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Effect of drought on growth, photosynthesis and total antioxidant capacity of the Saharan plant *Oudeneya africana*

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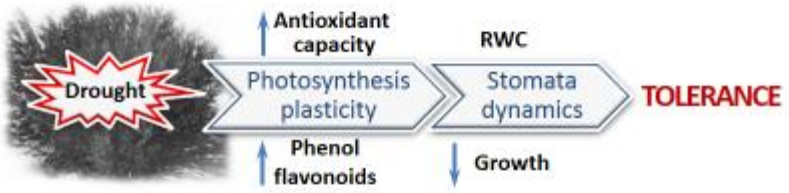
Graphical abstract



Highlights

- *Oudneya africana* responds to drought by increasing instantaneous and intrinsic water use efficiency, indicating that photosynthetic limitations could be due to stomatal closure.
- *O. africana* has a high capacity to restore photosynthesis after re-watering
- Polyphenols, flavonoids and total antioxidant capacity increased with increasing drought stress severity thus enabling the plant to survive under adverse environmental conditions

Abstract

Plants in arid and semi-arid regions are often exposed to adverse environmental conditions such as drought which can affect plant growth. In this study, we investigate the physiological responses of *Oudneya africana* to drought, using two different irrigation regimes (treatment 1: 50% field capacity; treatment 2: 25% FC), a 10- and 20-d time course analysis and a 5-d re-

watering period following drought. Our results show that water deficiency reduced growth mainly in T2 plants after 20 d of treatment, with a reduction of 26% in plant height, 64% in leaf numbers and of 39% in leaf area, as well as a significant decrease (50%) in the photosynthesis rate and chlorophyll content. While both instantaneous (A/E) and intrinsic (A/gs) water use efficiency were observed to increase by 96.3% and 173.20%, respectively, stomatal closure increased with time and the severity of drought, mainly in the abaxial side (50%), as evidenced by gs and Ci/Ca data. Polyphenols, flavonoids and total antioxidant capacity increased close to 2-3-fold, with increasing drought stress severity. Re-watering led to a recovery in most of the parameters analyzed, mainly the photosynthetic parameters, while antioxidant capacity remained high. Given these results, the plasticity of photosynthesis and the high antioxidant capacity of *O. africana* appear to contribute to its tolerance to drought.

Key words: antioxidants, desert plant, drought stress, *Oudneya africana*, phenols, photosynthesis

Abbreviations: A, photosynthesis rate; $ABTS^{\bullet+}$, 2,2′- azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical; Chl, chlorophyll; $DPPH^{-}$, 2,2-diphenyl-1-picrylhydrazyl; E, transpiration; FC, field capacity; gs, stomatal conductance; Ci/Ca, intercellular to ambient CO_2 ratio; TEAC, Trolox-equivalent antioxidant capacity; TPC, total phenolic content.

1. INTRODUCTION

In recent years, due to climate change challenges and harsh natural environments, a series of problems, such as declining vegetation cover, poor plant growth and widespread water stress, have arisen (Li et al., 2013). In particular, drought, one of the major abiotic stresses, reduces plant growth by affecting various physiological and biochemical processes, such as membrane integrity, pigment content, osmotic adjustments, water relations, secondary metabolism (Marchese et al., 2010; Chiappero et al., 2019), stomatal closure and, consequently, photosynthetic activity (Praba et al., 2009; Ma et al., 2016; Moualeu-Ngangue et al., 2017). Photosynthesis, the most fundamental and intricate physiological process in green plants (Ashraf and Harris, 2013), is highly susceptible to many environmental factors, including temperature, light intensity, CO_2 concentration, humidity and soil moisture (Ashraf and Harris, 2013). The decrease in the photosynthetic rate under environmental stress conditions is mainly attributed to stomatal regulation, with disruption in the supply of CO_2 caused by stomatal closure, non-stomatal regulation, mainly associated with reduced Rubisco activity, CO_2 availability in the chloroplast, and PSII photochemistry efficiency (Chaves et al., 2009; Xu et al., 2014; Wang et al., 2019). Under limited water conditions, plants undergo stomatal closure to prevent further water loss, which limits CO_2 availability for photosynthesis, thus giving rise to a reduction in NADPH (Kalefetoglu and Ekmekci, 2005). Besides stomatal limitations, impairments in leaf photochemistry and biochemistry are additional factors that cause a decrease in photosynthesis during water stress (Flexas et al., 2009). However, stomatal closure and mesophyll conductance are the principal factors limiting photosynthesis during the adaptation of plants to drought stress (Flexas et al. 2009). Ashraf and Harris (2013) have reported that drought and high-temperature stress adversely affect the functionality of both photosystems and reduce electron transport, giving rise to a reduction in ATP and NADPH.

The relative content of chlorophyll, one of the major components of chloroplast, is positively related to the photosynthetic rate. Numerous studies have shown that drought stress can significantly decrease photosynthetic pigment content (Chl a, Chl b and Chl a + b) (Dias et al., 2018; Semerci et al., 2016). Under drought stress conditions, the decrease in chlorophyll content could be considered a typical symptom of oxidative stress as a result of pigment photo-oxidation and chlorophyll degradation (Anjum et al., 2011; Ashraf and Harris, 2013). Drought causes oxidative stress through a reduction in CO_2 assimilation, which induces an excess of excitation energy and electron flux to O_2, giving rise to photo-oxidative stress and reactive oxygen species (ROS) overproduction (Zou et al., 2009). ROS are highly reactive species which damage proteins, chlorophylls, membrane lipids and nucleic acids (Halliwell and Gutteridge, 2007). Cells have developed an important, complex system of enzymatic and non-enzymatic antioxidant defenses to cope with the negative effects of ROS. Enzymatic antioxidants, including catalase (CAT), superoxide dismutases (SODs) and peroxidases (POXs), are among the most important antioxidants; ascorbic acid, glutathione, flavonoids, phenols and carotenoids are examples of non-enzymatic antioxidants which are more abundant in plants (Halliwell and Gutteridge, 2007).

Phenolic compounds and flavonoids are among the most important and widely distributed secondary products in plants (Ali and Abbas, 2003). These metabolites complement the enzymatic antioxidant system and have a considerable potential to reduce ROS and to prevent cell damage (Agati and Tattini, 2010). Their biosynthesis and accumulation are generally

induced in response to biotic and abiotic stimuli such as drought stress in plant tissues (Naczk and Shahidi, 2004). Under drought conditions, flavonoids can preserve the integrity of the chloroplast membrane through lipid remodeling in order to prevent oxidative damage (Inoue et al., 2011).

Oudneya africana is an endemic Saharan plant of the *Brassicaceae* family widely found in the Libyan, Tunisian, Algerian and Moroccan deserts (Quezel and Santa, 1963). The principal role of this desert plant is to stabilize mobile dunes (Chaieb and Boukhris, 1998) and is also used for medicinal purposes for digestive problems, colds, flu, fever and scorpion bites (Bouhadjera et al., 2005). Our overall goal was to study *Oudneya africana*, a good model to evaluate some of the survival mechanisms under extreme drought conditions, by analyzing its growth and several photosynthetic mechanimes, total non-enzymatic antioxidant capacity under moderate and severe stress conditions, as well as 10- and 20-day treatment periods. This study, which also analyzes the effect of plant re-watering following drought, should provide invaluable information to design new strategies for sustainable agriculture and ecosystem preservation in arid areas.

2. MATERIAL AND METHODS

2.1. Seed collection and site description

Dried *O. africana* seeds were collected from a natural population in Ksar Ghilen in southern Tunisia (Fig. 1 a). The site is characterized by an arid climate, with dry and hot summers and cold winters. Rainfall of less than 150 mm is irregular and sometimes absent for several years. The soil is sandy, with dunes reaching 12m in height (Fig. 1 b). The Ksar Ghilen site is dominated by *O. africana*, *Ephedra alata*, *Stipgrostis pun*gens, *Retama raetam* and *Calligonum azel.*

2.2 Growth conditions and treatments

Germination experiments were conducted in the dark in an incubator set at 18ºC. Pots (2L), containing dried sand and compost (2:1), with a drainage layer at the bottom of the pot, were weighed, and a nutritive solution was added; when the water was drained, the corresponding weight was estimated at 100% FC. Three germinated seeds were transplanted to each pot and three pots were used for each treatment. Plants were grown under greenhouse conditions: day/night temperatures of 23°C/18°C, 16 h photoperiod, photon flux density of 400 µmol. $m^{-2}.s^{-1}$ and relative humidity of 70-75%; the plants were irrigated every two days with a nutritive solution containing macronutrients: 0.52 mM KH_2PO_4, 6.75 mM $Ca(NO_3)_2$, H_2O, 1.63 mM $MgSO_4.7H_2O$, 0.085 mM $Mg(NO_3)_2.6H_2O$, 4.63 mM KNO_3, 0.05 mM KOH and SO_4H_2 to adjust pH; and micronutrients: 46.2 µM H_3BO_3, 10.18 µM $MnSO_4$, 0.45 µM $CuSO_4.5H_2O$, 1.53 µM $ZnSO_4.7H_2O$, 0.052 µM $(NH_4)_6Mo_7O_24.4H_2O$ and 80.08 g/L sequestrene 138 FeG 100. After two months of growth, the degree of drought stress was determined according to soil

moisture content. Watering treatment was of three types: normal watering, with soil moisture content at maximum 100% water-holding field capacity (control; 350ml); medium water deficit stress (T1), with soil moisture content at 50% of maximum water-holding field capacity (foregoing water for 8 days, 170ml); and severe water deficit stress (T2), with soil moisture content at 25% of maximum water-holding field capacity (foregoing water for 8 days, 85ml). Soil water content at field capacity was determined by weighing the pots. Overall, for the experiment, the nutrient solution was added to maintain the pots at 50% FC (medium stress) and 25% FC (severe stress) for 10 days for the first harvest and for 20 days for the second harvest. The third harvest was obtained after 5d of rewatering. The study was carried out using 3 extracts (one per pot) containing a mix of leaves from three different plants per extract (total of 9 plants) for each treatment and two independent experiments were carried out with at least 18 plants per treatment. Plant growth was monitored and sampled every two days over a period of 25 days by counting the number of green leaves and by measuring plant height, leaf elongation and leaf area (cm^2) by scanning leaves using Mesurium pro 6 software.

2.3. Leaf relative water content

Leaf relative water content (RWC) was analyzed as described by Talbi et al. (2015) using the formula provided by Schonfeld et al. (1988): RWC = (FW-DW) / (TW-DW) X 100. Fresh weight (FW) of leaves was measured by weighing the leaves which were then cut into small pieces, placed in glass tubes, covered with distilled water and kept at 4 ºC overnight. Turgid

weight (TW) was then calculated by weighing the pieces, which were then dried at 70 ºC for 72 h, followed by measurement of their dry weight (DW).

2.4. Chlorophyll and carotenoids

Leaf pigment content was determined according to the method described by Lichtenthaler and Welburn (1983). 0.1 g of leaves was extracted with 5 ml of pure acetone using a mortar. The pigment extracts were centrifuged at 2,500 rpm for 9 min. The resulting extracts were assayed spectrophotometrically. Concentrations ($\mu g.ml^{-1}$) of Chl a, Chl b and total carotenoids were determined using the following equations:

$$\mathbf{C_a = 11.75\ A_{662} - 2.350\ A_{645}}$$

$$\mathbf{C_b = 18.61\ A_{645} - 3.96\ A_{662}}$$

$$\mathbf{Car = (1000\ A_{470} - 2.270\ C_a - 81.4\ C_b) / 227}$$

2.5. Gas exchange parameters

The CO_2 assimilation rate was determined in the upper third fully expanded leaf of the control and treated plants. The photosynthesis rate (A), transpiration (E) and stomatal conductance (gs) were determined using a portable LI-6400 infrared gas analyzer (LI-COR Biosciences, Inc., Lincoln, NE, USA) by changing light intensities (light curves) with a range of 0 to 2000 quanta $m^{-2}s^{-1}$ or photosynthetic active radiation (PAR). The photosynthetic parameters were calculated using LI-6400 6.1 software. The intercellular to ambient CO_2 ratio (Ci/Ca) was determined, with the ambient CO_2 concentration being 380 $\mu mol.mol^{-1}$. Intrinsic water use efficiency (WUE_{int}) was calculated as the ratio of photosynthetic assimilation (A) to stomatal conductance

(gs) for water vapor, while instantaneous water use efficiency (WUE_i) was calculated as the ratio of photosynthetic assimilation (A) to transpiration (E) (Polley, 2002).

2.6. Stomatal assay

Abaxial and adaxial epidermal strips from the third, fully-expanded *O. africana* leaf of the control and treated plants were mounted on glass slides. Four images of each epidermal strip were taken using a Leica stereomicroscope connected to a Nikon NIS-F1 CCD camera and a Nikon DS-U3 controller (Nikon, Tokyo, Japan). The percentage of closed stomata (number of closed stomata / number of epidermic cells) x 100 and stomatal index (number of stomata / number of epidermal cells + number of stomata) x100 was calculated as described by Kubinova (1994).

2.7. Assays of antioxidant defense systems

2.7.1. Extraction of total phenolic contents and flavonoids

Air-dried and powdered *Oudneya africana* leaves (1g) were extracted three times with MeOH at room temperature. The combined extracts were evaporated to dryness using a rotary evaporator. The precipitate was dried, dissolved in 10 ml of absolute methanol and kept at -20°C.

2.7.2. Analysis of total phenolic compounds

The amount of total phenolics was determined with Folin–Ciocalteu reagent according to the method described by Lister and Wilson (2001). A standard curve with gallic acid was used. Different concentrations of gallic acid were prepared in methanol, and absorbance was recorded

at 760 nm. 100 µl of diluted sample (1:10) was dissolved in 500 µl of Folin–Ciocalteu reagent and 1000 µl of distilled water. The solutions were mixed and incubated at room temperature for 1 min. After 1 min, 1500 µl of 20% sodium carbonate (Na_2CO_3) solution was added. The final mixture was shaken and then incubated for 2 h in the dark at room temperature. The absorbance was measured at 760 nm using a Milton Roy 601 UV–vis spectrophotometer and the results are expressed in mg plant dry weight of gallic acid (GEA). Analyses were carried out in triplicate.

2.7.3. Estimation of total flavonoid content

Flavonoid content in extracts was determined spectrophotometrically according to the method described by Lamaison and Carnat (Quettier-Deleu et al., 2000) based on the formation of a flavonoid–aluminium complex, with maximum absorbance at 430 nm. Quercetine was used to obtain the calibration curve. 1 ml of the diluted sample (1:10) was separately mixed with 1 ml of 2% aluminum chloride methanolic solution. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm with a Milton Roy 601 UV–Vis spectrophotometer and flavonoid content was expressed in mg per g of Quercetine equivalent (QE). All measurements were performed in triplicate.

2.7.4. 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity

The scavenging activity on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined spectrophotometrically according to the method reported by Okonogi et al. (2007). The principle of the assay was based on the color change of the DPPH solution from purple to yellow

as the radical was quenched by antioxidants. The leaf extracts were mixed with methanol to prepare a stock solution of each leaf extract (10 mg/ml). 500µl DPPH (150 µM) was dissolved in methanol and mixed with a 500 µl aliquot of different leaf extract dilutions. The mixture was shaken vigorously and left to stand for 30 min in the dark at room temperature. The absorbance was recorded at 517 nm to determine the concentration of the remaining DPPH. All measurements were performed in triplicate. The radical-scavenging activity was calculated as a percentage according to the equation:

$$\textbf{DPPH radical-scavenging (\%)} = \left(\frac{A_0 - A_1}{A_0} \right) \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. The IC50 value denotes the concentration of the sample required to scavenge 50% of the DPPH free radicals. The lower absorbance of the reaction mixture indicates a higher level of free radical scavenging activity.

2.7.5. 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity

The 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$) assay was based on the slightly modified method described by Re et al. (1999). The $ABTS^{\cdot+}$ radical cation was produced by the reaction of 7 Mm ABTS solution with 2.45 mM potassium persulphate, and the mixture was left to stand in the dark at room temperature before use. The $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. After adding the 25 µl

sample or Trolox (used as standard) to the 2 ml diluted $ABTS^{\cdot+}$ solution, absorbance at 734 nm was measured after 5 min. Results were expressed as Trolox equivalent antioxidant capacity (TEAC).

2.8. Imaging of phenols and carotenoids by confocal microscopy

Handmade cross sections of O. *africana* leaves were placed between a slide and a cover slide. The distribution of carotenoids was imaged using an argon laser with excitation at 488 nmand emission at 500-580 nm. Chlorophyll was imaged using a He:Ne laser with 633 nm excitation and 650-700 nm emission. Phenols were imaged at 405 nm excitation and 448-483 nm emission.

2.9. Statistical assay

The data were analyzed using the ANOVA test with the aid of Info-Gen software (2012). When F-tests were significant, the differences between treatment means were compared using Tukey's multiple comparison tests at 0.05 probability. Correlation analyses of the different variables were performed. Significance levels were represented by * at $0.01 < p < 0.05$, ** at $p < 0.01$, and NS, Not significant. The data shown are mean values ± SE.

3. RESULTS

3.1. Effect of drought stress on plant growth

Plants were exposed to two different irrigation regimes for 10 and 20 days and then re-watered (RW) for 5 days. To study the effect of drought on *Oudneya* growth, we analyzed plant length, leaf expansion and leaf number (Figs. 2 a-c). At the beginning of stress, no significant effect of

drought on leaf number was observed, while leaf elongation (Fig. 2) and leaf area (Table 1) showed a significant reduction. The differences became more significant ($p<0.001$) and the effect of drought was stronger after 20 d of T2, with the highest reduction observed in the number of leaves (63.99 %), followed by leaf area (39.32%) (Table 1), leaf expansion (31.45 %) and plant length (26.45%) (Fig 2). After the re-watering period, a slight increase in leaf expansion was observed, while no significant change was observed in plant length or leaf number (Fig. 2).

3.2. Gas exchange responses during drought stress and recovery

Drought stress caused a significant reduction in net photosynthesis (A), stomatal conductance (gs) and the intercellular to ambient CO_2 ratio (Ci/Ca) after 10 to 20 days of drought ($p<0.001$) in T2 (Table 2). The value of A in well-watered plants, which was 21.7 µmol CO_2 m^{-2} s^{-1}; after 10 d of drought, declined by 4% and 20% in T1 and T2, respectively, and by 44,14% in T2 after 20 d of treatment (Table 2). The light-response curve (A/Q) at ambient CO_2 is shown in Fig 3 (a-c). Under control conditions (Fig. 3 a, b and c), plant photosynthesis reached a maximum of 21.7 ±1.05 µmol $m^{-2}s^{-1}$, 22.2 ±1.1 µmol $m^{-2}s^{-1}$ and 23.4 ±0.55 µmol $m^{-2}s^{-1}$ at 2000 µmol $m^{-2}s^{-1}$, respectively. Photosynthesis decreased slightly in relation to control, when T1 plants were watered at 50% FC (Figs. 3 a, b and c), reaching values of 20.76 ±0.96 µmol $m^{-2}s^{-1}$ and 19.86 ±0.59 µmol $m^{-2}s^{-1}$ after 10 and 20 days at 2000 µmol $m^{-2}s^{-1}$, respectively. Re-watering facilitated a very slow recovery to a value similar to that of the control at 2000 µmol $m^{-2}s^{-1}$. On the other hand, the photosynthetic capacity of plants subjected to T2 (25% of FC) for 20 days

was drastically affected. At intensities of 400 to 2000 µmol $m^{-2}s^{-1}$, net photosynthesis of T2 plants was reduced by 50%, similar to that of the well-watered plants (Fig. 3 a, b and c). Surprisingly, 5 days of re-watering induced a rapid recovery, reaching values similar to those recorded after 10 days in T2. Similarly, the transpiration rate decreased by 26 % after withholding water for 10 d and by 72% after 20 d in T2 (Fig. 3 d, e and f; Table1). The effect of T1 on E was visible after 20 days (Fig. 3 e and Table 1). The recovery in E at intensities of 400 to 2000 µmol $m^{-2}s^{-1}$ was very slow after T1 was applied, while recovery was faster in the T2 plants (Fig. 3 f). Drought caused a slight reduction in stomatal conductance (gs) after withholding water for 10 d in T1 as compared to control plants (Table 2). However, a sharp reduction of 42% and 80% was observed in T1 and T2, respectively, after 20 d of treatment (Fig. 3 f, h and i; Table 2). At higher intensities of 750 to 1500 µmol $m^{-2}s^{-1}$, a rapid increase in gs was observed after 5 days of re-watering of the T2 plants, which showed values above those obtained after 10 days, to finally reach similar gs at 2000 µmol $m^{-2}s^{-1}$ (Fig. 3). The intercellular to ambient CO_2 ratio (C_i/C_a) shows a similar pattern to that of gs, with a sharp decrease of ~75% observed after 20 days of T1 and T2 treatment, while the reduction reached ~20% after 10 days of treatment (Table 2). Interestingly, after 5 days of re-watering, CO_2 assimilation, E and gs gradually recovered, with a rapid recovery observed in C_i/C_a values to close to that recorded in control values (Table 1 and 2). Thus, in T2 re-watered plants, net photosynthesis was restored to 72.5 % of the control values, stomatal conductance to 60.8% (Table 2) and transpiration rose

to 68% (Table 1) of the levels recorded for the control plants. In addition, only a slight reduction in relative water content (RWC) was observed in T2 plants after 20 days (Table 1).

Instantaneous water use efficiency (WUE = A_{CO2} / E) and intrinsic WUE (WUE_i = A_{CO2}/gs) showed similar patterns, with a significant increase observed in T1 and T2 plants after 20 days. The most significant change was detected with respect to WUEi in T2 plants, which increased by 173% ($P < 0.01$; Fig. 4). WUE also increased significantly by 97% after 20 d. Following the re-watering period, WUEi and especially WUE reached values similar to those of control plants (Fig. 4).

3.3. Stomatal dynamics

The percentage of closed stomata was analyzed using stereomicroscopic images and the results are shown in Fig. 5. The percentage of closed stomata was similar in abaxial and adaxial leaf surfaces, whose values increased with the time and intensity of drought, although the largest changes were observed in the abaxial surface after 20 days of treatment (Figs. 5a and b). Images of the epidermis showing stomata in control and T2 plants after 20 days of drought are shown in Fig 5 C and D. The stomatal index (Suppl Fig. 1) did not vary greatly between the abaxial and adaxial surfaces, although the largest increase (4.33%) under drought conditions was observed in T1 plants after 20d in the adaxial surface.

3.4. Chlorophyll and carotenoid content

Analysis of chlorophyll and carotenoid content showed a similar pattern of changes associated with both time and intensity of drought treatment (Fig. 6). There was a significant increase in

concentrations of chlorophyll a and b in control plants throughout the period of analysis, while a significant reduction in chlorophyll a and b was observed in T1 and T2 plants after 20 days of treatment, with an increase observed in T1 plants after 10 d of treatment. Carotenoids followed a similar pattern to that of chlorophyll b, with a significant reduction in T1 and T2 observed only after 20 d of treatment, while re-watering did not significantly restore the values for chlorophyll and carotenoids (Fig. 6). Confocal microscopic imaging of carotenoids shows their localization in the cuticle, epidermis and vascular tissues (Figs. 7 a and b). In mesophyll cells, carotenoids were mainly located in chloroplasts, as shown by the overlap with the red emission of chlorophyll producing an orange color (Figs. 7 a-b).

3.5. Effect of drought on total phenolic and flavonoid content

Total phenolic content (TPC) varied with time and severity of drought treatment (Table 3), with values of 18.76 and 20.63 mg GAE.g^{-1} DW for T1 and T2, respectively, recorded after 10 days of treatment. However, the largest increase was associated with T2 plants after 20 days of treatment, with a 3.39-fold increase as compared to control plants during the same period of treatment (Table 3). On the other hand, re-watering considerably reduced TPC in both T1 and T2 plants as compared to the plants treated for 20 days (Table 3). Phenols were imaged in leaf cross sections by confocal microscopy at 448-483 nm emission. Most phenols were observed in the cuticle, epidermis and cell wall of vascular tissue, mainly in the xylem (Fig. 7 a and c). In mesophyll cells, the blue color, due to phenols, was associated with small non-identified organelles but was not observed in the vacuole (Figs 7 a and c).

Like phenols, flavonoids also increased significantly with both time and intensity of drought treatment. In T1 plants, flavonoid content was approximately 1.74- and 2.81-fold higher than in the control plants between 10d and 20d of treatment, respectively. In T2 plants, the increase was even higher (about 2.02- to 3.55-fold higher) than in the respective control plants at d 10 to d 20. Recovery from drought after 5 days of re-watering showed a 2.52- to 2.69-fold decline for T1 and T2 plants, respectively, as compared to the stressed plants (Table 3).

3.6. Effect of water deficit on antioxidant capacity

The capacity of *O. africana* leaf extracts to scavenge $DPPH^{+\cdot}$ free radicals, which has been used as a measure of total antioxidant capacity, is shown in Table 4. Leaf extracts from T2 plants after 20 d of treatment showed a greater capacity to scavenge free radicals (IC50 = 0.71 $mg.ml^{-1}$) as compared to control (IC50 = 3.3 $mg.ml^{-1}$) and T1 plants (IC50=0.77 $mg.ml^{-1}$). After re-watering, the IC50 value increased as compared to stressed plants (Table 4).

The results for Trolox equivalent antioxidant capacity (TEAC) are shown in Table 4. Drought stress induced an increase in TEAC associated with both length and intensity of water deprivation (Table 4). The largest increase (2.5-fold) was recorded after 20d in T2 plants.

4. DISCUSSION

4.1. Drought affects growth and photosynthesis of *Oudneya africana*

Drought, one of the most significant abiotic stresses in plants, is associated with arid and semi-arid areas (Weltzin and Tissue, 2003; Liu and Hwang, 2015). In deserts, water plays a crucial

role in sustaining the ecosystem, mainly with regard to vegetation (Huang et al, 2016). Desert plants exposed to a prolonged water deficit have developed different mechanisms, including morphological, physiological and biochemical modifications, to withstand adverse conditions imposed by drought (Farooq et al., 2012; Okunlola et al., 2017). In our experiment, water deficit caused a significant reduction in the growth of *O. africana*. Similar results were reported in different plant species such as Okra and maize (Gao et al, 2020; Jafarnia et al. 2018). The reduction in weight and leaf area can be explained by disturbances in both cell division and cell enlargement under drought stress conditions owing to turgor loss and decreased photosynthesis and energy supply (Taiz and Zeiger 2010; Luo et al, 2013). As the reduction in leaf area has been considered a survival strategy to reduce transpiration (Correia and Nogueira, 2004; Baerenfaller et al., 2012), leaf area plasticity can be regarded as an important strategy for controlling water use efficiency in these plants. We found a significant positive correlation between leaf area, leaf elongation, plant length, leaf number and gas exchanges (Table 5), which suggests that the limitation in plant growth caused by scarce water availability could be due, in part, to a reduction in carbon availability, as suggested by Claeys and Inzé (2013). Interestingly, RWC data did not vary significantly during treatment except in T2/20d-treated plants, suggesting that *O. africana* showed a better ability to maintain favorable water balance under drought conditions, which is considered a useful parameter for evaluating plant tolerance to drought (Bayoumi et al. 2008).

Photosynthesis is one of the most important indicators of physiological sensitivity to abiotic stress (Dikšaitytė et al., 2018). Sensitivity to environmental changes in photosynthesis varies among plant species and stress factors (Lin et al. 2017; Wang et al., 2019). Drought stress induced a decrease in photosynthetic machinery function in *O. africana*; however, despite the decline observed in net photosynthesis (A), 20 d of treatment were needed under the most severe drought conditions (T2) to cause a drastic reduction in CO_2 assimilation. Interestingly, after 5 d of re-watering, CO_2 assimilation, E, gs and Ci/Ca gradually recovered (Table 1 and 2). Thus, in T2 re-watered plants, net photosynthesis was restored to 72.5 % of the values for control plants, stomatal conductance to 60.8% and transpiration to 68% of control levels (Table 1 and 2). This highlights the capacity of *Oudneya africana* to tolerate drought over a long period of time and to rapidly restore its photosynthesis and transpiration capacity. This exceeds the stress threshold, demonstrating that *Oudneya africana* tolerates and adapts to these adverse conditions and may survive under more severe drought conditions, such as those prevailing in the desert where this plant is located, with an increase observed in the number of closed stomata in the abaxial and adaxial leaf surfaces. With regard to decreasing CO_2 assimilation under drought stress, Farooq et al, (2009) have suggested that it could be due to a restriction in CO_2 diffusion in the leaves and to an inhibition of ATP synthesis and Rubisco activity. The closure of stomata in *Oudneya* was correlated with a reduction in stomatal conductance (gs) which can protect plants against water loss and thus improve water use efficiency (Correia et al., 2018; Elferjani and Soolanayakanahally, 2018). However, the value of gs in most treatments exceeded 0.1 mol

$H_2O\ m^{-2}\ s^{-1}$ except in T2/20-d-treated plants, which, as reported by Flexas and Medrano (2002), is considered a low level of stress, while gs reached 0.045 in T2/20-d-treated plants which corresponds to severe drought. Sub-stomatal CO_2 concentration (Ci) levels declined after 10 days of drought treatment and even more sharply after 20 days, which implies that the Ci/Ca ratio decreased significantly. This decrease coincides with that in stomatal conductance (gs), suggesting that stomata limited photosynthesis (A) after 20 days of treatment, as can be seen in Table 2 and Figure 3 (Ort *et al*., 1994), while Ci fully recovered after re-watering. Additional parameters need to be analyzed in order to identify stomatal and non-stomatal limitations to photosynthesis in our experimental design (Varone et al., 2012). Interestingly, no significant changes were observed in the stomatal index during drought treatment. Meanwhile, *Populus* leaves grown under water-deficit conditions show lower stomatal indices than those under well-watered conditions (Hamanishi et al., 2012). Moreover, a reduction in stomatal density has been reported to increase drought tolerance in barley (Hughes et al., 2017).

Overall, drought stress effects can be either direct or indirect, with a decrease in CO_2 availability due to a reduction in stomatal aperture and mesophyll conductance being an example of a direct effect (Flexas et al. 2012; Zivcak et al. 2013), while indirect effects are more associated with changes in photosynthesis (Wang et al, 2019). Stomatal conductance (gs) was affected more than net photosynthesis by drought stress, while both intrinsic and instantaneous WUE increased when less water was available. This indicates an optimization of carbon uptake versus water loss, and the sharper reduction in gs shows that non-stomatal components could play an

important role in limiting photosynthesis when plants undergo prolonged water deficit in the field (Earl, 2002). It has been suggested that gs could be used as an indicator of WUE under water-limited conditions (Gulías et al., 2012; Liu et al., 2019). In addition, as reported by Flexas et al. (2014), the increase in intrinsic WUE is an adaptive strategy in Mediterranean plant species under drought stress conditions.

Re-watering gives rise to a partial recovery in photosynthetic machinery (Table 2) and a total recovery in both WUEs, with intrinsic WUE being reported to increase under water stress conditions (Flexas et al., 2002). This highlights the capacity of *Oudneya africana* to tolerate drought over a long period of time and to rapidly restore its photosynthesis and transpiration capacity. This capacity of *O. africana* to recover from drought could be especially useful to develop new drought tolerance breeding programs which will require further in-depth biochemical and molecular analysis.

Chlorophyll content is also a key factor in plant photosynthesis and closely reflects the photosynthetic capacity of plants (Takai et al., 2010). Drought stress induced a sharp increase in chlorophyll content after 10 d in T1 plants, although longer periods of treatment, which reduce the content of both chlorophyll a and b mainly under the most severe drought conditions, could be the main cause of inactivation of photosynthesis. Our results are consistent with those reported in tomato (Sánchez-Rodríguez et al., 2012) and in soybean (Wu and Zhang et al., 2019). A significant correlation was found between Chl a and A (0.76), Chl a and E (0.84), and Chl a and gs (0.87) (Table 5), which confirms the role of photosynthetic pigments in carbon

fixation. Furthermore, the decrease in chlorophyll content is also considered to be a typical symptom of oxidative stress and may be the result of pigment photo-oxidation (Dias et al., 2018; Liu et al., 2011; Semerci et al.,2016).

Carotenoids are among the most important quenchers of singlet oxygen which causes oxidative damage to chlorophyll and other pigments under drought conditions (Jahns and Holzwarth, 2012). Therefore, carotenoids can protect the photosynthetic apparatus from photooxidation by helping to dissipate excessive excitation energy in both PSI and PSII (Flowers and Colmer, 2008). However, our results show that carotenoids decreased after 20d, while re-watering did not significantly increase carotenoid content, suggesting that these compounds do not contribute to the drought tolerance of *O. africana*. Confocal analysis of these compounds, which are also found in the cuticle and xylem cell wall, showed co-localization with chloroplasts. Carotenoids are associated with plastoglobuli and chloroplasts in algae (Solovchenko, 2010).

4.2. Drought induces total phenolic content and antioxidant capacity in *O. africana* leaves.

Under stressful conditions, plants must prevent ROS accumulation or alleviate ROS-induced oxidative damage such as lipid peroxidation, protein and DNA oxidation (Cao et al., 2014). To cope with oxidative stress damage, complex antioxidant defense systems are activated to detoxify the harmful effects of ROS ; these include non-enzymatic systems, such as glutathione, a-tocopherols, carotenoids and polyphenols, and enzymatic systems such as superoxide dismutase, glutathione peroxidase and glutathione reductase (Gill and Tuteja, 2010). ROS production and oxidative damage are important components of the negative effects of

drought on plants, which may lead to oxidative damage to the photosynthetic apparatus (Noctor et al., 2014; Talbi et al., 2015). In a previous study, we demonstrated that increasing drought gives rise to oxidative stress, characterized by increased H_2O_2 content and lipid peroxidation (Talbi et al., 2015). In *O. africana*, drought induced an increase in total antioxidant capacity as measured by ABTS and DPPH methods which is proportional to the duration and intensity of water deprivation. A similar pattern was observed in the content of flavonoids and phenols, with a high correlation being observed with antioxidant capacity (Table 5). This agrees with the results reported for *Chrysanthemum morifolium* (Hodaei et al., 2018) and *Capsicum* species (Okunlola et al., 2017), olive leaves (Ben Abdallah et al., 2017) and *Amaranthus tricolor* (Sarker and Oba, 2018). The enhancement of phenolic compounds in plants is a common response to circumstances when photosynthesis is impaired by environmental constraints (Navarro et al., 2006), and Gao et al. (2020) reported that, as flavonoid and phenol content increases under water stress, the more plants adapt to drought. Production of phenolic compounds has been reported to be one of the strategies used by certain native xerophytic plant species, such as *Larrea divaricata* and *Lycium chilense* in Patagonian shrublands, under adverse environmental conditions to prevent oxidative damage caused by drought (Varela et al, 2016). However, Sánchez-Rodríguez et al., (2010) have observed a negative correlation between drought tolerance and phenol/flavonoid content in different tomato genotypes. Other studies have shown that osmotic stress inhibits phenolic compound synthesis in *Vitis amurensis* (Weidner et al., 2007) and *Vitis californica* (Weidner et al., 2011) germinating seeds. Therefore,

phenol accumulation could be a specific adaptive response of plants to drought conditions. In fact, we observed a decrease in total phenolic content during the recovery period following drought stress, which correlates with a reduction in lipid peroxidation during this period (Talbi et al., 2015), suggesting that oxidative stress declines under re-watering. In addition to their antioxidant properties, phenolic compounds are considered to be anti-inflammatory, anti-mutagenic and anti-carcinogenic and are also able to modulate certain key enzymatic functions in cells (Imrul et al., 2013). Some of these properties are associated with the medicinal properties of *Oudneya* in traditional North African culture due to the high concentrations of phenols in this plant (Bouaziz et al., 2009). With regard to phenol distribution in the leaf, confocal microscopy analysis showed an accumulation of phenols in the cuticle, epidermis, xylem cell wall and mesophyll cells (Fig. 7 a-c). The accumulation of phenolic compounds in the leaf cuticle, which can protect against excessive UV radiation, has been observed in different plant species (Solovchenko, 2010). In mesophyll cells, phenols, though detected in small non-identified organelles, were not present in the vacuoles. These results contrast with previous studies in which phenols are reported to be mainly associated with vacuoles (Solovchenko, 2010). In addition to phenols, a significant increase in enzymatic antioxidants, catalase, superoxide dismutase, peroxidases and glutathione reductase has previously been reported in the same species and under the same experimental conditions (Talbi et al., 2015). Glutathione and ascorbic acid have also been reported to contribute to *O. africana*'s tolerance to drought (Talbi et al., 2015). Therefore, the tolerance to drought stress in *O. africana* could

be due in part to its antioxidant capacity which in turn could protect photosynthesis. In fact, Flexas et al (2006) have reported that the photosynthetic rates of plants presenting higher antioxidant capacity recover to the fullest extent more rapidly. An example of this is the case of Mediterranean plants which are well equipped with photoprotection mechanisms, that are further enhanced during the summer drought period with mechanisms including xanthophyll cycle pigments (Galmés et al., 2007; Peguero-Pina et al., 2008) and an integrated antioxidant defense network (Peñuelas et al., 2004; Munné-Bosch and Lalueza, 2007).

5. CONCLUSION

In summary, increasing drought stress leads to a decrease in plant growth and produces disturbances in photosynthetic machinery. However, *O. africana* responds to these stressful conditions by increasing instantaneous and intrinsic water use efficiency, which indicates that photosynthetic limitations could be due to stomatal closure, although a non-stomatal dependence mechanism cannot be ruled out. It is worth highlighting the high capacity of *O. africana* to restore photosynthesis after re-watering, which could play an important role in the development of new strategies for sustainable agriculture and ecosystem preservation in arid zones. Another mechanism used by *O. africana* to withstand drought is to increase phenol and flavonoid content and hence total antioxidant capacity, thus enabling the plant to survive under adverse environmental conditions. In addition, high phenol and flavonoid content could add important pharmacological properties to this species.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

Author contributions

Sihem Talbi: Investigation, Writing - Original Draft and Formal analysi;s JA Rojas and M. Sahrawy, Investigation; M. Rodríguez-Serrano and K. Cárdenas: Investigation; D. Mohamed Supervision; and Luisa M. Sandalio: Conceptualization, Visualization, Supervision, Funding Acquisition and Writing - Review & Editing

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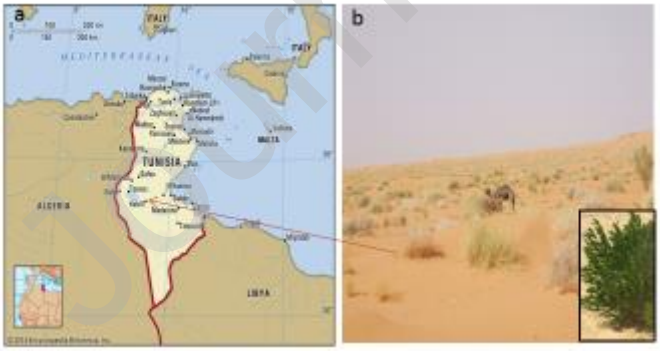

FIG.1 *Oudneya africana* collection site. a, map of Tunisia showing the location of Ksar Ghilen. b, picture showing plants growing in Ksar Ghilen. Insert: picture of Oudneya plant

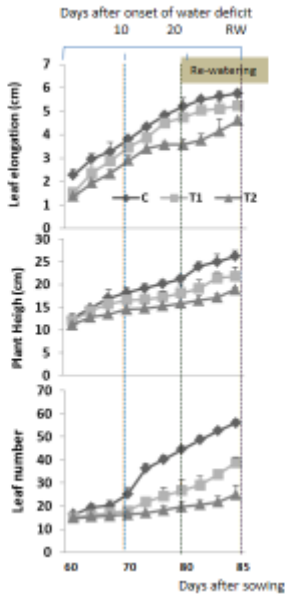

FIG. 2 Plant growth (leaf elongation, plant length and leaf number) of *Oudneya africana* under drought stress conditions; control (C), medium stress (T1) and severe stress (T2) at day 10 and 20 of drought stress and after 5 days of re-watering (RW). Values represent the mean of nine plants ± SE.

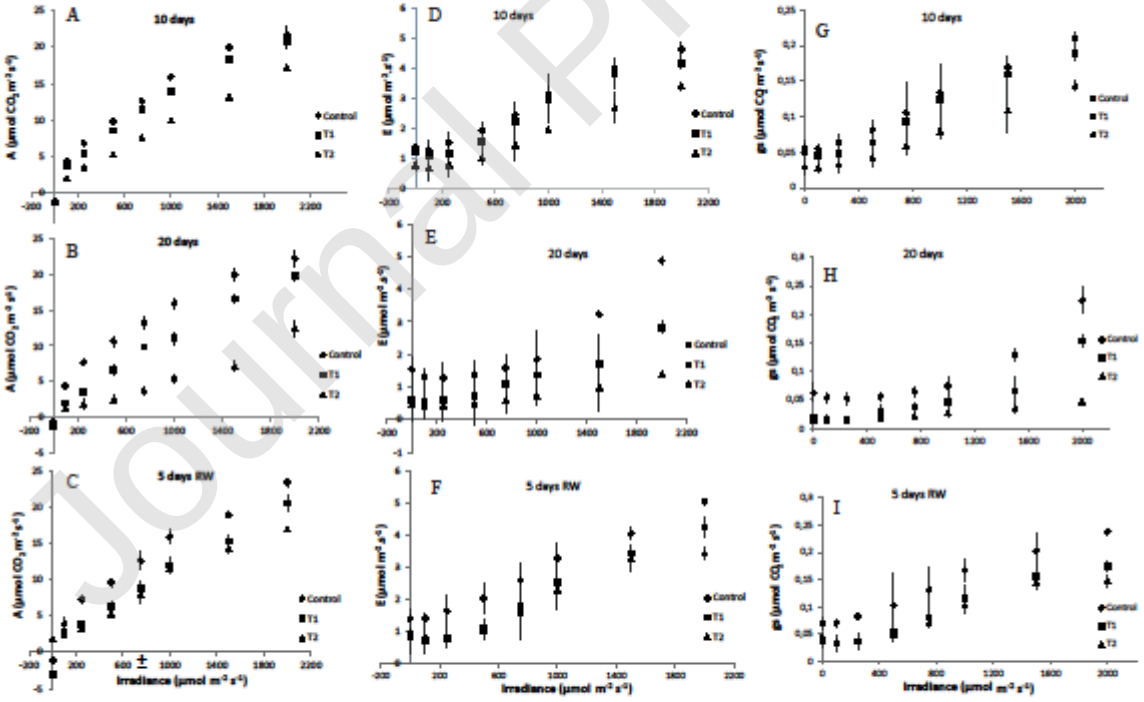

FIG. 3 Photosynthetic capacity of the plant species Oudeyna after 10 (a, d and g) and 20 days (b, e and h) of drought treatment and re-watering for 5 days (c, f and i). C: control, T1: medium stress, and T2:

severe stress. Photosynthetic carbon fixation rates were determined in the same leaf position as a function of increasing irradiance (a, b and c) at saturating CO2 (400 µ mol mol-1 ; A/Q response curve). Transpiration, E (d, e and f) and stomatal conductance, gs, (g, h and i) were determined in the same leaves. Values represent the mean of five plants ± SE.

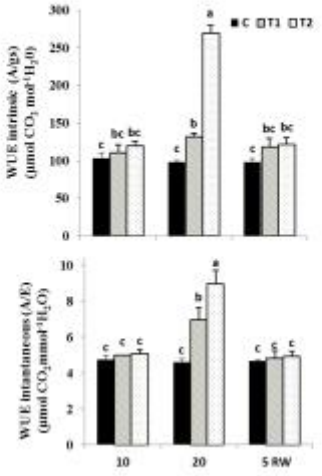

FIG.4 Intrinsic WUE (A/gs) and instantaneous WUE (A/E) under drought stress conditions; C: control, T1: medium stress and T2: severe stress at day 10 and 20 of treatment and after 5 days of re-watering. Data are the mean ± SE of six replicates. Means followed by different letters indicate statistically significant difference (Tukey test; $p < 0.05$).

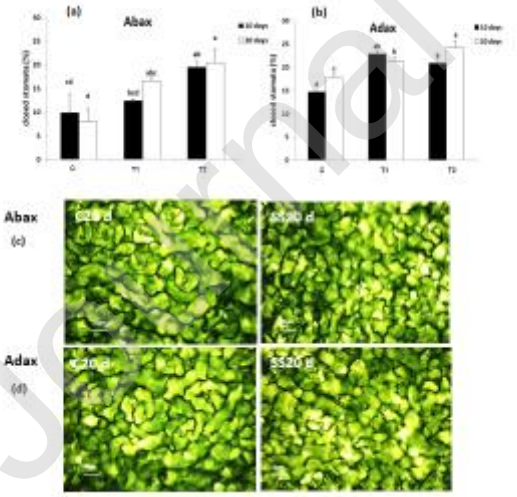

FIG.5 Percentage of closed stomata in abaxial (a) and adaxial (b) leaf surface of *Oudneya africana* under drought stress condition; c: control; T1: medium stress and T2: severe stress at day 10 and 20 of treatment. Data are the mean ± SE of three leaves per plant and three replicates. Means followed by different letters indicate statistically significant difference (Tukey test; $p < 0.05$). Picture of epidermic

stomata in abaxial (C) and adaxial (d) leaf surface under control (C) and severe stress (SS) conditions after 20 days of drought stress. Bars indicate 50 µm magnification.

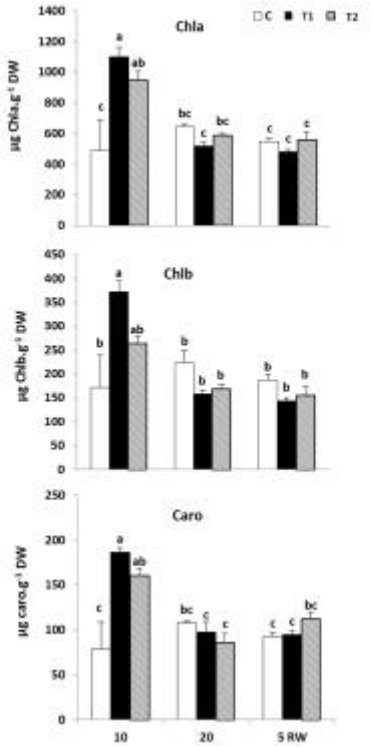

FIG.6 Chlorophyll a (Chla), Chlorophyll b (Chl b) and carotenoids (Caro) under drought stress conditions; C: control, T1: medium stress and T2: severe stress at day 10 and 20 of treatment and after 5 days of re-watering. Data are the mean ± SE of six replicates. Means followed by different letters indicate statistically significant difference (Tukey test; $p < 0.05$).

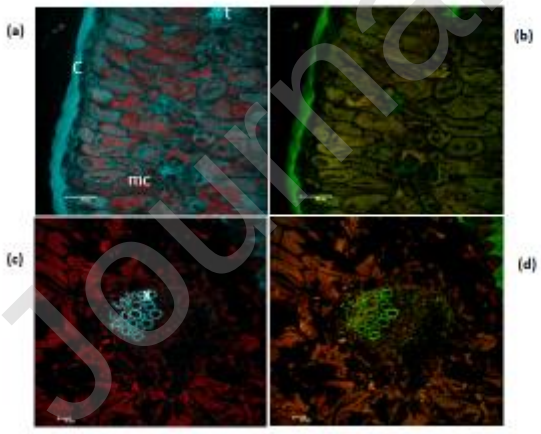

FIG. 7 Imaging of pigments and phenols in cross sections of *Oudneya africana* leaves. Images are projections of several optical sections collected by confocal microscopy showing the merging of chlorophyll (ex 543 and em 650-700, red colour, a-d) and carotenoids (ex 488 and em 500-580, green colour, a and b), and chlorohyll and phenols (ex 405 and em 448-483, blue colour, c and d). C: cuticle,

mc: mesophyll cells, t: trakeids, x: xylem vessels. Bars indicate 50 µm magnification in upper panels and 20µm in lower panels.

Suppl FIG. 1. Stomatal index (percentage) in abaxial (a) and adaxial (b) leaf surface of *Oudneya africana* under drought stress condition; c: control; T1: medium stress and T2: severe stress at day 10 and 20 and after 5 days of re-watering (RW). Data are the mean ± SE of three different leaves per plant and three replicates. Means followed by different letters indicate statistically significant difference (Tukey test; $p < 0.05$).

TABLE 1. Leaf area (LA, cm^2) ; E: transpiration, (mmol H_2O m^{-2} s^{-1}), and WRC (%) under drought stress conditions; C: control, T1: medium stress and T2: severe stress at 10 days, 20 days and after 5 days of re-watering. Analyze of variance for different parameter and interaction between time and treatment.

Parameter	**10 days stress**			**20 days stress**			**5 days re-watering**		
	C	**T1**	**T2**	**C**	**T1**	**T2**	**C**	**T1**	**T2**
LA	2.45±0.38bc	1.67±0.21de	1.47±0.04e	2.74±0.29ab	2.09±0.07bcd	1.66±0.24de	3.33±0.15a	2.71±0.22b	1.84±0.2cde
E	4.61±0,21ab	4.15±0.15b	3.39±0,15c	4.87±0.14a	2.82±0,19d	1.38±0. 04e	5.04±0.11a	4.25±0.33b	3.43±0.17c
RWC	80.35±1.12abc	79.11±0.48abcd	75.93±0.48cd	83.2±2.45ab	78.19±1.36bcd	72.14±1.68d	85.53±1.12a	81.53±1.42abc	80.91±0.98abc

Source of variance	**LA**	**A**	**E**	**gs**	**WRC**
Treatment	**	**	**	**	**
Time	**	**	**	**	**
Treatment x Time	ns	**	**	**	ns

**, Significant at $P < 0.01$. ns: non-significant.

TABLE 2. A: net photosynthesis, (µmol CO_2 m^{-2} s^{-1}); gs: stomatal conductance, (mol H_2O m^{-2} s^{-1}) and Ci/Ca: intercellular to ambient CO_2 ratio under drought

Parameter	**10 days stress**			**20 days stress**			**5 days re-watering**		
	C	**T1**	**T2**	**C**	**T1**	**T2**	**C**	**T1**	**T2**
A	21.7±1.05abc	20.76±0.96bc	17.15±0.15d	22.2±1.11ab	19.86±0.59c	12.4±1.05e	23.4±0.55a	20.56±1.06bc	16.95±0.11d
gs	0.21±0.009ab	0.189±0.01bc	0.14±0.007ef	0.225±0.007ab	0.15±0.01de	0.045±0.005g	0.23±0.006a	0.17±0.008cd	0.14±0.01fg
Ci/Ca	0.50±0.016cd	0.44±0.014bc	0.42±0.024b	0.52±0.06de	0.13±0.011a	0.14±0.011a	0.58±0.016e	0.58±0.019e	0.57±0.016de

Source of variance	**A**	**gs**	Ci/Ca
Treatment	**	**	**
Time	**	**	**
Treatment x Time	**	**	**

stress conditions; C: control, T1: medium stress and T2: severe stress at 10 days, 20 days and after 5 days of re-watering. Analyze of variance for different parameter and interaction between time and treatment.

TABLE 3. Effect of drought stress on total polyphenol content (TPC, expressed as mg gallic acid equivalent. g^{-1} DW) and flavonoids (expressed as mg quercetin equivalent. g^{-1} DW) of *Oudneya africana* under drought stress conditions; C: control, T1: medium stress and T2: severe stress at 10 days, 20 days and after 5 days of re-watering. Data are the mean of six replicates±SE. Means followed by different letters indicate statistically significant differences (Tukey test; $p < 0.01$)

	TPC (mg GAE.g^{-1} DW)			Flavonoids (mg QE.g^{-1} DW)		
	C	**T1**	**T2**	**C**	**T1**	**T2**
10 days	7.05±0.04e	18.76±0.70d	20.63±0.55c	6.88±0.53f	11.98±0.36e	13.92±0.5d
20 days	8.09±0.26e	22.91±0.27b	27.51±1.1a	7.23±0.11f	20.38±0.39b	25.49±0.59a
5 RW	8.82±0.06e	19.41±0.97cd	23.07±0.75b	7.46±0.3f	18.78±0.95c	20.06±0.72bc

Source of variation	TPC	Flav
Treatment	**	**
Time	**	**
Treatment*Time	**	**

**, Significant at $P < 0.01$

TABLE 4. Effect of drought stress on total polyphenol content (TPC, expressed as mg gallic acid equivalent. g^{-1} DW) and flavonoids (expressed as mg quercetin equivalent. g^{-1} DW) of *Oudneya africana* under drought stress conditions; C: control, T1: medium stress and T2: severe stress at 10 days, 20 days and after 5 days of re-watering. Data are the mean of six replicates±SE. Means followed by different letters indicate statistically significant differences (Tukey test; $p < 0.01$)

	TPC (mg GAE.g^{-1} DW)			Flavonoids (mg QE.g^{-1} DW)		
	C	**T1**	**T2**	**C**	**T1**	**T2**
10 days	7.05±0.04e	18.76±0.70d	20.63±0.55c	6.88±0.53f	11.98±0.36e	13.92±0.5d
20 days	8.09±0.26e	22.91±0.27b	27.51±1.1a	7.23±0.11f	20.38±0.39b	25.49±0.59a
5 RW	8.82±0.06e	19.41±0.97cd	23.07±0.75b	7.46±0.3f	18.78±0.95c	20.06±0.72bc

Source of variation	TPC	Flav
Treatment	**	**
Time	**	**
Treatment*Time	**	**

**, Significant at $P < 0.01$

TABLE 5. Matrix of correlations and statistical significances between growth parameters, photosynthesis and chlorophyll pigments under drought and rehydration conditions over the time. *, **: statistically significant at P<0.05; and P<0.01, respectively.

	PL	LN	LE	LA	Chla	Chlb	carot	A	E	gs	A/E	A/gs
PL	1											
LN	0,92**	1										
LE	0,86**	0,86**	1									
LA	0,89**	0,85**	0,81**	1								
Chla	0,45**	0,51**	0,23	0,57**	1							
Chlb	0,45**	0,5**	0,27	0,58**	0,92**	1						
Carot	0,49**	0,5**	0,43*	0,53**	0,65**	0,65**	1					
A	0,63**	0,64**	0,48*	0,7**	0,76**	0,72**	0,83**	1				
E	0,59**	0,61**	0,4*	0,61**	0,84**	0,78**	0,81**	0,91**	1			
gs	0,61**	0,66**	0,43*	0,69**	0,87**	0,84**	0,77**	0,92**	0,9**	1		
A/E	-0,38*	-0,38*	-0,22	-0,36	-0,71**	-0,61**	-0,74**	-0,7**	-0,91**	-0,69**	1	
A/gs	-0,43*	-0,45*	-0,31	-0,47*	-0,81**	-0,72**	-0,83**	-0,79**	-0,91**	-0,84**	0,91**	1

TABLE 6. Matrix of correlation and statistic significations between total phenolic contents, flavonoids and ABTS under drought stress conditions and rewatering. .

	TPC	FLAV	ABTS	IC50
TPC	1			
FLAV	0.94**	1		
ABTS	0.93**	0.98**	1	
IC50	-0.86**	-0.94**	-0.93**	1

**, Significant at $P < 0.01$.