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Physiological and ultrastructural responses of sour orange (C*itrus aurantium* L.) clones to water stress

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Abstract

Water stress is a major abiotic constraint leading to serious crop losses. Recently, in the Mediterranean region, water stress has become markedly sensed, especially in *Citrus* orchards. This study investigated the physiological responses of local sour orange (*Citrus aurantium* L.) clones to severe water stress. Water stress was applied by withholding irrigation during weeks, followed by a rewatering phase during three months. Under water stress, sour orange clones decreased their stomatal conductance, net photosynthetic rate, and transpiration rate. On the contrary, biomass was stable, especially in the Kliaa clone. In addition, reduced leaf water potentials (−3 MPa) and water contents were measured in most of the clones, except Kliaa which kept the highest water potential (−2.5 MPa). After rewatering, all clones recovered except of the Ghars Mrad (GM) clone. Ultrastructural observations of leaf sections by transmission electron microscopy did not reveal marked alterations in the mesophyll cells and chloroplast structure of Kliaa in comparison to the sensitive clone GM, in which palisade parenchyma cells and chloroplasts were disorganized. This contrasting behavior was mainly attributed to genetic differences as attested by molecular analysis. This study highlighted GM as the drought-sensitive clone and Kliaa as the tolerant clone able to develop an avoidance strategy based on an efficient stomatal regulation. Although a high percentage of polyembryony characterizes *C. aurantium* and justifies its multiplication by seeds, heterogeneous water-stress responses could be observed within sour orange plants in young orchards.

Additional key words: chloroplast ultrastructure; gas exchange.

Introduction

Water limitation is a major factor affecting the expansion of irrigated agriculture especially in semiarid and arid areas, where plants are often subjected to long drought periods simultaneously with a high evaporative demand. In the Mediterranean region, Tunisia belongs to the arid − semiarid bioclimatic range, characterized by frequent unpredicted or progressive water limitation and high temperature episodes. Under such conditions, transpiration often exceeds root water uptake, which exposes plants to a water deficit during several months (Rejeb 1992), consequently affecting the development and production of several fruit species. During the last decades, water stress effects became significant especially in Tunisian non-irrigated *Citrus* orchards. *Citrus* species are known to be water-demanding mainly in the Mediterranean region where rainfall is low and irregular (Loussert 1989). The theoretical water needs of *Citrus* species are approximately equal to 1,200 mm per year, markedly higher than mean annual rainfall in *Citrus* cultivated areas of Tunisia (400 mm per year). Rainfall irregularity affects also *Citrus* growth and development as well as yield and fruit quality. Within rootstock species, sour orange (*Citrus aurantium* L.) has been for a long time the main citrus rootstock in Tunisia, regarding its "traditional" tolerance to drought and salinity. Although a high polyembryony percentage characterizes this species (Aubert and Vullin 1997), seedlings cultivated in the orchards may show heterogeneity sometimes leading to variable behavior,

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Abbreviations: C − control, irrigated plants; C_i − intercellular CO_2 concentration; DM − dry mass; E − transpiration; EH − equivalent humidity; FM − fresh mass; GM − Ghars Mrad; g_s − stomatal conductance; IBA − indol-butyric acid; LWC − leaf water content; P_N − net photosynthetic rate; RAPD − random amplified polymorphic DNA; REW − rewatered plants; TEM − transmission electron microscopy; Wg − gravimetric soil water content; WS − water-stressed plants; ψ_w − leaf water potential.
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especially under abiotic constraints (Di Ferdinando *et al.* 2012). The behavior of such a rootstock species under water stress, in particular, still needs to be studied at the photosynthetic and ultrastructural levels.

Photosynthesis and cell growth are among the primary processes to be affected by water stress (Chaves *et al.* 2009, Aroca 2012). The effects of water stress on photosynthesis range from the reduction of CO_2 diffusion into the chloroplast, *via* limitations on stomatal opening and mesophyll transport of CO_2, to alterations in leaf photochemistry and carbon metabolism (Chaves *et al.* 2009). Water stress affects the yield and fruit quality, delays growth, increases fruit abscission, and decreases juice content and quality (García-Sánchez *et al.* 2007). The occurrence of morphological and physiological responses to water stress varies considerably among species (Souza *et al.* 2004). In this context, this study was carried out to evaluate the responses of four vegetatively propagated sour orange clones to a severe water stress, in terms of biomass and photosynthesis. Additionally, mesophyll ultrastructure investigations were simultaneously carried out in order to detect alterations at cellular level in water-stressed sour orange leaves.

Materials and methods

Plant material: Several *Citrus* orchard explorations were done in the "Cap Bon" region (semi-arid climate) in the North-East coast of Tunisia. In this study, four 80-year-old mother trees were selected in different *Citrus* orchards [Kliaa, Gaddour, Khsairi, and Ghars Mrad (GM): names of orchards where mother trees were prospected]. Shoot cuttings were sampled during the spring and immediately used for the vegetative multiplication of the selected mother plants. Two node-cuttings were soaked at their base in indol-butyric acid (IBA) and inserted in rockwool blocks saturated with a nutrient solution under 100% relative humidity in a controlled greenhouse. Within two months, roots and axillary buds began to develop. Rooted plantlets were subsequently acclimatized and individually transferred into pots filled with inert sand, irrigated using a nutrient solution, and cultivated under controlled greenhouse conditions.

Experimental conditions: Eighteen-month-old sour orange plants representing the four clones were transferred into 2.5-l plastic containers, filled with a native orchard soil (loamy-clay in structure) in a controlled greenhouse, where maximum/minimum temperatures were 26/20°C and relative humidity ranged from 60 to 70% during the course of the experiment. Twenty homogenous plants per clone were selected for water stress treatment. Ten sour orange control (C) plants were regularly irrigated to the field capacity [100% of equivalent humidity (EH)], while ten others were completely deprived of water for a period of three weeks (WS). The first harvest for biomass determination was performed at the end of water stress application on five C and five WS plants per sour orange clone. The remaining plants were subjected to the rewatering phase (REW) to the field capacity during three months under controlled greenhouse conditions in order to evaluate the recovery ability of each clone. The harvest for biomass determination was performed on five C and five REW plants per clone after a three-month rewatering period.

Symptoms such as leaf rolling, wilting, chlorosis, and desiccation were monitored during water stress and rewatering phases on five control and five stressed plants per clone.

Biomass: After three weeks of water stress, five C and five WS plants per clone were harvested. Leaves, shoots and roots were immediately and weighed for fresh mass (FM) determination using a precision balance *(Mettler Type PJ600,* Toledo). Plant organs were dried for seven days at 60°C in order to determine their respective dry masses (DM). Biomass measurements were also monitored for five C and five REW plants per clone after the three-month rewatering phase.

Leaf gas exchange: Net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E), and intercellular CO_2 concentration (C_i) were measured using *LI-COR 6200 (Li-Cor,* Lincoln, NE, USA). Two fully expanded mature leaves, about two-months old, from three C and three WS plants per clone were used. All measurements were made from 10:30 h to 11:30 h to avoid high afternoon temperatures and vapor pressure deficit.

Water relations: All measurements of leaf water potential (ψ_w) were done weekly, using mature leaves in the mid-stem region of each of the five replicate plants from each clone till the end of water stress and rewatering phases. All measurements were made from 11:00 h to 13:00 h using a Scholander pressure chamber (*PMS Instruments*, Corvallis, Oregon, USA).

At the end of water stress and rewatering phases, leaf water content (LWC) was calculated using three mature leaves per plant sampled on five C and five WS plants per clone based on the following formulae:

$$LWC\ [\%] = [(FM - DM)/FM \times 100].$$

Gravimetric soil water content (SWC) was determined at the end of the water stress (day 21) using five pots per treatment per clone. After the plants were removed, 500 g of soil samples from each pot were weighed (FM), dried at 65°C to a constant mass and reweighed again (DM). Gravimetric soil water content was then calculated according to Pérez-Pérez *et al.* (2007) as:

$$SWC = [(FM - DM) \times 100/DM].$$

Osmolyte contents: At the end of water stress and after rewatering, total carbohydrate contents were determined according to Staub *et al.* (1963). Extraction was performed in 5 ml of 80% ethanol, using 25 mg of dry powder leaf sample, in a 70°C water bath for 30 min. After extraction, sample tubes were centrifuged at 3,000 rpm for 15 min. The supernatant (25 µl) was collected in fresh tubes, kept in an ice bath, and mixed with 5 ml anthrone and 2.25 ml ethanol (80%), under agitation. Samples were then placed in a boiling water bath (100°C) for 10 min then immediately cooled down in an ice bath. The optical density of each sample was measured using a spectrophotometer (*Ultrospec 2000, Pharmacia Biotech,* USA) at 640 nm. Glucose (0.1 g l^{-1}) was used as a standard.

The extraction of proline was done using 100 mg of leaf dry powder according to Bates *et al.* (1973). Dry powder (100 mg) was homogenized in 2 ml of aqueous sulfosalicylic acid (3%). After the homogenates were centrifuged at 6,000 rpm at 4°C for 20 min, the supernatants (1 ml) were transferred to fresh 1.5-ml tubes adding 1 ml of ninhydrin reagent (1.25 g ninhydrin in 30 ml of glacial acetic acid and 20 ml of 6M H_3PO_4) and 1 ml of glacial acetic acid per sample. All sample tubes were incubated at 100°C for 1 h. After cooling the samples in an ice bath, 2 ml of toluene were added per sample tube and vigorously mixed. The organic phase of each sample was finally collected and optical density was measured using a spectrophotometer (*Ultrospec 2000, Pharmacia Biotech,* USA) at 520 nm. L-proline (1 g l^{-1}) was used as a standard.

Molecular characterization: DNA was extracted from young leaves of sour orange clones according to Dellaporta *et al.* (1983) with few modifications. Universal decamer oligonucleotides (*Operon Technologies Inc*. Alameda, USA) were used for random amplified polymorphic DNA (RAPD) analysis. Five primers (OPA-8, OPA-11, OPB-02, OPC-05, and OPC-06) were selected based on the reproducibility and polymorphism of the generated bands. A reaction mixtures (15 µl) was prepared using DNA templates (25−50 ng), primers (0.8 µM), Taq DNA polymerase (1 U) and dNTPs (200 µM). PCR was carried out in a *GeneAmp PCR System 9700 Thermocycler* (*Applied Biosystems,* USA) according to the following program: 3 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 37°C and 2 min at 72°C, and a final step of 7 min at 72°C. After 1.5% agarose gel electrophoresis of amplified products, staining and visualization under UV, imaging was performed using a *DI-01* system (*Major Science, USA*).

Amplified RAPD bands were numbered sequentially in a decreasing order according to their molecular mass. Polymorphic DNA bands were scored and data were analyzed using *SIMQUAL* module to generate a dendrogram using the *UPGMA* clustering algorithm (Sokal and Michener 1958).

Ultrastructural analysis: Leaves sampled for photosynthesis measurements were also observed by optical and electron microscopy. Leaf blade samples (2 × 2 mm) were excised from the mid-laminar region of both control and stressed plants of the four sour orange clones with a razor blade. The samples were immediately fixed for 2 h at 4°C in glutaraldehyde: cacodylate buffer (pH 7.4): distilled water (1:4:3, v/v/v), rinsed in the cacodylate buffer, post-fixed in osmium tetroxide (1%) for 2 h at 4°C, then dehydrated through a gradient of ethanol series (70, 95, and 100°), and finally embedded in *Epon* resin. After polymerization, the resin blocks were cut into 1 µm semithin, then 70-nm ultrathin sections using an ultramicrotome (*Reichert-Jung Ultracut E,* Vienna, Austria) photographed by a transmission electron microscope *JEM 1010* (*JEOL,* Tokyo, Japan) operated at 60 kV (Ben Salem-Fnayou *et al.* 2011). TEM observations focused on chloroplast ultrastructure.

Statistical analysis: Data were subjected to analysis of variance (*ANOVA*) using *STATISTICA* software. Mean comparisons were done using the *Duncan'*s multiple range test at 5% significance level.

Results

Soil water content*:* Within three weeks of water stress, SWC significantly decreased in pots of WS plants as compared to the C plants [from 9.8 to 10.4 g(H_2O) 100 g^{-1}(soil)], and did not exceed 1.6 g(H_2O) 100 g^{-1}(soil) for all the studied clones.

Symptoms: Plant response under water stress varied according to the clone. Kliaa plants were morphologically similar to the C during the first and the second week of water stress even though a slight rolling was noticed on adult leaves during the third week. In contrast, the GM plants exhibited leaf rolling since the first week of water stress followed by a progressive wilting, chlorosis, and desiccation at the end of the third week. Upon rewatering, desiccated GM plants did not exhibit any recovery symptoms, while the Kliaa and Gaddour plants were morphologically similar to the irrigated C plants.

Shoot and root biomass: All studied clones significantly decreased shoot biomass during water stress in comparison with the C plants. Kliaa and Gaddour clones had the highest shoot FM (91 and 89% of C, respectively) while the lowest values were registered for the Khsairi clone. Shoot FM increased in all the clones except in GM after rewatering. (Fig. 1).

Water stress did not significantly affect root FM of all the studied clones except in Khsairi. In the Gaddour clone, root FM significantly increased (15.17 g), compared with

control (13.32 g). After rewatering, root FM significantly increased (80% of C) in Khsairi clone (Fig. 1) while a significantly root FM decrease (36% of C) was measured in GM clone after rewatering (Fig. 1).

Gas-exchange rates: C_i of the WS plants was higher in the GM and Kliaa clones (305 and 260.8 μmol m^{-2} s^{-1}, respectively) compared with C, while C_i was zero in the Khsairi clone. After rewatering, all the sour orange clones showed C_i values insignificantly different from the C plants, except GM which had a C_i of 0 μmol m^{-2} s^{-1} (Fig. 2).

A significant decrease of P_N was measured in all the clones at the end of the water stress application. The highest P_N (17.3 % of C) was recorded for the Kliaa clone. The lowest P_N (2% and 0% of C) were recorded in GM and Khsairi clones, respectively. After a three-month rewatering period, P_N significantly increased in the Khsairi and Gaddour clones (2.85 and 3.24 μmol m^{-2} s^{-1}, respectively), whereas it was zero in the GM clone (Fig. 2).

The considered clones significantly decreased g_s in comparison to the C plants. Kliaa plants had the highest g_s (20.6% of C), while zero g_s values were registered in the Khsairi and GM clones. After rewatering, g_s significantly increased in all the clones and reached statistically similar values as the C plants in the Khsairi, Gaddour, and Kliaa clones (0.04 mol m^{-2} s^{-1}), while GM clone had zero g_s (Fig. 2).

E significantly decreased (19.6, 26.1, 5.5, and 0% of C) at the end of the water stress in Kliaa, Gaddour, GM, and Khsairi sour orange clones, respectively (Fig. 2).

Khsairi and Gaddour clones had the highest E after rewatering (88 and 94% of C, respectively), while the Kliaa plants had significantly lower E (74.1% of C), while E was zero in GM plants (Fig. 2).

Water relations: ψ_w significantly decreased in all the studied clones on the second week of water stress application. At the end of the third week, ψ_w reached the lowest values in the GM and Khsairi clones (−3 MPa) while a relatively higher ψ_w were measured in the Kliaa and Gaddour clones (–2.36 and –2.46 MPa, respectively) (Fig. 3). ψ_w decreased in all the clones except GM (Fig. 3) after rewatering but it was lower than the C plants after a three-month rewatering period.

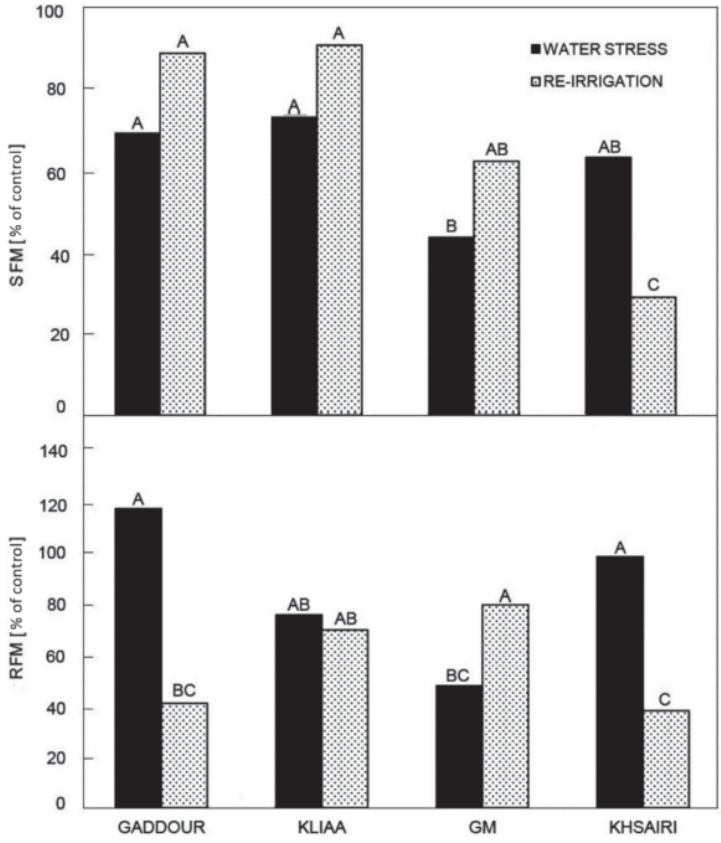

Fig. 1. Shoot and root biomass (FM) of the sour orange clones at the end of the water stress application and after a three-month rewatering period. Means followed by *different letters* are significantly different at 5% significance level (*Duncan*'s multiple range test).

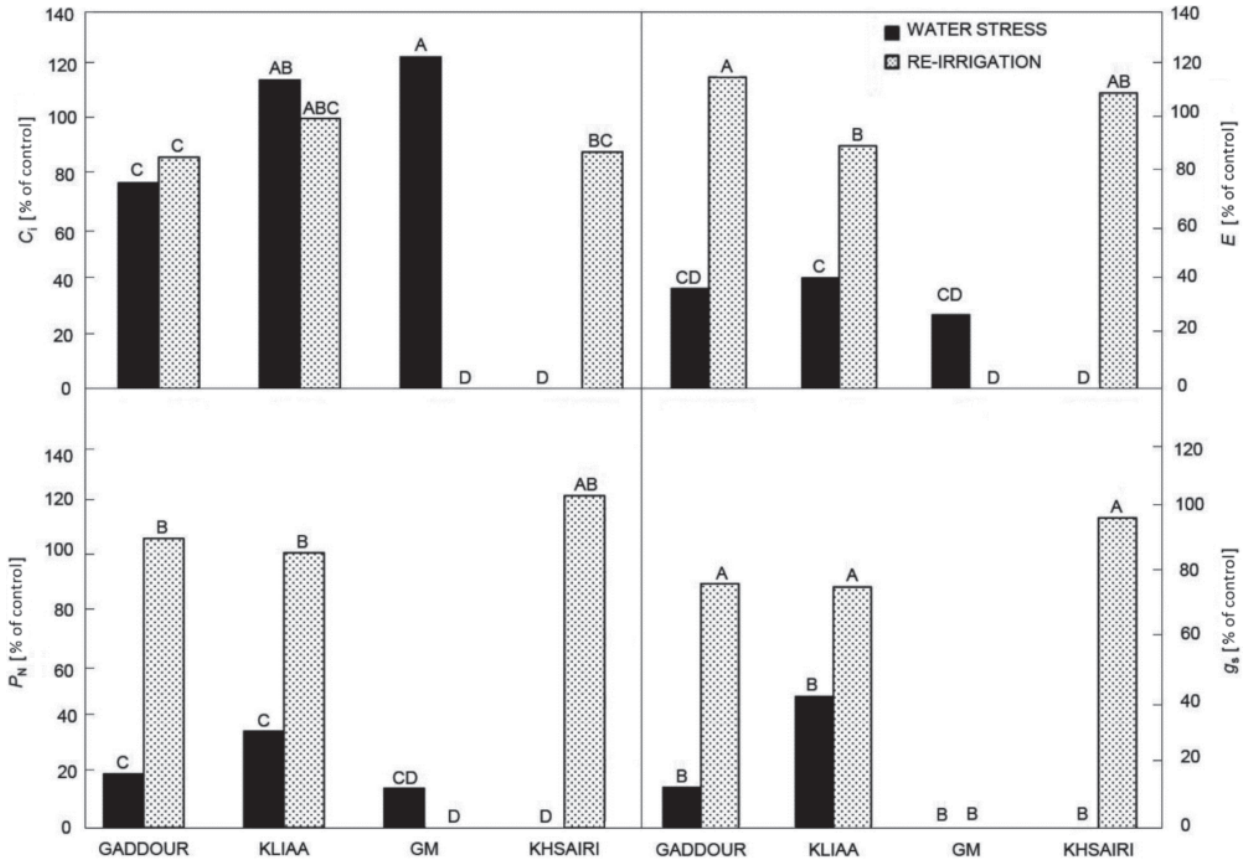

Fig. 2. Gas-exchange parameters in Gaddour, Kliaa, GM, and Khsairi sour orange clones after three weeks of water stress and three months of rewatering (in % of the control). Data are means of six values. Means followed by *different letters* are significantly different at 5% significance level (*Duncan*'s multiple range test). P_N − net photosynthetic rate; C_i − intercellular CO_2 concentration; E – transpiration rate; g_s − stomatal conductance.

LWC significantly decreased compared with the C plants in all sour orange clones (Table 1). The lowest LWC was monitored in the Kliaa clone (44.4%), while the highest in the Gaddour, Khsairi, and GM clones (54.3, 57.0, and 58.3%, respectively) (Table 1). After rewatering, LWC significantly increased (59.95 and 63.1% of the C) in comparison to WS plants in Gaddour and Kliaa, respectively. However, GM and Khsairi plants had significantly lower LWC (21.0 and 43.8%, respectively) than the WS plants (Table 1).

Osmolyte contents: The soluble sugar contents measured under water stress and after rewatering were not significantly different from C in the Kliaa and Khsairi clones (Table 2), while a significant decrease was observed in both Gaddour and GM clones (18.6 and 10.5% of C) under water stress. After rewatering, these contents increased to control values (Table 2).

No significant differences in the proline contents were observed between stressed and control sour orange clones, during drought and rewatering (Table 2).

Molecular polymorphism: A total of five primers were selected for their ability to generate consistently amplified band patterns and assess polymorphism in the studied clones. A total of 43 bands were recorded, 36 of which were polymorphic. Thus, sour orange clones are characterized by a large genetic diversity at the DNA level (Fig. 4). The relationships between the four studied clones were concluded based on the UPGMA dendrogram (Fig. 5) in which, three clusters were distinguished: the first composed of GM and Khsairi clones, the second including Gaddour clone, while Kliaa clone formed the third group.

Chloroplast ultrastructure: In C plant, leaves of both Kliaa and GM clones were lens-shaped and elongated with a well developed internal membrane system. Most of the chloroplasts contained starch granules (Fig. 6*A*,*B*). Under water stress, mesophyll cell alterations were observed in the GM clone (Fig. 6*E*). In particular, chloroplasts showed disintegrated membranes. Moreover, they contained large intrathylakoid spaces (Fig. 6*F*), and numerous plastoglobules. On the contrary, the leaf ultrastructural

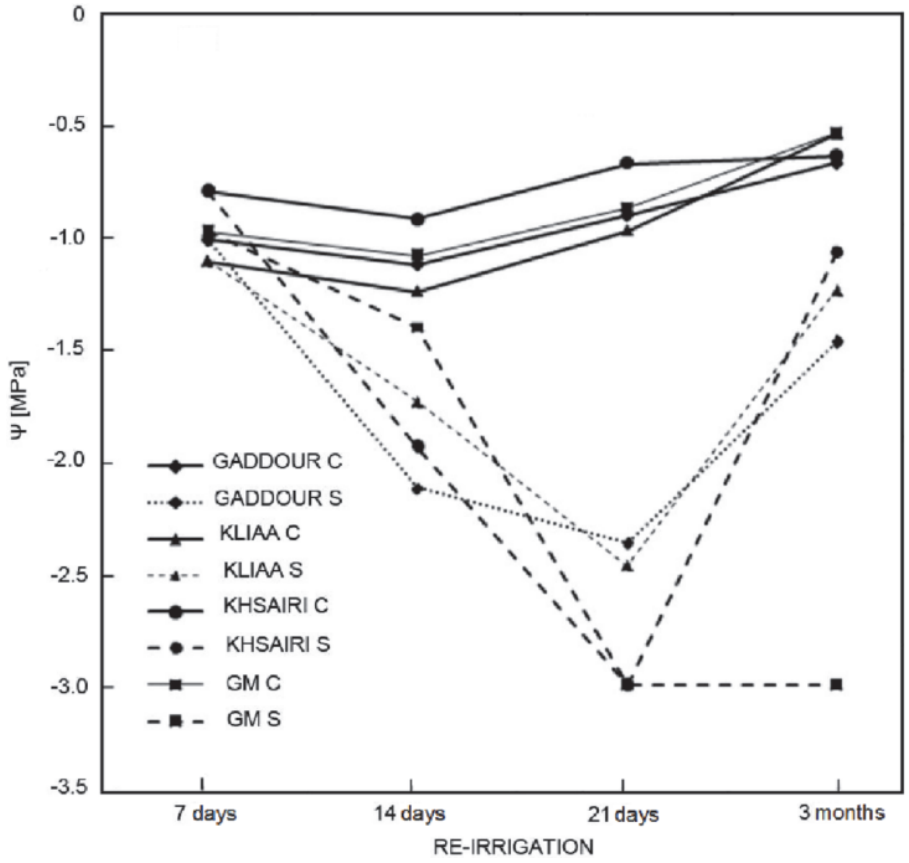

Fig. 3. Leaf water potential (ψ_w) (MPa) in control and water-stressed plants from Gaddour, Kliaa, GM, and Khsairi sour orange clones, during the constraint and upon rewatering.

Table 1. Leaf water content (LWC) in control, water-stressed, and rewatered plants from Gaddour, GM, Kliaa and Khsairi sour orange clones. Data are means of ten values. Means followed by *different letters* are significantly different at 5% significance level (*Duncan*'s multiple range test).

LWC [%]	Gaddour	Kliaa	Khsairi	GM
Control	68.601^{B}	67.112^{Bc}	68.309^{Bc}	76.42^{A}
Water stress	54.333^{E}	44.38^{F}	57.031^{De}	58.308^{De}
Rewatering	59.958^{Cde}	63.149^{Bcd}	43.842^{F}	20.975^{G}

observations of the Kliaa clone under water stress showed a similar structure as the C plants (Fig. 6*C*). Even though, few insignificant alterations in relation mainly with plastoglobuli and starch granules were noticed in WS Kliaa leaf cells (Fig. 6*C*), chloroplast ultrastructure was similar to C with stacked thylakoids and well organized grana (Fig. 6*D*).

Discussion

Under water stress, plant symptoms varied significantly in the tested clones. The Kliaa plants were morphologically similar to the control during the first and second week of water stress. Nevertheless, a slight rolling was noticed during the third week of the stress experiment. In contrast, GM plants exhibited leaf rolling since the first week of water stress followed by a progressive wilting, chlorosis and desiccation at the end of the third week. Based on such observations, a preliminary idea could be formulated on the tolerance/susceptibility of sour orange clones in terms of plant growth and biomass values in response to water stress. Our results showed relatively stable shoot and root biomasses in Kliaa and Gaddour clones under water stress, as compared with C. On the contrary, leaf biomass decreased in the GM clone under water stress, while root biomass was not significantly different from the C plants. Our results showed that water stress generally affected gas-exchange parameters in all studied clones. In Gaddour, GM, and Khsaïri plants, the decreased P_N under water stress may be attributed to stomata closure (Chaves *et al.* 2003; Isarangkool *et al.* 2011). A similar result was reported in *Citrus macrophylla* L. seedlings under drought (Gimeno *et al.* 2014). This led to a decreased C_i, particularly in the Khsairi clone. However, GM clone had

Table 2. Soluble sugars and proline content in control, water-stressed, and rewatered plants from Gaddour, Kliaa, Khsairi, and GM sour orange clones. Data are means of three values. Means followed by *different letters* are significantly different at 5% significance level (*Duncan*'s multiple range test).

Parameter	Clone			
Soluble sugars [mmol g^{-1}(DM)]	Gaddour	Kliaa	Khsairi	GM
Control	23.389^{Ab}	23.208^{Ab}	21.447^{Bc}	27.079^{A}
Water stress	18.577^{C}	24.076^{Ab}	21.917^{Bc}	10.051^{D}
Rewatering	22.412^{Bc}	24.848^{Ab}	21.109^{Bc}	22.4^{Bc}
Proline [mmol kg^{-1}(DM)]				
Control	106.047^{Ab}	107.682^{Ab}	103.855^{Abc}	96.333^{C}
Water stress	105.722^{Ab}	103.867^{Abc}	108.042^{Ab}	100.631^{Bc}
Rewatering	109.422^{A}	108.621^{Ab}	110.639^{A}	108.923^{Ab}

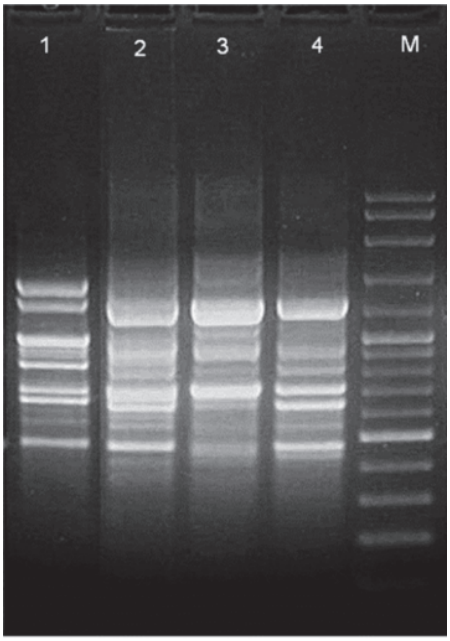

Fig. 4. Random amplified polymorphic DNA (RAPD) patterns of different *C. aurantium* clones generated using RAPD primers OPA-08. *Lane 1*: Khsairi; *Lane 2*: GM; *Lane 3*: Kliaa; *Lane 4*: Gaddour; M: 1 KB ladder (*Promega*, USA) as a molecular mass marker.

a zero P_N but a high C_i which may be attributed to mesophyll cell alterations. Pérez-Pérez *et al.* (2007) related the increased C_i simultaneously with the decreased P_N in Carrizo citrange plants under water stress to non-stomatal limitations inducing photosynthesis inhibition. On the other hand, a high C_i under water stress suggests the occurrence of an alteration in the CO_2 assimilation mechanism, inducing a low carboxylation efficiency, as observed Flexas and Medrano (2002). Challabathula *et al.* (2012) showed that P_N decrease under water stress is due to a limitation in the NADH generation. Our results showed a significant g_s decrease under water stress in comparison with control plants. The highest g_s was measured in the Kliaa clone in comparison to GM and Khsairi, plants which had null conductances indicating a stomatal closure. Mohd *et al.* (2004) showed that photosynthesis is more affected under drought by stomatal than nonstomatal limitations. On the contrary, Boujnah *et al.* (2004) linked photosynthesis decrease in *Olea europaea* (Chemlali variety) under water stress to nonstomatal limitations, namely to mesophyll cell alteration. In the Kliaa clone, g_s was high as compared to the other clones allowing a sufficient CO_2 diffusion for photosynthesis justifying also the E values (25% of control). This behavior demostrated the capacity of Kliaa clone to control stomata under water stress, indicating an avoidance strategy and a water saving characteristics.

Following a three-week water stress, all clones recovered within the fourth week of rewatering except the GM clone. This recovery period depended on the constraint severity, probably inducing biochemical and/or photochemical damages at the cellular level (da Silva *et al.* 2005). The considered clones significantly increased g_s, P_N, and E to control values except the GM clone which was irreversibly affected by water stress.

Our results showed a progressive ψ_w decrease in all studied clone as water stress progressed. This is attributed to the combined effects of E and soil water limitations (Kramer and Boyer 1995; Toumi 2008). A decreased ψ_w under water stress could be also related to osmotic adjustment (Hummel *et al.* 2010). Midday leaf water potentials in WS plants of all sour orange clones were similar to those reported in other *Citrus* species under severe water stress (Mohd *et al.* 2004). The Kliaa and Gaddour clones had a relatively high ψ_w (–2.5 MPa) compared to Khsairi and GM clones (–3 MPa) after a three-week water stress. In this context, Ali and Ashraf (2011) reported that the stability of plant water status is fundamental for growth under harsh environments. In *Citrus* hybrid Cleopâtra mandarin × *Poncirus trifoliata* (Forner–Alcaide), Rodríguez-Gamir *et al.* (2010) observed progressively decreasing (ψ_w), reaching –3.76 MPa after 18 d of drought stress. After rewatering, a recovery was observed in all sour orange clones except GM, as reported by Pérez-Pérez *et al.* (2007).

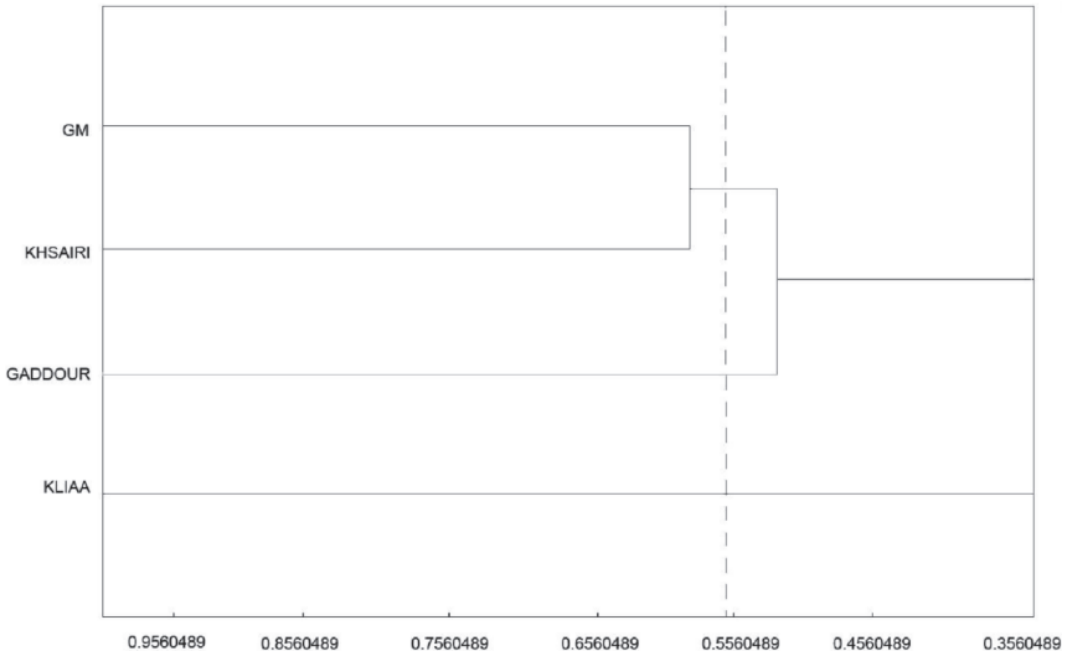

Fig. 5. Clusters of studied sour orange clones represented as a UPGMA dendrogram.

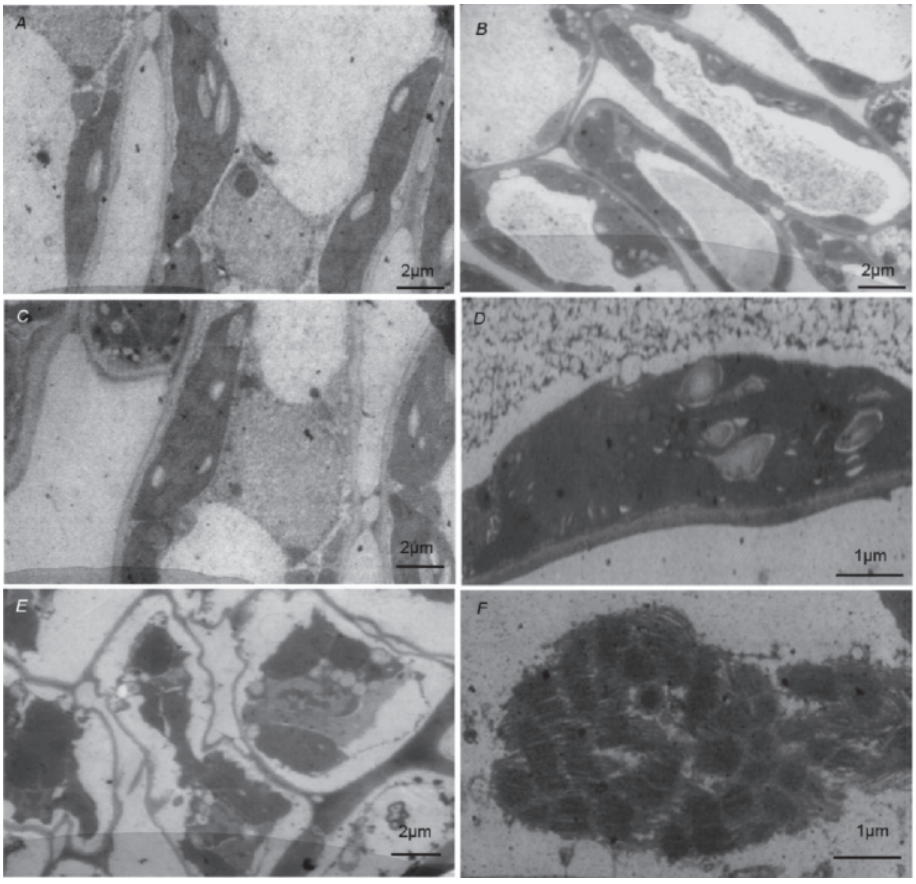

Fig. 6. Transmission electron microscopy (TEM) micrographs of leaves from Kliaa and GM plants under control and water-stress conditions: palisade parenchyma cells of Kliaa (*A*) and GM (*B*) plants under control conditions; palisade parenchyma cells of Kliaa under water stress (*C*); chloroplast details from Kliaa under water stress (*D*); palisade parenchyma cells of GM under water stress (*E*); chloroplast details from GM under water stress (*F*).

According to Aroca (2012), the recovery after a severe dehydration consists of a two-stage process: the first occurs during the first days upon rewatering and is associated to recovery of water status and stomatal reopening, whereas the second stage lasts several days and requires *de novo* synthesis of photosynthetic proteins.

As compatible osmolytes, soluble sugars did not seem to be involved in the response of sour orange clones to water stress. Pérez-Pérez *et al.* (2007) reported a significant decrease of soluble sugar contents in comparison with control plants in Carrizo citrange plants cultivated under water stress. In contrast, Wu *et al.* (2008) showed significantly higher soluble sugar contents in young *Poncirus trifoliata* plants subjected to water stress, in comparison with control. On the other hand, our results revealed that no proline was synthesized under water stress. However, Carrizo citrange plants had significantly higher proline contents under water stress (Pérez-Pérez *et al.* 2007). Similar results were reported in young *Poncirus trifoliata* plants under drought (Wu *et al.* 2008).

At the leaf ultrastructural level, details of mesophyll cell and chloroplasts in particular were observed as these organelles are commonly the site of the earliest abiotic injury visible in plant ultrastructure (Shao *et al.* 2016). TEM micrographs of the Kliaa clone exhibited a similar mesophyll cell ultrastructure as control, while marked alterations were observed in the GM clone, with disintegrated chloroplast membranes. Liu *et al.* (2010) reported similar results in *Cucumis sativus* L. varieties under PEG-induced osmotic stress, with leaves exhibiting disintegrated thylakoid membranes. The presence of plastoglobules would be related to membrane disintegration (Bondada and Syvertsen 2005). Przybył and Idzikowska (2003) reported that reduced chloroplast size, degeneration of the membrane systems of thylakoids (swelling and disappearance), and increased electron density of plastoglobuli are some of the senescence-related changes.

In conclusion, this study highlighted different adaptive strategies to water stress in sour orange. In fact, the active stomatal regulation observed in the Kliaa clone subjected to a 3-week water stress period indicated an avoidance strategy. However, the GM clone did not show a strategy to face severe water stress as stomata were closed, photosynthesis affected and water potential declined. This contrasting behavior of the Kliaa and GM clones towards water limitation could be attributed to genetic differences as confirmed by RAPD. Finally, sour orange multiplication by seeds seems to generate a noticeable variability of responses to water stress within plants in young *Citrus* orchards.

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