\mu$ L of crude enzyme extract. Superoxide-dependent NBT reduction was followed by reading absorbance at 560 nm in the reaction solution for 15 min. SOD activity was measured as ability of plant extracts to inhibit  $O_2^{\bullet}$  dependent NBT reduction compared to a blank sample.

CAT and POD activities were measured according to Chance and Maehly (1955). The solution to determine CAT activity contained 50 mM SPB (7.8 pH) and 100  $\mu$ L of enzyme extract.  $H_2O_2$  was added to a final concentration of 5.9 mM to trigger the reaction. Depletion of  $H_2O_2$  due to CAT activity was followed by recording absorbance at 240 nm. Similarly, POD activity was determined as the  $H_2O_2$ -dependent reduction of guaiacol by reading absorbance of reduced product at 470 nm. The reaction mixture contained 20 mM guaiacol, 50 mM SPB (7.8 pH), and 100  $\mu$ L enzyme extract. Finally,  $H_2O_2$  was added to final concentration of 40 mM to trigger reaction.

Ascorbate peroxidase (APX) activity was measured according to Nakano and Asada (1981), following the reduction in absorbance at 290 nm due to  $H_2O_2$ -dependent enzymatic oxidation of ascorbate. The solution contained 0.5 mM ascorbate, 0.1 mM EDTA, and 100  $\mu$ L enzyme extract in 50 mM SPB (pH 7.0). Reaction was triggered by adding  $H_2O_2$  to final concentration of 1.2 mM.

To determine glutathione reductase activity (GR), the enzyme-dependent oxidization of NADPH was followed in the reaction mixture by recording the absorbance at 340 nm, as described in Foyer and Halliwell (1976). Reaction mixture consisted of a solution containing 0.5 mM oxidized glutathione, 0.2 mM NADPH, and 2 mM EDTA in 100 mM potassium phosphate buffer (PPB) (7.8 pH). To trigger reaction, 100  $\mu$ L of crude enzyme extract was added.

To determine total soluble proteins (TSP), 0.5 g of leaf and root samples were homogenized in 1 mL of PSB at pH of 7.2 (Sambrook and Russell, 2006). Samples were then centrifuged at 10,000 rpm for 5 min to pellet debris, and supernatant was recovered for determination. Briefly, 0.2 mL of supernatant was combined with 0.78 mL of deionized water and 0.02 mL of Coomassie blue dye solution (Bradford, 1976). Formation of protein-dye complexes was recorded at 595 nm. The quantitation of protein concentration was achieved by interpolating absorbance at 595 nm of extracts in a calibration curve made with bovine serum albumin as standard and analyzed similarly.

#### *Determination of osmoprotectants and chlorophyll 'a' and 'b'*

Proline (PRO) analysis was performed following (Bates *et al.*, 1973). Briefly, 0.5 g of powdered, frozen plant material was homogenized in 10 mL of a 3% sulfosalicylic acid solution. It was centrifuged to remove tissue residues. The supernatants were recovered and combined with ninhydrin reagent and glacial acetic acid following a 1:1:1 ratio. Reaction mixtures were incubated at 100 °C in water bath for 1 h, then quickly cooled in ice bath. After cooling, the reaction mixture was partitioned twice against 4 mL of toluene to recover dye. Finally, organic layers were recovered, and absorbance at 520 nm was read against blank. Tissue PRO contents were determined by external calibration made with standard proline and following the same procedure.

Glycine betaine (GB) analysis was performed according to Grieve and Grattan (1983). Briefly, 0.5 g of powdered, frozen leaves or roots were homogenized in distilled water and centrifuged. Supernatant (1 mL) was combined with 1 mL of hydrochloric acid and 0.2 mL of potassium triiodide. The solution was placed in ice bath under continuous stirring for 90 min. The tubes were kept cold to allow the periodide complex to separate from the acid medium. Once this happened, the solution was supplemented with 2 mL of cold distilled water and 20 mL of chilled 1,2-dichloroethane to dissolve periodide crystals. Organic layer absorbance was measured at 365 nm. To achieve quantitation of GB, a reference standard curve was prepared in 1 N  $H_2SO_4$  and processed similarly.

To quantify chlorophylls 'a' and 'b' in leaves, the method described in Harborne (1973) was utilized. Briefly, 0.5 g of leaf samples was homogenized in 10 mL of 80% cold acetone overnight and in the dark. After this time, supernatants were recovered, and 645 and 663 nm absorbance was measured against a blank. Chlorophyll concentration was calculated (Arnon, 1949).

#### *Determination of H<sub>2</sub>O<sub>2</sub>, malondialdehyde, total phenolic content and Antioxidant capacity*

For spectrophotometric determination of H<sub>2</sub>O<sub>2</sub> and malondialdehyde (MDA) (mol g<sup>-1</sup> FW), 0.2 g of frozen powdered plant material (leaves or roots) was homogenized in a 0.2% trichloroacetic acid solution. For H<sub>2</sub>O<sub>2</sub> estimation, 0.5 mL of extract was combined with a reaction mixture containing 1 mL of 1 M potassium iodide in 0.5 mL of 10 mM PPB. Absorbance was then read at 390 nm against a blank (Velikova *et al.*, 2000). Quantitation was achieved by interpolating absorbance reads in a calibration curve made with standard H<sub>2</sub>O<sub>2</sub> and analyzed identically. Similarly, MDA was analyzed using 0.5 mL of extract but added to solution containing 0.5% thiobarbituric acid and 20% trichloroacetic acid. Solution was heated to 100 °C for 1 h and immediately cooled in ice bath. The absorbance of the derivatized MDA was read in the reaction mixture at 532 and 600 nm comparison to blank. MDA content was calculated using the equations described in Heath and Packer (1968).

Total phenolic content (TPC) or antioxidant capacity (AC) were estimated according to Özgen *et al.* (2010). Briefly, 0.5 g of frozen powdered plant material (roots or leaves) was homogenized in a solution containing 70% ethanol, 29% distilled water or 1% acetic acid. After centrifugation of extracts, TPC concentration was estimated by combining 1 mL of Folin-Ciocalteu's reagent plus 20 mL of distilled water, 10 mL of 7% sodium carbonate, and 1 mL of the plant extract. The solution was placed at room temperature for 120 minutes, and absorbance was read at 750 nm against blank. TPC content was determined as gallic acid equivalents by interpolating absorbance values in a calibration curve analyzed identically. For the determination of AC, 0.03 mL of plant extract was combined with 2.97 mL of a solution containing 0.1 mM 2,2-diphenyl-1-picrylhydrazyl, mixed thoroughly, and placed in the dark for 10 minutes, after which absorbance at 515 nm was recorded. To assess antioxidant capacity of samples by both methods, absorbance values were compared with those derived from standard curves of Trolox (10–100 µmol L<sup>-1</sup>). Antioxidant capacity values were expressed as Trolox equivalents (mmol 100 g<sup>-1</sup>).

#### *Statistical analysis*

Statistical data analyses were accomplished utilizing Statistix 8.1 (Analytical Software, Tallahassee, FL, USA). The results of the two-way analysis of variance (ANOVA) are presented in Table S1. Least significant difference (LSD) test with a significance level of P 0.05 was employed to compare means (Table S2). A comparison of variable values between both rootstocks under water deficit conditions is shown in Table 1. For a more insightful comparison, the increase and decrease in percentage were calculated as follows:

Increase = value on measured day – value on day zero

Increase in percentage (%) = increased value of measured day × 100 / value on day zero

Decrease = value on day zero – value on the measured day

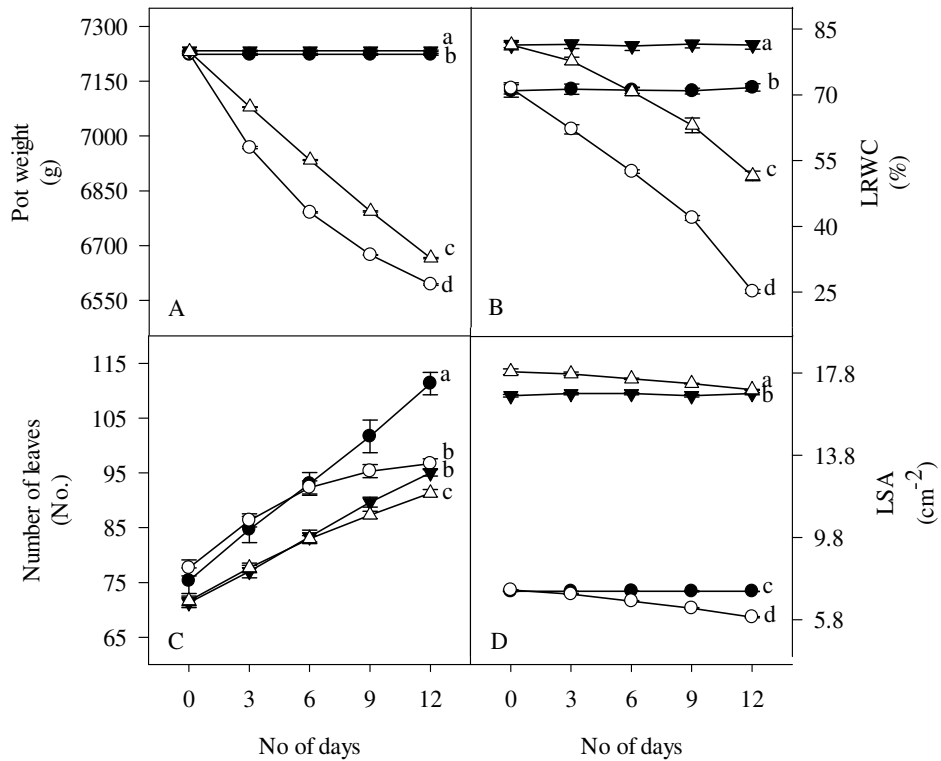
Decrease in percentage (%) = decreased value of measured day × 100 / value on day zero

## **Results**

#### *Pot weight and leaf morphological parameters*

In the present study, the decrease in pot weight was higher in trifoliolate orange compared to rough lemon. This decrease was primarily due to water loss through transpiration and, from the pots, through evaporation. Compared to their controls, maintained at field capacity, both rootstocks lost a significant amount of water under water deficit conditions. Initially, water loss was faster and higher in trifoliolate orange than rough lemon.

In this genotype, water loss was slower and had little impact on plant growth compared to trifoliolate orange, likely due to its high initial water loss (Figure 1). Under control conditions, both rootstocks showed a constant value of LRWC and LSA. At the same time, a parallel decline in LRWC was observed under water deficit conditions. Although pot water content reduction followed almost an identical pattern in pots with each rootstock genotype, LRWC showed a different trend. In this respect, and despite differences existing between control plants of both genotypes, the adverse effects of drought, particularly on LRWC, were always more pronounced in trifoliolate orange (64.8% vs 36.7% of decrease for trifoliolate orange and rough lemon, respectively, Table 1). Differences in LSA over time attributable to water stress were observed only in trifoliolate orange (Figure 1). Leaf number increased in plants of both rootstocks but with a less pronounced trend in plants subjected to water deficit conditions.



**Figure 1.** Pot weight and leaf morphological parameters of trifoliolate orange (T.O) and rough lemon (R.L) under control and water deficit conditions (A) Pot weight; (B) LRWC; (C) Number of leaves; (D) LSA. Values are mean  $\pm$  S.E. at  $p < 0.05$ . ● = T.O control; ○ = T.O water deficit condition; ▼ = R.L control; ▲ = R.L water deficit condition

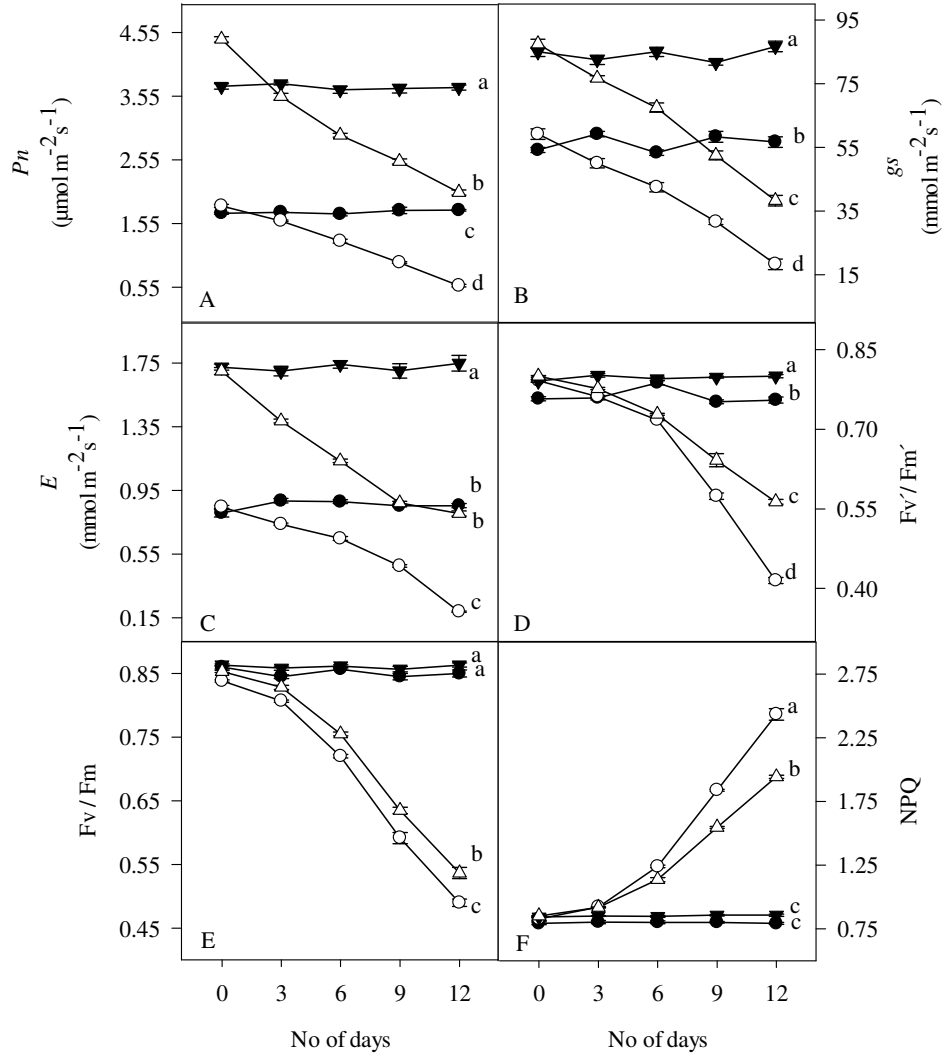
**Table 1.** Comparison between variables of trifoliolate orange and rough lemon under water deficit conditions

Variable	Increase/Decrease	Percentage	
		Trifoliolate orange	Rough lemon
Pot weight	Decrease	8.75	7.82
Leaf relative water content	Decrease	64.81	36.70
Leaf surface area	Decrease	18.18	4.92
Number of leaves	Increase	24.46	27.44
Photosynthesis	Decrease	68.43	54.10
Stomatal conductance	Decrease	69.01	56.19
Transpiration rate	Decrease	77.73	52.62
Chlorophyll fluorescence in light-acclimated leaves	Decrease	47.58	29.58
Chlorophyll fluorescence in dark-acclimated leaves	Decrease	41.55	37.11
Non-photochemical quenching	Increase	194.36	128.18
Superoxide dismutase in leaves	Increase	40.67	99.25
Superoxide dismutase in roots	Increase	29.18	48.91
Peroxidase in leaves	Increase	34.30	52.12
Peroxidase in roots	Increase	29.27	42.52
Catalase in leaves	Increase	30.50	48.73
Catalase in roots	Increase	49.94	96.94
Glutathione reductase in leaves	Increase	107.58	133.27
Glutathione reductase in roots	Increase	199.56	253.35
Ascorbate peroxidase in leaves	Increase	42.41	43.85
Ascorbate peroxidase in roots	Increase	45.47	71.15
Total soluble proteins in leaves	Increase	88.32	88.59
Total soluble proteins in roots	Increase	48.04	81.10
Proline in leaves	Increase	52.05	68.40
Proline in roots	Increase	64.83	83.44
Glycine betaine in leaves	Increase	42.85	63.92
Glycine betaine in roots	Increase	26.13	33.20
Chlorophyll 'a' in leaves	Decrease	57.13	44.06
Chlorophyll 'b' in leaves	Decrease	52.49	43.48
Hydrogen peroxide in leaves	Increase	77.65	36.34
Hydrogen peroxide in roots	Increase	101.34	62.87
Malondialdehyde in leaves	Increase	383.66	230.65
Malondialdehyde in roots	Increase	466.55	318.80
Total phenolic content in leaves	Increase	68.95	73.51
Total phenolic content in roots	Increase	58.49	73.62
Antioxidant capacity in leaves	Increase	217.26	247.93
Antioxidant capacity in roots	Increase	122.96	124.65

*Chlorophyll fluorescence and gas exchange parameters*

Water stress induced significant Pn, gs, and E reductions in both genotypes, but rough lemon always showed higher values than trifoliolate orange (2.3, 1.6, and 1.9 times, respectively) (Figure 2 and Table 1). As for gas exchange parameters, water stress also induced a decline in chlorophyll fluorescence parameters of light-

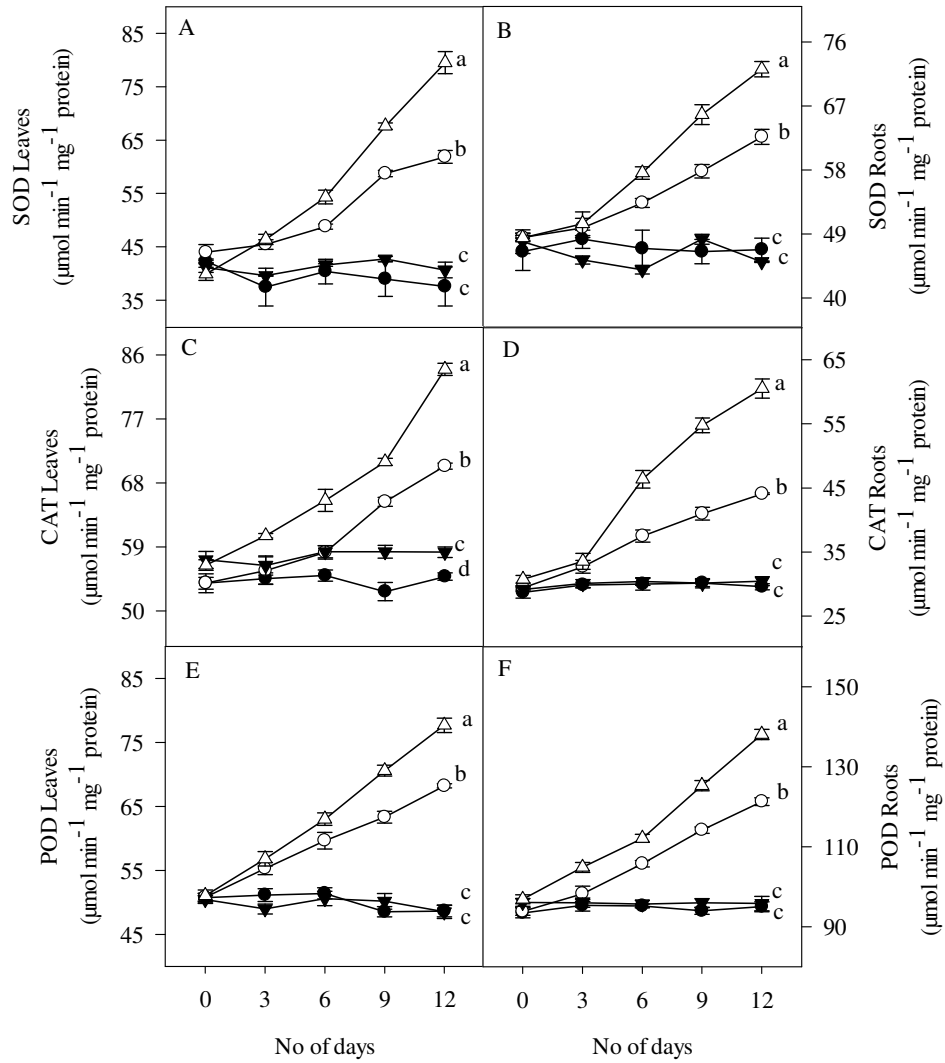
acclimated ( $F_v'/F_m'$ ) or dark-acclimated leaves ( $F_v/F_m$ ) in both rootstocks, but trifoliolate orange showed a more pronounced effect than rough lemon. Moreover, a parallel increase in NPQ was observed in both rootstocks subjected to water stress, significantly higher in trifoliolate orange than in rough lemon (Figure 2 and Table 1).



**Figure 2.** Gas exchange and chlorophyll fluorescence in leaves of trifoliolate orange (T.O) and rough lemon (R.L) under control and water deficit conditions (A)  $P_n$ ; (B)  $g_s$ ; (C)  $E$ ; (D)  $F_v'/F_m'$ ; (E)  $F_v/F_m$ ; (F) NPQ. Values are mean  $\pm$  S.E. at  $p < 0.05$ . ● = T.O control; ○ = T.O water deficit condition; ▼ = R.L control; ▲ = R.L water deficit condition.

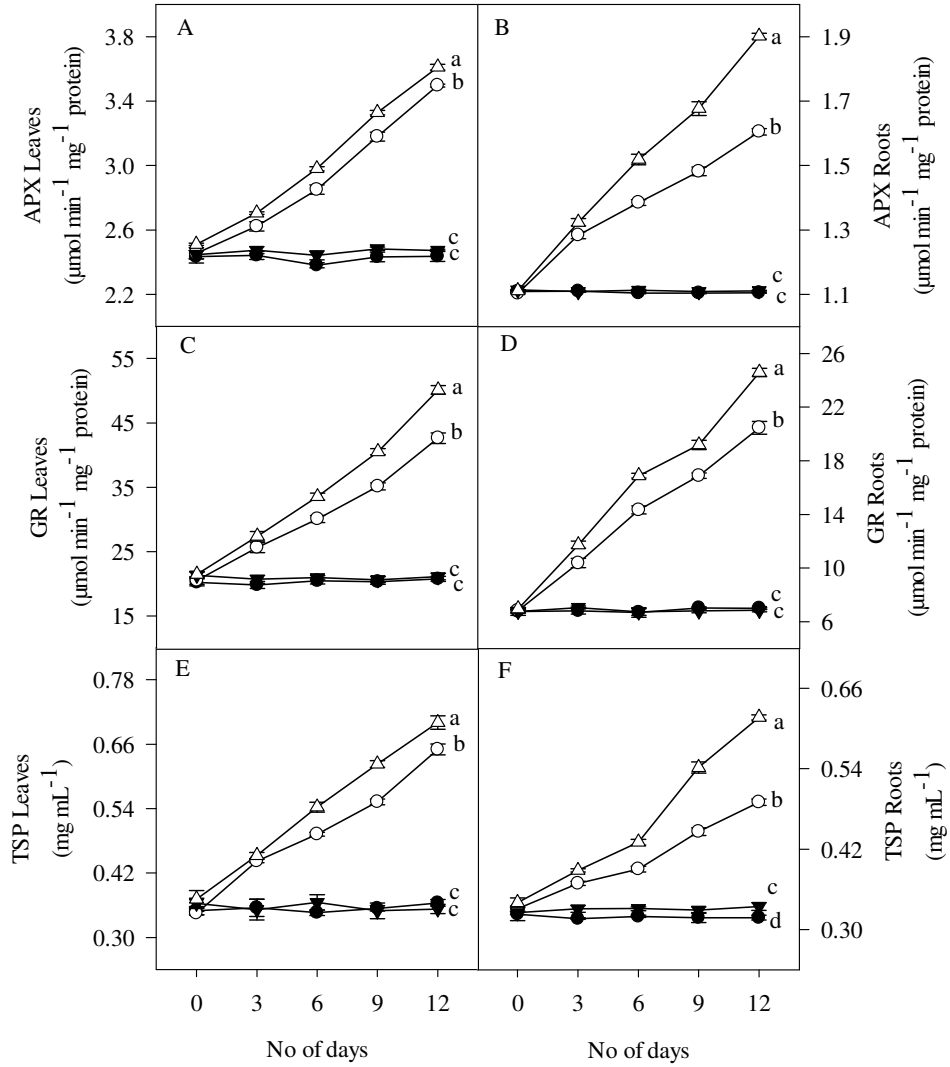
*Antioxidant enzymes activity and total soluble proteins*

Total soluble proteins and antioxidant enzyme activity (CAT, SOD, POD, and GR) values in leaves or roots of both rootstocks raised in response to water deficit conditions compared to control, but to a greater extent in rough lemon than in trifoliolate orange (Figures 3 and 4 and Table 1).



**Figure 3.** Antioxidant enzymes activity in leaves or roots of trifoliate orange (T.O) and rough lemon (R.L) under control and water deficit conditions (A) SOD-L; (B) SOD-R; (C) CAT-L; (D) CAT-R; (E) POD-L; (F) POD-R. Values are mean  $\pm$  S.E. at  $p < 0.05$ . ● = T.O control; ○ = T.O water deficit condition; ▼ = R.L control; △ = R.L water deficit condition.

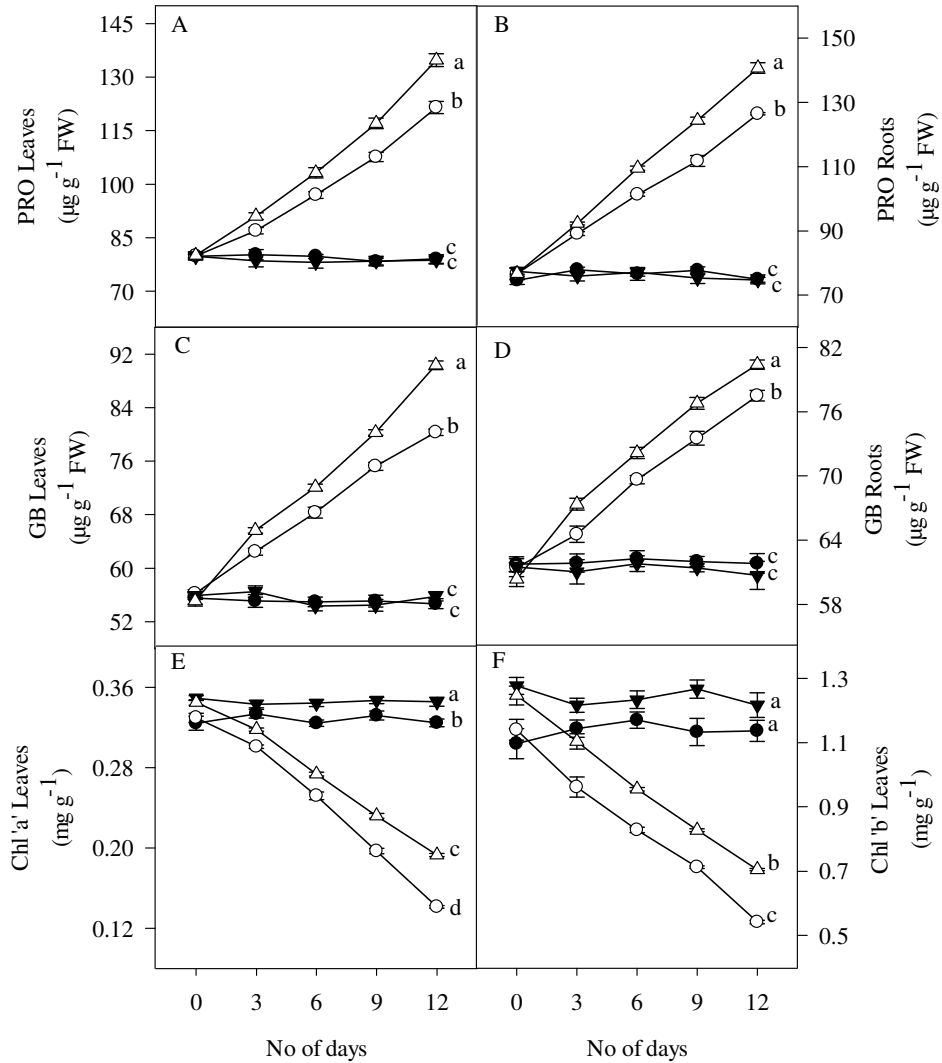
The differences in APX activity and TPS among stressed plants of the two genotypes were less pronounced in leaves than in roots, even though rough lemon always showed significantly higher values than trifoliate orange (Figure 4 and Table 1).



**Figure 4.** Antioxidant enzymes activity and total soluble protein in leaves or roots of trifoliate orange (T.O) and rough lemon (R.L) under control and water deficit condition (A) APX-L; (B) APX-R; (C) GR-L; (D) GR-R; (E) TSP-L; (F) TSP-R. Values are mean  $\pm$  S.E. at  $p < 0.05$ .  $\bullet$  = T.O control;  $\circ$  = T.O water deficit condition;  $\blacktriangledown$  = R.L control;  $\triangle$  = R.L water deficit condition.

*Osmoprotectants and chlorophyll 'a' and 'b'*

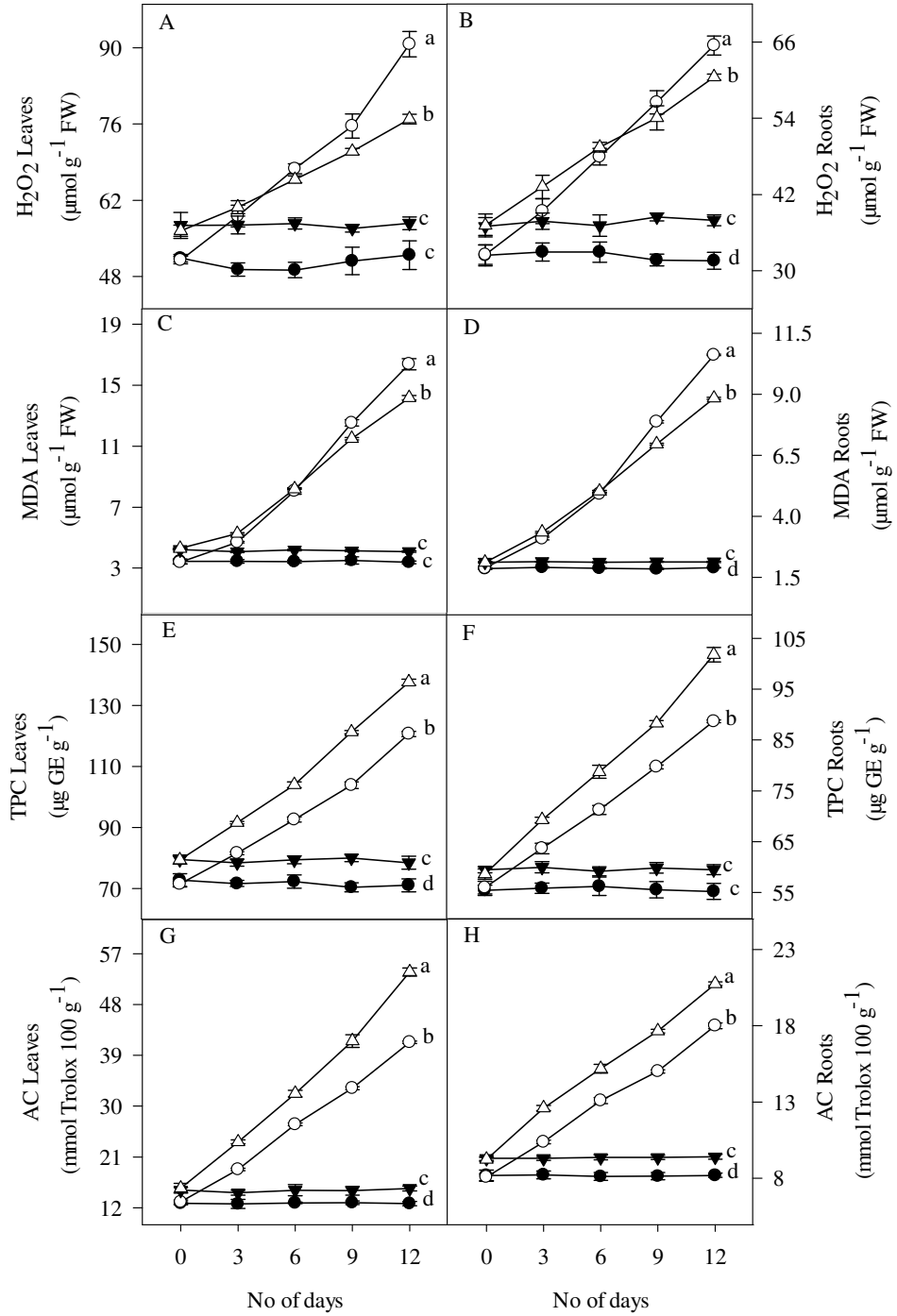
Water stress induced accumulation of osmoprotectant compounds (proline or glycine betaine) in tissues of both genotypes. However, this accumulation was more pronounced in rough lemon than in trifoliate orange (Figure 5 and Table 1). Moreover, both rootstocks showed a decline in leaf chlorophyll 'a' and 'b' levels in response to a water deficit that was more severe in trifoliate orange than in rough lemon (Figure 5 and Table 1).



**Figure 5.** Osmoprotectants in leaves and roots and chlorophyll 'a' and 'b' in leaves of trifoliate orange (T.O) and rough lemon (R.L) under control and water deficit condition (A) PRO-L; (B) PRO-R; (C) GB-L; (D) GB-R; (E) Chl 'a'-L; (F) Chl 'b'-L. Values are mean  $\pm$  S.E. at  $p < 0.05$ .  $\bullet$  = T.O control;  $\circ$  = T.O water deficit condition;  $\blacktriangledown$  = R.L control;  $\Delta$  = R.L water deficit condition.

*Hydrogen peroxide, malondialdehyde, total phenolic content and antioxidant capacity*

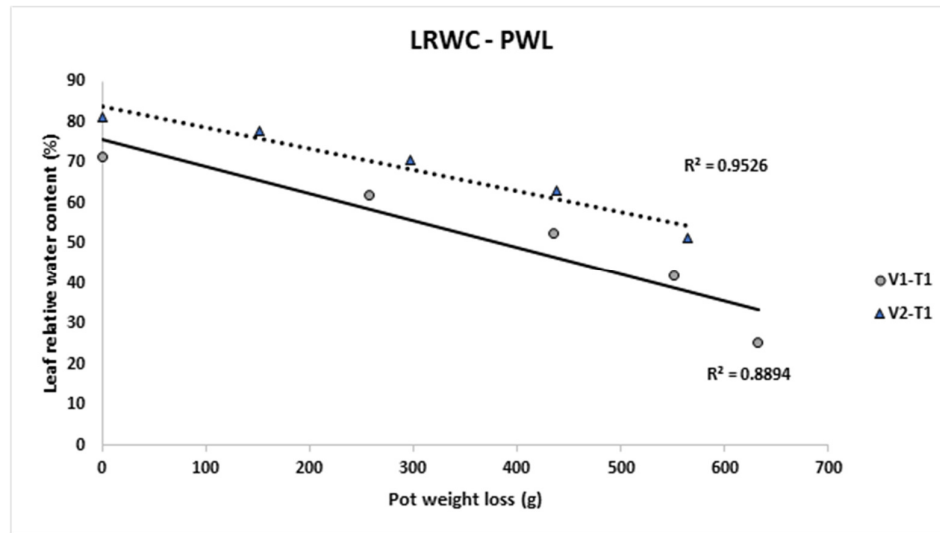
Water stress increased  $\text{H}_2\text{O}_2$  or MDA concentrations in leaves or roots of both genotypes, although higher values were found in trifoliate orange compared to rough lemon (Figure 6 and Table 1). Total phenolic content (TPC) or antioxidant capacity (AC) in both rootstocks increased in tissues of both genotypes in response to water stress, with rough lemon showing significantly higher levels (Figure 6 and Table 1).



**Figure 6.** Hydrogen peroxide, malondialdehyde, total phenolic contents, and antioxidant capacity in leaves or roots of trifoliate orange (T.O) and rough lemon (R.L) under control and water deficit condition (A) H<sub>2</sub>O<sub>2</sub>-L; (B) H<sub>2</sub>O<sub>2</sub>-R; (C) MDA-L; (D) MDA-R; (E) TPC-L; (F) TPC-R; (G) AC-L; (H) AC-R. Values are mean  $\pm$  S.E. at  $p < 0.05$ .  $\bullet$  = T.O control;  $\circ$  = T.O water deficit condition;  $\blacktriangledown$  = R.L control;  $\triangle$  = R.L water deficit condition.

*Relationship among variables*

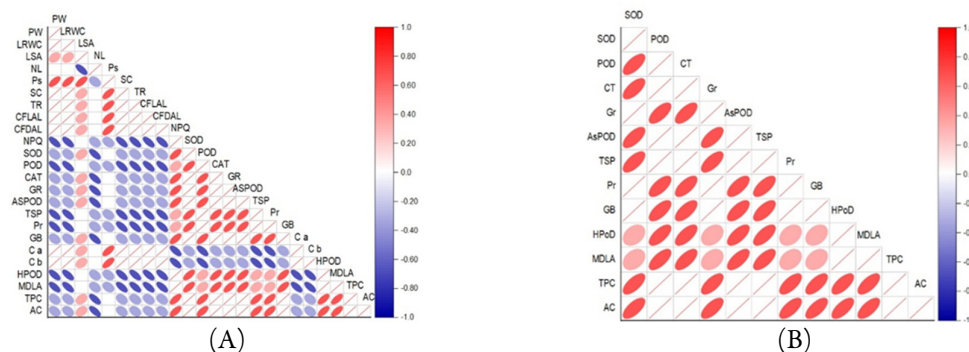
The regression of leaf relative water content (LRWC) on pot water loss (PWL) showed a significant linear correlation between the two parameters, with a more pronounced negative slope in trifoliolate orange (Figure 7).



**Figure 7.** Linear model regression of LRWC over Pot weight loss (PWL) in trifoliolate orange and rough lemon grown under water deficit conditions  
V1-T1 = trifoliolate orange under water deficit condition, V2-T1 = rough lemon under water deficit condition.

*Pearson's correlation analysis*

Figure 8 (A) and (B) shows the relationship among different parameters measured in leaves and roots. Pot weight, Photosynthetic rate, transpiration rate, stomatal conductance, leaf surface area, leaves relative water content, number of leaves were significantly and positively correlated with each other, while superoxide dismutase, peroxidase, catalase, ascorbate peroxidase, total soluble protein and total phenolic content, hydrogen peroxide and malondialdehyde were significantly and negatively correlated with photosynthetic rate, transpiration rate, stomatal conductance, leaf surface area, leaves relative water content and number of leaves. In roots all the parameters are positively correlate with each other (Figure 8).



**Figure 8.** Pearson's correlation matrix among different parameters of drought tolerant and drought sensitive citrus rootstocks under control and water deficit condition  
(A) = PCA for leaves parameters, (B) = PCA for roots parameters.

## Discussion

Water stress limits plant development and growth or lowering yield (Santos *et al.*, 2014; Nilsen *et al.*, 2014). Plants can cope with drought stress through physiological, biochemical, anatomical, or morphological modifications. In citrus, an essential aspect of orchard management is suitable rootstock selection. It is important to carefully evaluate all agronomic and physiological information available for each accession to attain this. This is done by considering the pedoclimatic conditions of the region where the orchard is. Therefore, we experimented with investigating effect of water deficit conditions on two different rootstock genotypes: trifoliolate orange or rough lemon, which are widespread in many citrus-producing areas and known to have distinct and divergent responses to water stress. Our objective was to decipher the physiological or biochemical determinants of drought sensitivity and tolerance in both genotypes at the seedling stage.

As expected, both rootstock genotypes exhibited divergent behavior under water deficit conditions, particularly when comparing water loss and LRWC depletion, which were more pronounced in trifoliolate orange (Figure 1), which exhibited evident symptoms of stress earlier than rough lemon, such as MDA and H<sub>2</sub>O<sub>2</sub> accumulation in plant tissues (Figure 6). These results are in line with previous work showing that drought stress tolerance in citrus, irrespective of the mechanism to maintain tissue water content, is related to a higher capacity for detoxification of ROS (Hussain *et al.*, 2018; El-Beltagi *et al.*, 2024). In this work, maintaining an adequate water balance was also associated with an improved ability to reduce ROS production. In citrus and other plant species, the abiotic stress pressure is related to oxidative stress (Merah, 2001; Zandalinas *et al.*, 2017). In our study, both rootstock genotypes exhibited a reduction in LRWC when subjected to water deficit conditions. However, trifoliolate orange exhibited higher reduction in this parameter than rough lemon (Figure 1), indicating a more reduced capacity to maintain water balance in this genotype.

Similarly, LSA was also evident in trifoliolate orange due to its inability to maintain proper water content. These differences were also probably associated with an arrest in new leaf growth and an increase in leaf drop due to water stress. In line with its higher tolerance, variations in leaf number were less affected in rough lemon, likely due to a reduced leaf drop (Figure 1 and Table 1).

Consistent with the hypothesized lower tolerance of trifoliolate orange to water stress conditions, gas exchange parameters were also more severely affected in trifoliolate orange than in rough lemon (Figure 2). In response to salinity, gas exchange parameters also correlated with citrus stress tolerance (López-Climent *et al.*, 2008). In this respect, all measured gas exchange parameters were reduced in parallel to water depletion in the soil. This indicates that this was probably the main driver in their regulation through the modulation of abscisic acid, as mentioned in citrus under various stress conditions (Arbona *et al.*, 2017). It is generally accepted that stomatal closure, which seeks to minimize water loss, is the primary physiological reaction to water deficit (Chaves *et al.*, 2009), potentially contributing to reduction in Pn due to the restriction of CO<sub>2</sub> entry into leaf mesophyll (Osório *et al.*, 2011). However, combining gas exchange and chlorophyll fluorescence parameters indicated that g<sub>s</sub> was not the only contributor to Pn decay. Other biochemical factors are involved, as observed in citrus subjected to salinity (López-Climent *et al.*, 2008; El-Beltagi *et al.*, 2022c). Examining impact of fast and slow water deficit on diploid or tetraploid Volkamer lemon, Khalid *et al.* (2021) obtained similar results. In this work, trifoliolate orange showed more significant water loss per plant than rough lemon (Figure 9), indicating higher whole-plant transpiration under water deficit conditions than rough lemon. Under water deficit conditions, Fv/Fm and Fv'/Fm' decreased in both rootstocks, indicating that the stress conditions imposed were damaging to PSII performance and photochemistry. Decrease in Fv/Fm and Fv'/Fm' could be attributed to photoinhibition damage caused by water stress and a reduction in photosynthetic electron flow downstream PSII (Bjorkman and Demming, 1987).

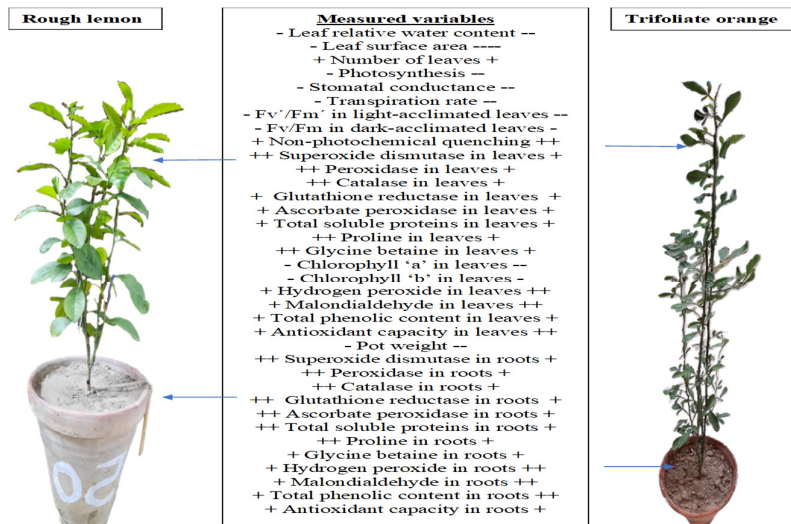
Nevertheless, trifoliolate orange showed a more significant decline in both parameters than rough lemon (Figure 2), indicating it is more stressed. This is consistent with the observed parallel increases in NPQ as an excess energy dissipation mechanism to prevent further photosystem damage (dos Santos *et al.*, 2019). Hence,

as expected, an increase in NPQ was observed in both rootstocks subjected to water stress that was significantly higher in trifoliate orange than rough lemon (Asada, 1999; Khoshbakht *et al.*, 2018).

During photosynthesis and respiration, plant cells experience oxidative stress due to increased flow of electrons toward O<sub>2</sub>, which enhances ROS generation (Asada, 1999). During abiotic stresses, ROS or oxidative damage at cellular level are the leading causes of chlorophyll degradation (Smirnoff, 1993). Under water deficit conditions, both rootstocks showed a decline in chlorophyll 'a' or 'b' in leaves, which was more severe in trifoliate orange than in rough lemon (Figure 5). Some ROS, such as superoxide and H<sub>2</sub>O<sub>2</sub>, damage proteins, lipids, or DNA, resulting in plant cell death (Mittler, 2002; Simova-Stoilova *et al.*, 2008).

In our experiment, the increase in H<sub>2</sub>O<sub>2</sub> was faster and more pronounced in trifoliate orange than in rough lemon (Figure 6), in line with observed reduction in gas exchange and chlorophyll fluorescence parameters. Plant antioxidant defense system regulates ROS generation and consumption to ensure optimal plant growth. As observed previously, SOD, CAT, and POD are essential antioxidants in citrus and other plant species' ROS defense (Noctor and Foyer, 1998; Simova-Stoilova *et al.*, 2008). Plants with higher antioxidant enzyme activities and osmoprotectant concentrations are generally more resistant to adverse conditions, such as water stress (Demiral and Türkan, 2005; Oustric *et al.*, 2019). Plants of rough lemon subjected to water stress exhibited more excellent ROS scavenging enzyme activities and increased amounts of PRO and GB than trifoliate orange. This indicates that these could be critical factors in defining its higher stress tolerance (Figure 3-5). Previous studies have shown that citrus genotypes that are tolerant to different abiotic stress conditions are more likely to have higher antioxidant activity (enzymes and antioxidant metabolites), as well as a higher accumulation of osmoprotectants than sensitive ones (Demiral and Türkan, 2005; Oustric *et al.*, 2019; Zandalinas *et al.*, 2017;). Similarly, AC and TPC increased significantly in rough lemon than in trifoliate orange, in line with the observed antioxidant enzyme activity and compatible osmolyte concentration (Figure 6).

The evaluation of PWL and LRWC in both genotypes showed that trifoliate orange and rough lemon operate through different mechanisms when subjected to water shortage. Rough lemon retains higher LRWC despite a smaller PWL, preserving pot water content. As a result, transpiration is reduced by rapidly closing stomata, resulting in much lower E and g<sub>s</sub> in trifoliate orange than in rough lemon (Figure 9).



**Figure 9.** Comparison between variables of trifoliate orange and rough lemon in leaves or roots under water deficit conditions. For numerical values, please check Table 1

## Conclusions

*Poncirus* seedlings showed a faster and greater pot soil water content depletion than rough lemon in our experimental conditions. This might be the factor behind this genotype's earlier development of water stress symptoms. Therefore, reducing water loss through transpiration and maintaining tissue water content seems to be an advantage of rough lemon over trifoliolate orange. Deep-rooted genotypes, such as rough lemon, are usually less affected by soil drying and are more tolerant to water stress. In contrast, *Poncirus* genotypes, which typically have a shallow root system, are less efficient at exploring the soil searching for water and hence more sensitive to variations in soil water availability than other genotypes, such as rough lemon. Besides, rough lemon also possesses biochemical machinery that is better prepared to protect cells from damaging effects of stress, such as an enhanced antioxidant system and osmoprotectant metabolite buildup.

## Authors' Contributions

Conceptualization: MS, HSE-B, MFK; Methodology: MS, SH, AH; Software: EA, SA, MNS; Validation: VA, HSE-B; Investigation: MS, HSE-B, SH, MNS; Resources: MFK, SH, AH; Data curation: MS, SH; Writing original draft preparation: MS, SH, MNS; Writing—review and editing: MS; Funding acquisition: HSE-B. All authors read and approved final manuscript.

## Ethical approval (for researches involving animals or humans)

Not applicable.

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## Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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