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EFFECT OF WATER DEFICIT STRESS ON ELECTROLYTE LEAKAGE AND MINERAL CONTENTS IN CITRUS ROOTSTOCKS

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Abstract

This study evaluated the physiological performance, electrolyte leakage, and mineral content of trifoliate orange (*Poncirus trifoliata*) and rough lemon (*Citrus jambhiri*) one-year-old seedlings under control and water- deficient conditions for 12 days. The experiment was conducted using a completely randomized design with two treatments and three replications. Under water deficit stress, sodium content in leaves increased by 39.42% in rough lemon compared to 35.81% in trifoliate orange leaves. Conversely, trifoliate orange roots reduced sodium levels more than rough lemon (27.27% vs. 23.03%). Leaves and roots of both rootstocks lost chloride content under water deficit stress, with rough lemon showing a stronger decrease than trifoliate orange (9.97% vs. 8.85% in leaves and 8.03% vs. 6.78% in roots). Calcium content in both rootstocks' leaves and roots increased under water deficit stress. In leaves, the increase was greater in trifoliate orange (11.58%) compared to rough lemon (10.59%), while in roots, rough lemon had a larger increase than trifoliate orange (22.89% vs. 21.39%).

Under drought stress, trifoliate orange exhibited lower nitrogen, phosphorus, and potassium content in leaves and roots compared to rough lemon, except for potassium content in roots (51.29% vs. 50.60%, 27.28% vs. 25.64%, 47.55% vs. 44.08%, and 33.63% vs. 33.42%). Under water deficit stress, trifoliate orange leaves and roots leaked more electrolytes than rough lemon leaves and roots (72.94% vs. 53.42% and 54.90% vs. 34.07%). On day 12 of the study, both rootstocks had a higher plant height, root length, fresh weight, dry weight, and diameter than on day zero, regardless of water treatment. However, rough lemon increased more than trifoliate orange, except for plant dry weight (18.49% vs. 14.32% for plant height, 9.04% vs. 8.03% for root length, 14.05% vs. 12.35% for plant fresh weight, 16.34% vs. 17.59% for plant dry weight, and 16.28% vs. 11.42% for plant diameter). Water deficit decreased leaf greenness in both rootstocks, but trifoliate orange decreased more than rough lemon (27.77% vs. 50.16%). Our results indicate that rough lemon plants cope better with short-term water deficit by maintaining growth and mineral balance.

Key words: Citrus rootstocks; Water deficit; Mineral content; Electrolyte leakage; Plant growth

Introduction

Drought is widely considered the most critical environmental condition that inhibits plant growth, reproductive development, and survival. These aspects of stress are related to water supply constraint, which is regarded as the complete cessation of water delivery and a persistent deficit in water throughout the stages of growth, reproduction, or development (Flexas & Medrano, 2002; Martínez-Cuenca *et al*., 2016).

Normally, plants sense water stress in two ways: either because the water supply to their roots has become limited or because the transpiration rate has exceeded the absorption rate. When a plant is subjected to water stress, its water potential and cell wall pressure decrease to the point where it has difficulties carrying out its usual physiological processes (Kumar *et al*., 2019). In addition, plants will have a low chance of surviving, developing, and growing if they are subjected to prolonged drought stress and a chronic lack of water. The absence of water in the soil is the most common cause of drought, although excessive evapotranspiration can also contribute to the severity of the problem (Lipiec *et al*., 2013). This stress is caused by a mismatch between the amount of water taken in from the soil and the amount of water lost by evapotranspiration flow (Younis *et al*., 2020). It is essential to consider water stress when planning irrigation in arid and semi-arid regions. Water deficit might substantially impact crops' yield response during certain phenological phases. In perennial fruit crops, the presence of water stress before, during, or after the flowering and post-bloom periods can harm yields by causing a decrease in the total number of fruits produced as well as a reduction in cell number and size responsible for fruit growth (García-Tejero *et al*., 2010). In most cases, a late- season deficit will significantly affect final fruit size or quality more than total yield (Kumar *et al*., 2019). Additionally, drought stress affects plants' mineral nutrition, leading to secondary detrimental effects to fitness. For instance, drought stress reduces the translocation of minerals and nutrients from the roots to the shoots by decreasing transpiration rates and altering the function of membrane transporters. Among the mineral nutrients, macronutrients

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are essential structural components of plants, and their deficiency-induced sensitivity in plants can be readily observed and targeted for corrective measures. In contrast, micronutrients may directly or indirectly affect plant susceptibility to stress factors by altering enzyme activity, modulating signal transduction pathways, and producing specific metabolites (Hajiboland, 2012).

Mineral nutrients are required for proper plant growth and development, usually absorbed from soils as inorganic ions. Soil water status affects nutrient availability by influencing nutrient movement within the soil. When the soil dries, the reduction in water content is accompanied by an increase in mechanical impedance, adversely affecting potassium (K^+) mobility in the soil and K^+ uptake by the plant (Dogan & Akinci, 2011). In turn, nutrient deficiency can affect water uptake and induce rapid changes in root hydraulic conductivity by altering cell membrane permeability and regulating aquaporin activity (Maurel *et al*., 2008).

Under drought conditions, nutrient uptake is impaired due to reduced soil moisture, leading to slower diffusion of mineral nutrients from the soil to the root surface; hence, translocation to the leaves is also reduced. Drought induces early stomatal closure, thereby reducing transpiration and limiting nutrient transport from the root to the shoot. Thus, drought stress reduces the availability and transport of nutrients in the soil matrix and plant tissues (Silva *et al*., 2011). Despite various reports available on the effects of nutrient supply on plant growth under drought conditions, it is generally accepted that increasing nutrient supply will not improve plant growth if adequate nutrient content already exists in the soil (Hu and Schmidhalter, 2005). The relationship between water status and the acquisition and distribution of mineral nutrients within the plant is mutual, simply because the same organ (i.e., roots) acquires both resources. For example, it was previously reported that nitrogen uptake decreased in soybeans (*Glycine max* L.) under water-stress conditions (García-Sánchez *et al*., 2007); and in the case of cotton (*Gossypium hirsutum* L.), nitrogen deficiency aggravated sensitivity to water stress (Singh & Gupta, 1993).

Plants' major solutes for osmoregulation are K^+, Cl^-, and malate, which induce water flow through the membrane matrix, particularly the aquaporins (Moshelion *et al*., 2002). Potassium-sufficient plants' improved drought resistance (Umar, 2002) and photosynthetic efficiency (Sen Gupta *et al*., 1989) can be related to several causes, including oxidative stress avoidance, stomatal regulation, and high vacuolar osmotic potential maintenance. Nevertheless, Na^+ can replace K^+ as a cheap osmoticum in the generation of turgor and cell expansion and may even surpass K^+ in this respect because it accumulates preferentially in the vacuoles (Broadley *et al*., 2012). Under certain conditions, Na^+ can partially substitute for K^+ in osmotic adjustment and may contribute to maintenance of plant water status during drought, but K^+ remains the primary ion regulating stomatal movements and water-use efficiency.

Under biotic and abiotic stress, plants activate a wide range of stress-responsive mechanisms to reestablish homeostasis, protect and repair damaged proteins or membranes, and enhance tolerance to such stress (Vinocur & Altman, 2005). Stress tolerance involves maintaining membrane integrity under osmotic stress (Kirch *et al*., 2004). Measuring solute leakage from plant tissue is a long-standing method for estimating membrane permeability and the degree of damage related to different stresses. Estimating the number of ions a plant sample releases is called electrolyte leakage (Whitlow *et al*., 1992).

The ability of citrus to cope with the negative impact of water deprivation is also related to the genotype of the plant, resulting in the following order of drought resistance: high resistance: mandarins > rangpur lime > rough lemon > sour orange > Citrus macrophylla; medium resistance: lemon > trifoliate orange > citrange hybrid > Citrus chuana; poor tolerance: sweet orange > Citrus verrucose > grapefruit (Rodríguez-Gamir *et al*., 2011; Zaher-Ara *et al*., 2016).

Using different rootstocks with varying levels of drought tolerance will help elucidate the mechanisms underlying that rootstock's drought tolerance. Drought tolerance of different rootstocks has been previously evaluated by studying the physiological and biochemical processes rootstocks undergo at different levels of water stress. The role of mineral nutrients in increasing or decreasing drought stress tolerance of plants has been studied by several researchers; however, it is still inadequate and somewhat elusive (Santana-Vieira *et al*., 2016; Silva *et al*., 2023; Morade *et al*., 2025). Increasing our understanding of the roles of nutritional networks under drought stress is important because it will support the refinement and adoption of best management practices to reduce the damage caused by drought and subsequent nutrient deficiency (Marschner, 2012; Ramegowda & Senthil-Kumar, 2015).

Therefore, the objective of this study was to evaluate the water stress tolerance of two different citrus rootstocks with varying drought tolerance levels by measuring mineral contents, physical growth, and electrolyte leakage in their leaves and roots. As mentioned above, rough lemon has superior drought resistance compared to trifoliate orange because of its deep root system, which enables plants to absorb water and nutrients from the soil. We hypothesized that the rootstocks used in this study would perform differently regarding electrolyte leakage and mineral uptake from the soil under water deficit stress. Also, rough lemon benefits from a deep root system, enabling the plants to absorb water and nutrients more effectively from the soil.

Materials and Methods

Plant material: The present study was conducted in the greenhouse of the Department of Horticulture, Bahauddin Zakariya University, Multan, at approximately 30.1999° N and 71.4783° E. Rough lemon and trifoliate orange seeds were obtained from healthy, disease-free fruits at the Citrus Research Institute, Sargodha. Before sowing, the seeds were treated with copper-based fungicide (1% solution for 1 hour). These seeds were grown in 2.5 x 2.5-inch plastic containers under greenhouse conditions. After two months of germination, seedlings of both rootstocks were transplanted into 12-inch earthen pots. The plants were kept in the greenhouse for the next ten months. A half-strength

Hoagland solution was used to optimize seedling growth, and seedlings were maintained at field capacity (50% of soil saturation). The solution was added to the pots when needed. All the plants were treated on the same grounds.

Morphologically identical and vigorous seedlings of one year of age were used to assess the impacts of water deficit stress on electrolyte leakage and mineral contents, e.g., nitrogen, phosphorus, potassium, calcium, sodium, and chloride in citrus rootstocks. From each rootstock, 30 seedlings were selected and divided into groups (15 for control and 15 for water deficit stress for a total of 60 experimental units (n=60). During the experiment, control seedlings were maintained at field capacity with Hoagland nutrient solution, and water deficit stress was applied by withholding irrigation. Day and night mean temperatures were 27°C and 14°C, respectively. Relative humidity was maintained between 45% and 75%. The experiment lasted 12 days until wilting symptoms appeared in the plants. The experiment consisted of two treatments, each with three replications and five intervals. Five seedlings were used as experimental units in each replication, and data were collected every three days starting from day zero. One seedling from each experimental unit was harvested at intervals (0, 3, 6, 9, and 12 days) to estimate mineral contents and other physical parameters.

Mineral contents: At each interval, freshly harvested leaves (at the middle of the stem) and roots (primary and secondary) of both rootstocks were oven-dried for two hours at 70ºC to measure the different mineral contents. For calcium (Ca), phosphorus (P), sodium (Na^+), nitrogen (N), and potassium (K^+), wet digestion was performed; for chloride (Cl) analysis, dry digestion was used. For wet digestion, 0.1 g of plant dry samples were digested in 2 mL sulfuric acid at 300ºC for 1 hour. After cooling the samples at room temperature, 0.2 mL H_2O_2 was added to stabilize the reaction. This process was repeated until the samples were colorless in appearance. Total N was measured using the method described by Martin *et al*., (1983) (colorimetric method). P in plants was estimated using a malachite green method, as Ohno & Zibiliski (1991) reported. Na^+, Ca, and K^+ were measured using a flame photometer (PFP7, Jenway UK) according to the method of Ryan *et al.*, (2001). For dry digestion, 0.1 g samples were ashed in a muffle furnace at 400ºC for 1 hour, and Cl analysis was carried out by chloride electrode (Orion 9617BNWP, Thermo Fisher Scientific).

Electrolyte leakage: Electrolyte leakage in the leaves of both rootstocks treated was measured using an E.C. electrode (HI763100, Hanna, U.S.A.) according to the method reported by Yahmed *et al*., (2016). After harvesting, leaves of both rootstocks were placed on ice until the treatment started (<1h). Two leaf discs of 100mg weight were cut from each experimental unit. These discs were plunged into 2 mL of a 300 mM NaCl solution at an osmotic pressure of -1.38 MPa and -0.92 MPa for 20 h at ambient temperature (26°C). Both discs were taken from the same leaf. After the stress period, the discs were rinsed with deionized water and transferred into new tubes containing 20 mL of deionized water. Using a conductivity meter, the first conductivity measurement was taken immediately after transferring the discs to the tubes (T0). After 1 hour (T1), the solution was homogenized rapidly with a vortex, and the conductivity was measured again. Finally, the tubes were autoclaved for 20 minutes at 121°C; the last measurement was performed after cooling (25°C) and homogenization. This measure corresponds to the maximum ion release (Tf). The release of ions E was calculated using the following equation:

$$\mathrm{E} = \frac{\left(\frac{\mathrm{T1} - \mathrm{T0}}{\mathrm{Tf}}\right) \times 100}{\mathrm{p}}$$

This equation provides the percentage of ions released in the first hour after rehydration based on the total ions released per disc's weight (p). The same protocol was used to measure electrolyte leakage in roots using 100mg of dry root tissue.

Plant physical parameters: At each interval, one plant from each experimental unit was harvested to measure its fresh weight using a digital scale. The same plants used to calculate the fresh weight were used to measure dry weight. Plants were oven-dried at 70ºC for two days until constant weight was achieved.

For plant height measurement, one plant from each experimental unit was tagged at the start of the experiment. A mark was placed at the base of the plant stem for plant height and diameter. Plant height was taken from the mark to the top of the leaf. Plants used for fresh weight measurement were also used to quantify root length. Root length was measured in centimeters by using a measuring tape. The same plants used to measure the plant's fresh weight were also used to measure the plant's diameter. Plant diameter was measured using a Vernier Caliper in millimeters. At intervals, leaf greenness index was measured using a chlorophyll meter (SPAD 502, Konica Minolta, Japan) from previously-tagged plants in each experimental unit.

Statistical analysis

The experiment was arranged under a completely randomized design. The data were statistically analyzed using standard techniques in Statistix 8.1. Mean values for different parameters of both rootstocks under control and water deficit conditions are presented in Table 2. SigmaPlot software was used to create figures. For a more insightful comparison between treatments (i.e., water level and rootstocks), the increase and decrease in percentage of different parameters 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

Mineral contents in citrus rootstocks under water deficit conditions: Regarding the sodium contents in leaves (mg g^{-1}), both rootstocks showed a significant increase of 39.42% in rough lemon and 35.81% in trifoliate orange under water deficit conditions. In contrast, under control conditions, no significant change was observed in either rootstock from day zero to day twelve. On the other hand, sodium content in roots decreased under water deficit; however, this decrease rate was lower in rough lemons than in trifoliate oranges (23.03% vs 27.27%) (Fig. 1A and B, Table 1).

Chloride content (mg g^{-1}) in the leaves and roots of both rootstocks decreased under the water deficit treatment. However, the decrease was higher in rough lemon than in trifoliate orange (9.97% vs 8.85% in leaves and 8.03% vs 6.78% in roots). In the control treatment, chloride content remained approximately constant in both rootstocks from day zero to day 12, at study termination (Fig. 1C and D, Table 1).

Calcium content (mg g-1) in leaves under control conditions remained the same and constant in both rootstocks for the duration of the study. In water deficit conditions, with respect to percent increase and decrease, an increase was observed, with a greater increase in trifoliate orange than rough lemon (11.58% vs 10.59%) compared with the control treatment for both rootstocks. Similarly, for root calcium content, a significant increase was observed in both rootstocks under water-deficit conditions, with a greater increase in rough lemon (22.89%) than in trifoliate orange (21.39%) (Fig. 1E and F, Table 1).

Regarding the leaf's nitrogen content, a significant decrease was observed in both rootstocks under water deficit conditions, with a slightly greater reduction of trifoliate orange (51.29%) than rough lemon (50.60%), while under control conditions, the nitrogen content in both rootstocks remained constant during all sampling intervals, with different values that were statistically different at the end of the study. A similar trend of 27.28% in trifoliate orange and 25.64% in rough lemon was observed for root nitrogen content in both rootstocks (Fig. 2A and B, Table 1).

Phosphorus content in leaves for both rootstocks remained constant in all intervals under control conditions (Fig. 2C). In contrast, under water deficit conditions, a significant decrease was observed in both rootstocks, with a greater reduction of trifoliate orange than rough lemon (47.55% vs 44.08%). Roots' phosphorus contents under water deficit conditions also decreased in both rootstocks, with a little bit more decrease in trifoliate orange than rough lemon (33.63% vs 33.42%), while under control conditions, no changes were observed in both rootstocks through sampling intervals, but final phosphorus content differed between rootstocks (Fig. 2D, Table 1).

Regarding the leaf's potassium content, a significant decrease was observed in both rootstocks under water-deficit conditions, with a greater reduction in trifoliate orange than in rough lemon (20.15% vs 18.76%), whereas under control conditions, the potassium content in both rootstocks remained unchanged across all intervals. Similarly, a decrease in root potassium content was observed with a slightly more significant reduction in rough lemon than in trifoliate orange (10.01% vs 11.13%) compared to their control values (Fig. 2E and F, Table 1).

Electrolyte leakage and plant physical parameters: In leaves, a significant increase was observed in electrolyte leakage under water deficit conditions, with a greater increase in trifoliate orange (72.94%) than rough lemon (53.42%) compared to their counterparts in the control treatment. Under control conditions, electrolyte leakage in the leaves of both rootstocks was approximately constant throughout the experiment. A similar trend for electrolyte leakage in roots (54.90% increase in trifoliate orange vs. 34.07% increase in rough lemon) was observed in both rootstocks under control and water deficit conditions, as observed in leaves (Fig. 3, Table 1).

Regarding the plant height of both rootstocks, an increase was observed under the control treatment, with trifoliate orange growing taller regardless of water treatment. Under water deficit conditions, plant height was greater for rough lemon than for trifoliate orange during the study duration (18.49% vs 14.32%, Fig. 3C). Similarly, root length was greater in rough lemon under water-deficit conditions than in trifoliate orange (9.04% vs 8.03%). On the other hand, under control conditions, root length was slightly higher in trifoliate orange than in rough lemon but statistically similar (Fig. 3D, Table 1).

Regarding the plant's fresh weight, a significant increase was observed in both rootstocks under control conditions (i.e., well-watered), with a greater increase in rough lemon than in trifoliate orange, while under water deficit conditions, the increase in plant fresh weight was less compared to the control treatment. However, the increase was higher for rough lemon (14.05%) than for trifoliate orange (12.35%). In the trifoliate orange, the weight was increased till the 9th day; after that, it decreased. On the other hand, a slow but continuous gain in fresh weight was observed in rough lemon throughout the experiment. A similar trend was observed in the dry weight of both rootstocks under control conditions. In contrast, under water deficit conditions, the percent increase in dry weight was higher in trifoliate orange (17.59%) than in rough lemon (16.34%) (Fig. 3F, Table 1).

An increase in plant diameter in both rootstocks was observed under control and water-deficient conditions. However, the increase in plant diameter of both rootstocks was higher under control conditions with respect to water deficit conditions. Under water deficit treatment, the increase was more in rough lemon (16.28%) than in trifoliate orange (11.42%) from the start to the end of the trial (Fig. 4A, Table 1).

Both rootstocks showed different leaf greenness (SPAD) values. Under control conditions, leaf greenness exhibited the same constant pattern throughout the experiment for both rootstocks. However, under water deficit conditions, a significant decrease in SPAD was observed in both rootstocks, with a greater reduction for the trifoliate orange than the rough lemon, starting on day 3 of the experiment. The percent decrease in leaf greenness in trifoliate orange was double (50.15%) that of rough lemon (27.76%) (Fig. 4B, Table 1).

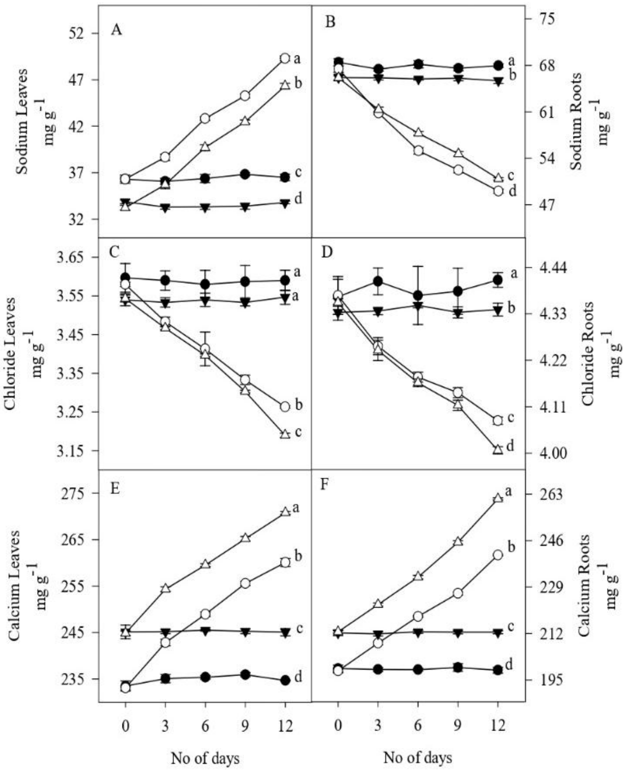

Fig. 1. Mineral content in leaves and roots of trifoliate orange (T.O.) and rough lemon (R.L.) under control and water deficit conditions. (A) Sodium content in leaves; (B) Sodium content in roots; (C) Chloride content in leaves; (D) Chloride content in roots; (E) Calcium content in leaves; (F) Calcium content in roots. Values are mean ± S.E. at $p<0.05$. ● = T.O control; O = T.O water deficit condition; ▼ = R.L control; Δ = R.L water deficit condition. Different lowercase letters indicate significant differences among treatments within plant organs on day 12 of the experiment.

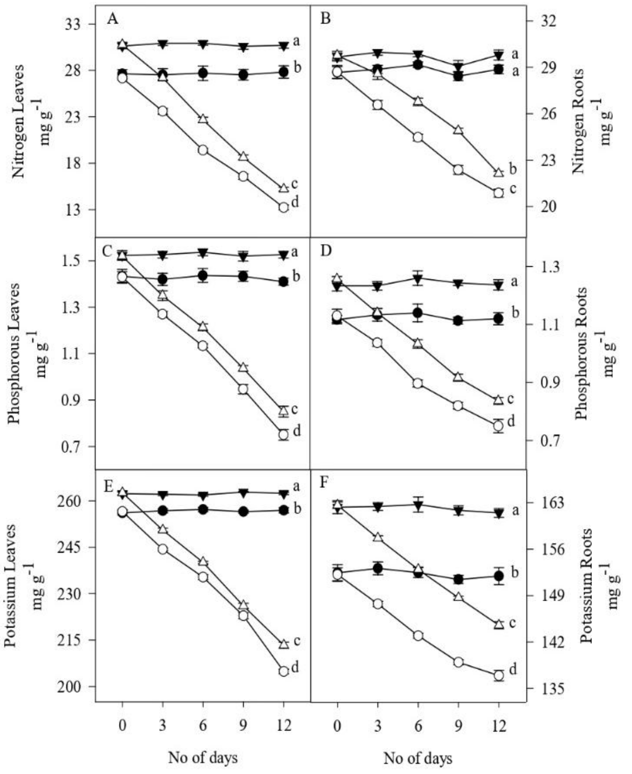

Fig. 2. Mineral content in leaves and roots of trifoliate orange (T.O.) and rough lemon (R.L.) under control and water deficit conditions. (A) Nitrogen content in leaves; (B) Nitrogen content in roots; (C) Phosphorous content in leaves; (D) Phosphorous content in roots; (E) Potassium content in leaves; (F) Potassium content in roots. Values are mean ± S.E. at $p<0.05$. ● = T.O control; O = T.O water deficit condition; ▼ = R.L control; Δ = R.L water deficit condition.

Table 1. Comparison between trifoliate orange and rough lemon regarding their measured variables under water deficit conditions on the basis of percent increase and decrease.

Variables	Increase or decrease	Values in percentage for	
		Trifoliate orange	Rough lemon
Sodium contents in leaves	Increase	35.81	39.42
Sodium contents in roots	Decrease	27.27	23.03
Chloride contents in leaves	Decrease	8.85	9.97
Chloride contents in roots	Decrease	6.78	8.03
Calcium contents in leaves	Increase	11.58	10.59
Calcium contents in roots	Increase	21.39	22.89
Nitrogen contents in leaves	Decrease	51.29	50.60
Nitrogen contents in roots	Decrease	27.28	25.64
Phosphorous contents in leaves	Decrease	47.55	44.08
Phosphorous contents in roots	Decrease	33.63	33.42
Potassium contents in leaves	Decrease	20.15	18.76
Potassium contents in roots	Decrease	10.01	11.13
Electrolyte leakage in leaves	Increase	72.94	53.42
Electrolyte leakage in roots	Increase	54.90	34.07
Plant height	Increase	14.32	18.49
Root length	Increase	8.03	9.04
Plant fresh weight	Increase	12.35	14.05
Plant dry weight	Increase	17.59	16.34
Plant diameter	Increase	11.42	16.28
SPAD	Decrease	50.16	27.77

Table 2. Mean values for different parameters of trifoliate orange and rough lemon under control and water deficit conditions.

Parameters	**Control**		**Water deficit**	
	Trifoliate orange	**Rough lemon**	**Trifoliate orange**	**Rough lemon**
Sodium contents in leaves	36.500c	33.800d	49.300a	46.333b
Sodium contents in roots	67.967a	65.700b	49.067d	50.900c
Chloride contents in leaves	3.5900a	3.5467a	3.2633b	3.1900c
Chloride contents in roots	4.4100a	4.3400b	4.0767c	4.0067d
Calcium contents in leaves	234.67d	245.07c	260.07b	270.77a
Calcium contents in roots	198.57d	212.47c	240.71b	261.27a
Nitrogen contents in leaves	27.833b	30.700a	13.233d	15.200c
Nitrogen contents in roots	28.867a	29.800a	20.867c	22.133b
Phosphorous contents in leaves	1.4100b	1.5267a	0.7500d	0.8500c
Phosphorous contents in roots	1.1200b	1.2367a	0.7500d	0.8367c
Potassium contents in leaves	256.93b	262.40a	204.83d	213.30c
Potassium contents in roots	151.93b	161.47a	136.93d	144.53c
Electrical leakage in leaves	4.5167c	4.3200c	7.5400a	6.7300b
Electrical leakage in roots	5.5267d	5.7700c	8.4367a	7.6333b
Plant height	62.123a	51.113c	55.577b	45.637d
Root length	23.800a	22.633b	21.067c	21.300c
Plant fresh weight	43.603b	51.233a	34.597d	42.490c
Plant dry weight	14.917c	18.550a	13.303d	16.633b
Plant diameter	7.3533a	7.0800b	6.6000c	6.2600d
SPAD	79.233a	49.433b	39.667c	37.200d

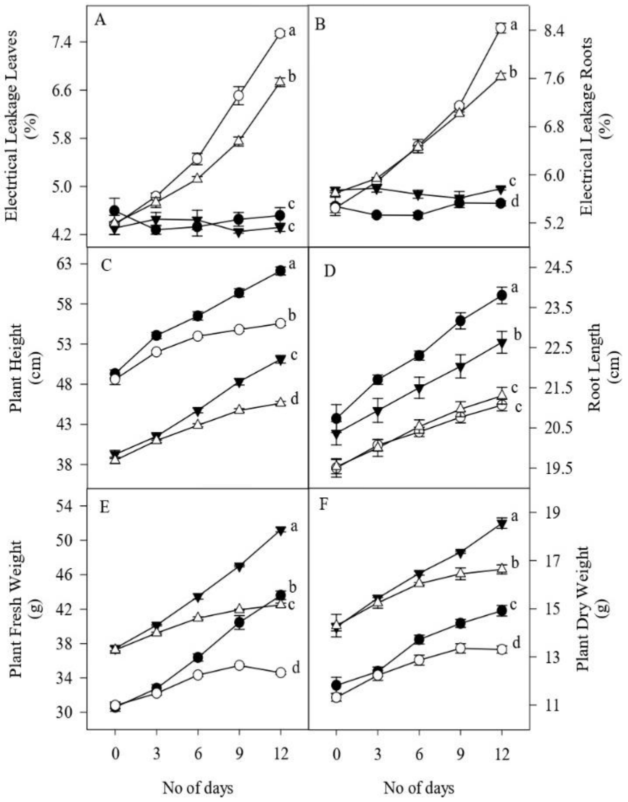

Fig. 3. Electrolyte leakage and plant physical parameters of trifoliate orange (T.O.) and rough lemon (R.L.) under control and water deficit condition. (A) Electrolyte leakage in leaves; (B) Electrolyte leakage in roots; (C) Plant height; (D) Root length; (E) Plant fresh weight; (F) Plant dry weight. Values are mean ± S.E. at $p<0.05$. ● = T.O control; O = T.O water deficit condition; ▼ = R.L control; Δ = R.L water deficit condition.

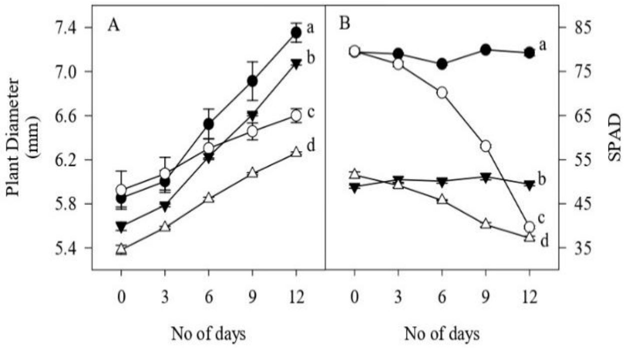

Fig. 4. Plant diameter and leaves greenness (SPAD) of trifoliate orange (T.O.) and rough lemon (R.L) under control and water deficit conditions. (A) Plant diameter; (B) SPAD. Values are mean ± S.E. at $p<0.05$. ● = T.O. control; O = T.O. water deficit condition; ▼ = R.L. control; Δ = R.L. water deficit condition.

Discussion

Due to climate change, drought stress has emerged as the most common stress on existing agroecosystems, and it is one of the major concerns of the scientific community (He *et al.*, 2014). Stress tolerance mechanisms become critical for plant survival or efficient recovery from stressful situations if the stress becomes more severe and the plant can no longer maintain enough water or ion homeostasis. Cell dehydration tolerance mechanisms are characterized by the accumulation of osmoprotectants, antioxidants, and reactive oxygen species (ROS) scavengers, as well as the biosynthesis of cell-protecting proteins, such as heat shock proteins (HSPs) and hydrophilins. Plants respond by overproducing antioxidant enzymes, such as superoxide dismutase, catalase, and peroxidase, and metabolites, such as ascorbate and glutathione, to reduce ROS toxicity. The ROS response correlates favorably with the degree of resistance of citrus plants (Balal *et al*., 2012; Seday *et al*., 2014). It is well established that antioxidant defenses are essential for tolerance to abiotic stress. Hydrophilins, such as late embryogenesis abundant (LEA) proteins, help cells survive protoplasmic water depletion. Several studies have shown that hydrophilins and HSP protection proteins have a role in citrus plants' responses to water stress (Podda *et al*., 2013; Xiao *et al*., 2017).

Various experimental evidence has shown that drought tolerance in Citrus spp., related genera (e.g. Poncirus), and their hybrids (e.g. citranges and citrumelo) is based mainly on avoidance mechanisms that include stomatal closure, and hence decreased transpiration and CO_2 assimilation (Pérez-Pérez *et al*., 2009; Rodríguez-Gamir *et al*., 2010; Pedroso *et al*., 2014), reduced leaf area, with concomitant decreased vegetative growth and yield (Pérez-Pérez *et al*., 2008; Melgar *et al*., 2010). On the other hand, some evidence suggests that citrus plants also exhibit some tolerance mechanisms to drought, such as osmotic (Pérez-Pérez *et al*., 2009; Rodríguez-Gamir *et al*., 2010) and cell wall elastic adjustments (Savé *et al*., 1995). Another striking feature observed in citrus under field conditions is that drought tolerance is a characteristic usually conferred by the rootstock (Barry *et al*., 2004; Romero *et al*., 2006). For instance, the rootstock 'Rangpur' lime is known to confer enhanced drought tolerance to sweet orange scions under field conditions as compared with other citrus rootstocks, such as 'Swingle' citrumelo, 'Sunki' mandarin, 'Cleopatra' mandarin, and Poncirus trifoliata (Cantuarias-Avilés *et al*., 2011). The outstanding performance of 'Rangpur' has been related to its high root hydraulic conductivity (Medina *et al*., 1998), enhanced root growth (Magalhães *et al*., 2008), and remobilization of carbohydrate reserves to the roots (Pedroso *et al*., 2014) under drought-stress conditions. Rootstock selection and improvement to increase plant water-use efficiency are effective strategies to minimize the effects of climate change on plant production (Berdeja *et al*., 2015). Superior rootstocks increase the resistance of citrus crops to biotic and abiotic factors (Machado *et al*., 2013). In the case of drought, this resistance is due to physiological changes that lead to changes in leaf water potential, stomatal conductance, and root hydraulic conductance (Romero *et al*., 2006; Rodríguez-Gamir *et al*., 2011).

A decrease in nutrient availability in a plant under drought stress could result from impaired nutrient absorption, reduced unloading mechanisms, and reduced transpiration. The external supply of nutrients can increase, decrease, or have no effect on a plant's fitness under water stress, depending on the severity of the drought, the concentration of the elements in the soil, and other conditions (Hu & Schmidhalter, 2005). K^+ is an essential component of osmotic balance in plants (Ahanger & Agarwal, 2017; Zahoor *et al*., 2017), and its positive effects in plant homeostasis are especially pronounced under drought stress (Hosseini *et al*., 2016). Aside from K^+, Na^+ can also play a similar role to K^+ to some extent regarding osmotic balance (Erel *et al*., 2014). Na^+ can be partitioned into vacuoles (Apse & Blumwald, 2007), and the partitioned Na^+ can be used for osmotic adjustment to reduce water potential and increase drought resistance (Kang *et al*., 2015; Cui *et al*., 2019).

In our experiment, the Na^+ content in the leaves of both rootstocks increased under drought stress, with a greater increase in rough lemon, which helps with osmotic adjustment to reduce water potential and increase drought resistance. Tadayyon *et al*., (2018) found that drought stress significantly affected Na^+ concentration. The concentration increased as the drought stress increased.

Chloride content in the leaves and roots of both rootstocks decreased under water deficit conditions compared to day zero. Chloride content in rough lemon decreased by 9.97% and 8.03% in leaves and roots, respectively, on day 12. While in the trifoliate orange, an 8.85% decrease in leaves and a 6.78% decrease in roots, respectively, were measured on day 12. Results indicated that rough lemon compartmentalized more chloride ions than trifoliate orange, performing better under water deficit stress. Tolerant citrus rootstocks exclude chloride ions more efficiently than sensitive rootstocks (Hussain *et al*., 2012).

Calcium ion (Ca^{+2}) is a vital signaling messenger that mediates the actions of many hormones and environmental factors, including interactions with biotic and abiotic stress factors. Calcium is needed to help plants recover from dehydration. Many studies have reported that calcium can alleviate and postpone drought-related oxidative damage (Palta, 2000). In our experiment, calcium content in the leaves of both rootstocks increased under drought stress. Compared to day zero, the increase in calcium content in leaves on day 12 was 11.58% in trifoliate orange, while in rough lemon, it was 10.59% higher.

Similarly, calcium contents in roots increased 22.89 % in rough lemon and 21.39% in trifoliate orange from the start to the end of the experiment. In agreement with our results, Khalid *et al*., (2021) also found that calcium content increased in citrus under water deficit stress. Tadayyon *et al*., (2018) found that drought stress increased Ca^{+2} and Na^+ contents in castor plants.

Nitrogen is one of the main macro elements required for proper plant growth and development (Smithson & Sanchez, 2001). Nitrogen application enhances water use efficiency and alleviates drought stress effects on plant growth (Saneoka *et al*., 2004). In our experiment, nitrogen content in the leaves and roots of both rootstocks decreased significantly under drought stress, with a greater decrease in trifoliate orange than in rough lemon. Compared to day zero, the reduction in nitrogen content in leaves on day 12 was 50.60% in rough lemons, while a 51.29% decrease was observed in trifoliate oranges on day 12. Nitrogen content in the roots of both rootstocks also decreased at day 12 compared to day zero. The decrease was higher in trifoliate orange (27.28%) than in rough lemon (25.64%). Results showed that drought stress significantly reduced the plants' absorption of soil nitrogen.

Phosphorus plays a vital role in the preservation and transfer of energy in plant metabolism (Wang *et al*., 2015). Drought stress reduces phosphorus transfer from the soil to the root and its subsequent transport to the stem (Cramer *et al*., 2009). Studies indicate that many species' drought tolerance and water use efficiency can be improved by enhanced phosphorus nutrition (Singh & Sale, 1998; Garg *et al*., 2004; Waraich *et al*., 2011). In our experiment, phosphorus contents in the leaves and roots of both rootstocks decreased significantly under drought stress, with a more significant decrease in trifoliate orange than rough lemon. Compared to day zero, the reduction of phosphorus content in leaves on day 12 was 44.08% in rough lemon. In contrast, in trifoliate orange, a 47.55% decrease was observed.

Phosphorus content in the roots of both rootstocks was also decreased at day 12 compared to day zero. The decrease was 33.63% and 33.42% in trifoliate orange and rough lemon, respectively. Our results are similar to those reported by Khalid *et al*., (2021). Nitrogen and phosphorus content in leaves and roots of both rootstocks were decreased by low soil moisture in the water deficit treatment (Cramer *et al*., 2009; Sardans & Penuelas, 2012).

Potassium maintains plant water status, stomatal movements, enzyme activity, osmoregulation, and membrane stability (Ahmad *et al*., 2014; Jatav *et al*., 2014; Erel *et al*., 2015). Under drought conditions, the soil potassium availability for plants is lowered, and that limits its uptake by the root, ultimately affecting its root-shoot translocation (Wang *et al*., 2013). In our experiment, potassium content in the leaves of both rootstocks decreased under drought stress, with a greater decrease in trifoliate orange than in rough lemon. Compared to day zero, the potassium content in leaves on day 12 was reduced by 18.76% in rough lemon and by 20.15% in trifoliate orange. On the other hand, potassium content in the roots of both rootstocks also decreased on day 12 compared to day zero, but the decrease was higher in rough lemon (11.13%) than in trifoliate orange (10.01%). Results showed that rough lemon roots transfer more potassium to leaves than trifoliate orange roots, helping rough lemon plants maintain stomatal opening and osmotic adjustment. These results indicate that rough lemon performed better under drought stress and was more tolerant than trifoliate orange to imposed water stress. When plants are exposed to water-deficient conditions, potassium concentration significantly decreases, resulting in stomata closing and oxidative damage (Khalid *et al*., 2021).

In our experiment, a significant increase in electrolyte leakage in the leaves and roots of both rootstocks was observed in the water deficit treatment. In trifoliate orange, a 72.94% increase in leaves and a 54.90% increase in roots were observed on day 12 as compared to day zero, while in rough lemon, a 53.42% increase in leaves and a 34.07% increase in roots were observed on day 12 compared to day zero. Higher electrolyte leakage values in trifoliate orange indicate that trifoliate orange is more sensitive to water deficit stress than rough lemon. Under water deficit stress, rough lemons perform better, and our results agreed with Khalid *et al*., (2021) findings, who observed that under rapid water deficit stress, citrus plants have more electrolyte leakage than the control treatment. Yahmed *et al*., (2016) also found that electrolyte leakage in citrus leaf discs increased under drought stress. Sensitive rootstocks showed more electrolyte leakage than tolerant rootstocks (Santos *et al*., 2019).

In this study, plant height and root length increased on day 12 compared to day zero. However, growth was higher in rough lemon than in trifoliate orange. At the start of the experiment, plant height and root length grew faster in trifoliate orange, but after day 9, the increase was very slow, while a constant growth was observed in rough lemon's plant height and root length from day zero to 12. The increase in plant height in rough lemon was 18.49%; in trifoliate orange, the increase was 14.32%. Similarly, the increase in root length of rough lemon was 9.04%, and in trifoliate orange, the increase was 8.03%. Results indicate that rough lemon performed better under drought stress than trifoliate orange.

In our experiment, the fresh and dry plant weight of both rootstocks was increased under control conditions and water deficit stress; the increase was slow under water deficit stress. The increase in plant fresh weight on day 12 was 14.05% in rough lemon, while in trifoliate orange it was 12.35%. Plant diameter increased more in rough lemon (16.28%) than in trifoliate orange (11.42%) on day 12 compared to day zero, showing the better performance of rough lemon under water deficit conditions than trifoliate orange. Carr (2012) found that in citrus crops, water scarcity negatively affects plant growth and impairs cell metabolism, affecting the overall tree growth.

Leaf greenness (SPAD) is an important attribute when comparing the performance of both rootstocks under drought stress. In our experiment, leaf greenness decreased 27.77% in rough lemon and 50.16% in trifoliate orange, which showed that rough lemon performed better (i.e., greener leaf tissue) under water deficit conditions than trifoliate orange. Our results are similar to those of Khalid *et al*., (2021), who observed that under water deficit stress, the decrease in leaf greenness was less in tetraploid plants than in diploid plants.

Conclusion

Rough lemon and trifoliate orange plants exhibited differences in their anatomical and physiological processes, e.g., rough lemon had a deep root system, which helped rough lemon plants to absorb water and uptake essential minerals from deep soil layers. In contrast, trifoliate orange had a shallow root system and thus faced drought stress earlier, with detrimental impacts on the traits measured. As our previous results showed, the larger leaves of rough lemon improve photosynthesis in rough lemon plants more than the narrow leaves of trifoliate orange. Rough lemons have a waxy coating on their leaves, which helps conserve water by reducing water loss from leaves through transpiration. They also showed different tolerance mechanisms; for example, rough lemon adopted a drought tolerance mechanism, while trifoliate oranges adopted a drought avoidance mechanism against water scarcity. Rough lemon plants showed more tolerance by maintaining their physical growth, better conductivity, and mineral translocation. In drought-tolerant species, the focus lies on limiting drought-induced damage during dry periods, while in drought-avoidant species, the focus lies on avoiding tissue water deficits as the dry period exceeds the damage caused by the water deficit stress increase, which is irreversible. Under water deficit conditions, rough lemon plants had more mineral content, e.g., nitrogen, phosphorus, and potassium, which helped rough lemon to maintain its growth. Under water stress, electrolyte leakage was also lower in rough lemon seedlings compared with trifoliate orange, indicating that rough lemons tolerate better water deficit stress by maintaining osmotic adjustment. In the future, transcriptome profiling/RNAseq will be done for both rootstocks to investigate the genes functioning in the tolerance of rough lemon under water deficit stress. These genes can be engineered in important commercial rootstocks to improve their performance under water-deficient conditions.

Author's Contribution: MS: Conceptualization, methodology, software, validation, formal analysis, writing-original draft preparation; **MNS:** Formatting and proofreading. **JRC:** Review and Proofreading; **SA:** Review, editing, visualization; **SH:** Software, validation, formal analysis, data curation.

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